

PERSPECTIVE / REVIEW / OPINION / HYPOTHESIS:

Ancient Origin Properties of Natural Exosomes Contribute to Their Therapeutic Superiority Compared to Artificial Nanoparticles

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Short title:

Superiority of therapy with natural ancient physiologic exosomes compared to artificial nanoparticles

Keywords:

exosomes; micro vesicles; extracellular vesicles; mesenchymal stromal cells (MSC); miRNA; cell therapy; artificial nano particles

Abbreviations:

Ab. Antibody

Ag, antigen

APC, antigen presenting cells

CS, contact sensitivity

DTH, delayed-type hypersensitivity

EV, extracellular vesicles

FLC, free light chains of antibodies

OMV, outer membrane vesicles released by bacteria

PEGylated, linked with synthetic polymer poly-ethylene glycol

RES, reticulo endothelial system

ABSTRACT

Extracellular vesicles (EV) such as exosomes, are newly recognized fundamental, natural and physiologic particles of life that seemingly are involved all biologic processes and clinical diseases. Due to their universal involvements, understanding the nature and the potential therapeutic uses of these nano-vesicles requires innovative experimental approaches, in virtually every field. Of the EV group, exosome nano-vesicles and larger companion extracellular micro vesicles (MV) can mediate completely new phenomena dependent on intercellular transfer of proteins and selected RNAs; particularly miRNAs, between donor and targeted cells to elicit epigenetic alterations inducing functional cellular changes. These recipient acceptor cells are nearby (paracrine transfers) or far away after distribution via the circulation (endocrine transfers).

The major properties of such vesicles seem to have been conserved over eons, suggesting that they may have ancient evolutionary origins arising perhaps even before cells in the primordial soup from which life evolved. Their potential ancient evolutionary attributes may be responsible for the ability of some modern day exosomes to withstand unusually harsh conditions; perhaps due to unusual membrane lipid compositions. This is exemplified by maternal milk exosome survival of the neonatal acid/enzyme rich stomach. It is postulated that this also applies to their durable presence in phagolysosomes; suggesting unique intracellular release of contents. A major issue discussed is the generally poorly realized superiority of these naturally evolved nano vesicles to therapies compared human engineered artificial nanoparticles; say for treatment of cancers.

INTRODUCTION:

Ancient superior qualities of extracellular vesicles, such as exosomes

Exosomes are fundamental natural and physiologic particles of life. Compared to artificial human-engineered nanoparticles they can readily penetrate tissues, have long duration of in vivo actions, can readily cross the blood brain barrier and very importantly as natural, exosomes easily evade the reticuloendothelial system, and acid/enzyme environments like mediating stomach digestion and in the tissue microenvironment of cancers. Compared to the hubris of attempting to artificially create potentially therapeutic nano particles aimed at imitating or improving on the superb optimal evolutionary development of extracellular vesicles [EV] like exosomes, artificial alterations of natural exosomes seems to be a superior therapeutic approach. This enables harnessing the inherent ancient superior properties of exosomes for modern therapies.

NATURAL EVOLUTION-DERIVED EXOSOMES

Universal presence and participation of EV like exosomes likely in all of biology and clinical medicine

Consideration of the evolutionary origins of EV like exosomes gives insight into their unusual properties, that are different than their producing cells and not true of human investigator-engineered artificial nanoparticles. The natural EV like exosomes are part of a family of subcellular released vesicular particles that are universal to life and arguably in some instances essential for all life. They seem to be made in some form by all cells as related to the: last universal common cell

ancestor [1],[2], and in all species, down to yeast [3], and fungi [4], and yet further to analogous outer membrane vesicles (OMV) produced by bacteria [5].

Relationship of EV like exosomes to evolution and procells at the origin of life

We propose that the family of minute extracellular vesicles that are currently able to exchange genetic information between cells via transfers of various RNAs and active proteins like enzymes, are related to and likely descendent from ancient universal particles of life that preceded eukaryotic and even prokaryotic cells. Alternatively, they could be related to such ancient primordial vesicles by having arising again via convergent evolution. This universality of exosomes and their likely ancient origin are compelling reasons to prefer use of these vesicles, as opposed to to the hubris of currently created human artificial nano particles for treatment of diseases. The exosome RNA content is of course even more ancient and dates back to near the origin of life. This is known as the “RNA world” [6],[7].

Current concepts derived from contemporary experiments suggest that the origins of these minute natural vesicles are related to proto-cell or pre-cell vesicles postulated to be involved in the origin of life [8],[9]. These potential vesicle predecessors of cells that are postulated to have arisen from near in time to the origin of life, are thought related to postulated primordial lipid membrane vesicles spontaneously formed in the noxious and highly diverse “primordial soup” that preceded life [10]. As evolution originating, they developed properties according to this early environment. Thus, the prebiotic chemistry in this paleoenvironmental context, was constrained by geologic and geochemical conditions [11]. At their origin, in this diverse pre-life primordial soup environment, available natural lipids are postulated to have spontaneously formed into vesicles with unilamellar membranes, This is analogous the generation of bubbles in a bath, as can currently be simulated in the laboratory [10],[12],[13].

Subsequently, over eons later, instead of one vesicle separating into two vesicles, a few randomly turned the daughter vesicle inside the other to form sturdier bilamellar vesicles with consequent greater life span [13]. Over time, this perhaps was due to a change of incorporated available lipid subtypes that preferentially allowed transformation into formed sturdier vesicles with bilamellar membranes. Subsequently, such originally rare vesicles with bilamellar membranes were then naturally selected for their stability. Subsequently, perhaps there was emergence of a subset, via yet more natural selection, that evolved to have advantageous interactions with ancient primitive RNA nucleotides [9].

Potential acquisition of RNA polynucleotides in evolutionarily primitive prehistorical nanovesicle procells

Individual RNA nucleotides were formed in the natural primordial soup by spontaneous chemical processes made possible by the extreme harsh physical and chemical conditions that acted on available constituent atoms to form individual pre-RNA nucleotides via energy available from toxic chemicals like cyanides [14],[15] and free radicals [16]. These individual RNA nucleotides that came to be present in the ancient vesicles, likely passed into and then through and out of the early unilamellar vesicles described above, and were transiently trapped inside the bilamellar vesicles when these were formed. Then as time advanced, when the vesicles had evolved to have the more advantageous bilamellar membranes, composed of unusual individual lipids in dense compositions, resulting in particularly high viscosity that enabled optimal withstanding of the noxious environment in the harsh primordial seas [17],[18].

Eventually over time, these intra-vesicular single nucleotides spontaneously and non-enzymatically reacted to form into polyribonucleotide chains [19],[20], based on their chemical nature, as well as the harsh environment and trace metal availability [15],[16]. Randomly, some of these still short RNA polyribonucleotides with particular sequences happened to have enzymatic activity of RNase ribozymes [21],[22],[23]. By natural selection these rare small macromolecules were favored to survive because of their ability to destroy non-enzymatic companion polyribonucleotide chains also present in the vesicles. Current day experiments have shown that the minimal number of bases in an RNA chain to have at least some enzymatic activity is only five bases [24]. Over yet more eons, similar molecular natural selection may have favored survival of greater lengths of RNA polynucleotides with progressively greater RNase activity to persist in the vesicles compared to those of less enzymatic activity.

As time advanced, multiple individual different advantageous RNA polynucleotides with differing RNase activities attained the ability to reproduce themselves [25],[26]. This was achieved through the binding of reverse sequence polyribonucleotides of optimal three dimensional conformation via reciprocal base pairing of sense sequences. There was thought to be multiple joining of the enzymatic base pairs. Thus, the original sense polyribonucleotides may in some rare cases have been guided by a matched set of such multiple RNases that now via enzymatic activity that could induce formation of reverse sense polyribonucleotide polymers that are anti-sense to the existing

chain sequences. The RNA replication cycle generally requires cooling to moderate temperatures for the copying, punctuated by periods of high temperature for strand separation. Lakes in geothermal active areas provide an environment with fluctuating temperatures, such that they are cool in winter and otherwise, as within hydrothermal vents that emit streams of very hot water for transient high temperature exposure that would promote RNA strand separation. These RNA and RNase carrying small vesicles that have been called proto cells [10],[13],[14], could be quickly mixed with surrounding cold water so contained delicate RNA molecules would not be destroyed by heat; over all this favored base pairing replication [19],[20], [24],[25],[26].

Postulated evolutionary origins of EV, such as exosomes from procells to OMV of prokaryotes and then to exosomes in eukaryotes._____

Yet later in evolution when eukaryotic cells emerged, these RNA-containing ancient vesicles may have been retained as secreted EV of the terminal endosomal pathway, like exosomes have become and further, as companion micro vesicles (MV) that bud from the eukaryotic cell surface, much like OMV bud from bacteria; as useful for intercellular communication. Note that MV of current eukaryote animal cells pinch off of the cell surface very much like OMV bud from the surface of prokaryotes [27]. The alternate and dominant process resulting in generating exosomes in eukaryotic cells in part employs generic intracellular mechanisms of vesicle formation. This involves an early endosomal pathway for deriving exosome vesicles by pinching off of these intracellular membranes, into an expanding intracellular space at the cell periphery called a multiple vesicular body (MVB). Finally, there is eventual release extracellularly by compound sequential exocytosis of the MVBs; thus the name exosomes for these particular intracellular arising EV [28].

This alternate process of endosomal exosome EV formation and extracellular release is progressively found in ancient archaea [2],[27], current yeast [4],[28], plants [29], vertebrate fish [30], shell fish [31], insects [32], nematodes [33], reptiles [34], birds [35], and finally in all mammals. Such conservation across such a broad range of species strongly suggests essential usefulness of such EV for all forms of life. Alternatively, ancient RNA-containing vesicles that evolved in harsh primordial conditions may have developed yet again in prokaryotic cells and/or eukaryotes due to convergent evolution, because of these very useful properties of communicating genetic functions between cells.

As noted, such vesicles likely were naturally selected for ability to uniquely serve intercellular export and receipt of RNAs and proteins between eukaryotic cells, to mediate intercellular communication of

molecules for epigenetic regulation. This likely allowed for successful competition by natural selection, compared to other cells without such capacities. In modern organisms, their related EV like micro vesicles and exosomes, comprise in fact the principal means of performing these essential functions of intercellular genetic communication. Other less emphasized pathways include non-exosomal RNA carriage via chaperones like argonautes [36],[37],[38], and by hydrophobic high density proteolipids [39]. Interestingly, in the end these non-exosomal carried RNAs can become functional by transfer in vivo into host exosomes, that then mediate inter cellular transfers [38].

Current bacterial outer membrane vesicles (OMV) seen as evolved from primordial procell vesicles of ancient times toward current day exosomes

Over many many evolutionary centuries, the first prokaryotic bacterial cells likely emerged, perhaps from conserved proposed RNA polynucleotide containing ancient vesicle procells. Subsequently, these vesicles, are thought to have evolved to retain subcellular exosome-like MV particles, by a natural selection preference. OMV are generated individually in bacteria by ballooning out of their external membrane and then pinching off of these EV at the bacterial surface. In these prokaryotes the OMV are diverse and very useful indeed, this was useful for their advantageous properties of intercellular communication in early organisms like bacteria and archaea, that now have such vesicles known as important retained microbial outer membrane vesicles (OMV) [5],[6],[41],[42],[43]. In addition to containing LPS to induce the systemic inflammatory response syndrome [44], OMV carried bacterial toxins can served for their protection [45],[46] or induce diseases [47],[48],[49] or cancer pathogenesis [50],[51], also importantly can contain RNAs [52],[53],[54], release RNAs [55],[56]; and transfer RNAs amid other factors of pathogenicity to hosts [57],[58],[59],[60],[61], and like current day mammalian exosome subpopulations also can resist harsh conditions [62],[63].

In current modern times, these OMV are generated individually in bacteria by ballooning out of their external membrane and then pinching off of these OMV at the bacterial surface. In these prokaryotes the OMV are diverse and very useful. They mediate horizontal transfer of information transfer like genes [64],[65],[66], and importantly promote some infection driven clinical diseases by transporting DNA genetic virulence factors to less able co-hort bacteria [67],[68], that predominantly are OMV carried virulence factor DNA genes, with similar effects transferred by OMV from archaea [69]. Further, besides general innate [70], and acquired immunomodulatory actions of OMV [71],[72],[73] those from OMV of bacteria [74] and exosomes of yeast [75] that can promote allergy driven

pathologies; perhaps from release of their RNAs [76]. This includes OMV from staphylococcal [74], and fungi [75],[76], in the skin that can participate in the induction of allergic atopic dermatitis.

Beside transfer of genetic information like RNAs to mediate epigenetic effects there can be important OMV transfers to other bacteria [64] of genes encoding antibiotic resistance [77],[78], such as transferring plasmid-borne gene for β -Lactamase or this gene produced β -Lactamase itself for resistance to amoxicillin [79],[80],[81],[82],[83],[84]. Overall, OMV and exosomes are now understood to molecularly connect among, and between biological kingdoms [85][86]; such as harboring viral genomes [87],[88], exchanges between plants and associated fungi that depend on EV exchange [89]. and transfer of miRNAs connecting parasites with the host to induce a cytokine environment favorable to the helminths [33].

Particular subsets of exosomes resist harsh conditions

It is postulated that there are subsets of exosomes that come from particularly activated cell donors that have membranes composed of unusual lipids. It is proposed that these lipids likely account for unusual properties of some exosome subsets. Outstanding is survival under harsh conditions; including strong acid/digestive enzyme environments; like in the stomach and in hypermetabolic sites of growing cancer cells. Further exosomes from immune activated T or B lymphocytes exhibit unusual surface binding of Ab FLC and moreover ability to associate with a given added miRNA, while EV from non-immune animals do not have these activation properties [40],[90],[91],[92]. We call this special subset “activated exosomes” as they often are derived from especially stimulated donor cells, but note that they are mixed, as perhaps a minor subpopulation, together with the great mass of EV derived from non-activated cells, that are without these special resistance and binding properties. The unusual properties of resistance to harsh conditions of these “activated exosomes” are postulated to particularly reflect the origins of their ancestors in the ancient harsh primordial environment that closely followed the origin of life.

Exosome resistance to harsh conditions may in part be due to special lipid properties of their membranes acquired by some exosome subsets

In some cases, membrane biophysical analysis determining lipid composition and fluidity have found a particular lipid content in such activated exosomes, often released at low pH, with high rigidity and viscosity that likely are at least partly responsible for the unusual surface properties of such resistant subsets [17],[18]. Interestingly, the overall lipid composition of exosome membranes can be very different than membranes of the host vesicle-producing cells. This is due to the intracellular endosomal membrane becoming the outer membrane of the released exosomes, due to the terminal

endosomal intracellular budding, with enrichment of certain phospholipids for an unusual membrane organization of unique viscous lipids induced by activation of the producing cell [17],[18],[89],[90],[91],[92]. This suggests that these variations in lipid composition have a targeting and perhaps functional purpose when these particular exosomes are transferred to acceptor cells. As noted above, these special exosome surface lipids are thought to be a major contributor of resistance to harsh conditions continuing on from primordial times and seem to be at least in part responsible for unusual properties of exosomes produced by certain cell activators or in tissue sites with special properties. Other factors contributing to the resistance properties of exosome membranes include endosomal origin, that not only brings an unusual lipid composition different from the cell membrane, but also increased diverse tetraspanins like CD9, CD63, and CD81 that enforce the unusual stability of the membrane.

Our own studies are a particular example of these issues concerning exosome subsets with special membrane properties.

In High dose Ag-induced immune tolerance, induced Ag-specific CD8^{pos} suppressor T cell-derived exosomes are a subpopulation differing from exosome cohorts derived from normal donor non-activated cells. They are isolated from the exosome total by specific Ag or anti-CD9 Ab affinity column chromatography separation. They are repeatedly able to absorb immunoglobulin Ab FLC, but not Ab heavy chains nor whole IgG on to their surface and further to associate with added chosen miRNA; properties not found in exosomes from non-activated normal donors [40],[90],[91],[92].

Lipid membranes with unusual properties in subpopulations of current day exosomes may derive from their ancient evolutionary beginnings

Current concepts derived from contemporary experiments suggest that the origins of these minute natural EV are related to proto-cell or pre-cell vesicles postulated to be descending and evolving since the origin of life [9],[10]. These potential EV predecessors of whole cells are postulated to have arisen from ancient time near to the origin of life. They are thought related to primordial lipid membrane pro cell vesicles spontaneously formed in the noxious and highly diverse “primordial soup” that preceded the origin of life [11],[93],[94]. Natural lipids that were available in the primordial soup are postulated to have over time spontaneously formed into vesicles with unilamellar and then bilamellar membranes, in this pre-life diverse noxious environment; analogous to generation of bubbles in a bath. This process can currently be simulated in the laboratory [11],[17].

The unusual lipid composition of some current exosomes derived from their ancient origins likely results in increased size and most importantly distinctly greater viscosity and stiffness of produced activated exosome membranes [17],[18]. These unusual membrane constituents may account for the unusual properties of some exosomes we call “activated” compared to those from non-stimulated cells; such as noted; surface binding of Ag-specific Ab free light chains (FLC) [90],[91],[92], and the ability of such exosome subsets to associate with in vitro added selected miRNA for added gene-specific functional delivery of particular epigenetic alterations in surface Ag-specific exosome Ab targeted acceptor cells [40],[90],[91],[92].

Attempts to demonstrate special lipid properties in subpopulations of activated exosomes

Our ideas about a role for special constituent membrane lipids in exosomes from activated cells, that we call “activated exosomes,” come from prior studies of similarly unusual exosomes that are able to surface bind of Ab FLC due to altered membrane lipid constituents [96],[97],[98],[99]. This concept was sustained by our preliminary unpublished studies examining Ab FLC binding to individual lipids per se. Firstly, monoclonal Ab FLC were shown to have stronger binding to certain individual lipid components, such a Phosphatidylethanolamine (PE). This was determined with an ELISA-type assay using different single simple lipids adsorbed on micro plate wells and then bound by Ab FLC. The amount of FLC bound was quantitated by employing a second binding of anti-FLC monoclonal antibody (mAb)-linked with a detecting enzyme. Secondly, liposomes then were constructed at exosome size, and dominantly composed of the best individual FLC lipid binders, like PE, vs. less and enriched with of poor FLC binders like sphingomyelin; and were found able to bind the Ab FLC, detected by Western blotting. Third, these Ab FLC-associated liposomes had biological properties that were not present compared to routinely composed liposomes [Hutchison AT and Askenase PW, unpublished].

An outstanding example of strong resistance to harsh conditions, is dietary milk exosomes that can survive harsh environment of the stomach

Overall, some EV such as activated exosomes, can play special newly recognized biologic roles under harsh conditions, compared to mediating previously unrecognized physiological processes. An important example is the unique ability of some exosomes to function in milk for neonatal gastric passage. Exosome survival in milk is resistance to highly acidic pH of even 1.0-4.0 and a strong

mixture of digestive enzymes that cells cannot survive, nor can artificial exosome-imitating nano vesicles [99],[100],[101].

This powerful example allows gastrointestinal tract passage of oral administered exosomes in mothers' milk and in cows' milk, across the stomach for subsequent absorption in the small intestine [102],[103]. This results in a natural process of exosome vesicle transfer of contained miRNAs into off spring, to likely participate in neonatal development after systemic absorption. The milk exosomes contain about 14,000 maternal RNA transcripts representing the milk transcriptome, that likely transfers the mothers genetic information to neonates during breastfeeding [104],[105]. Together the RNAs in maternal milk can regulate development of important neonatal protective systems like: innate [106] and acquired immunity, [107],[108],[109],[110]; as well as immunoregulation relevant to development of atopic allergies [111], and immune relationships to their newly acquired microbiome [112],[113],[114]. Further, mothers milk exosomes also have effect on the development of several other systems such as: bone formation [115],[116]. and development of the endocrine/metabolic systems [117] and the nervous systems [118],[119]; such as delivery of specific miRNAs to specific tissues, such as the neonatal intestine [120]. Importantly, the miRNAs in bovine milk exosomes are bioavailable in humans across species, but as external RNAs do not elicit an increase of plasma cytokines following oral administration [121].

Further, and very importantly for current day EV treatments, there is survival of therapeutic exosomes after oral administration, also surviving the strong acid/enzyme mixtures of the stomach [122]. In our work, this allows for transfer to adult recipients of systemically-acting Ag-specific exosomes delivering carried specific functional miRNA after oral treatment [91],[92]. Properties of such resistance to these current day harsh conditions are not possible for in any given cells that are all digested, nor any of the various investigator designed artificial nano particles.

Further there is an ability of certain exosomes to survive by resisting detergents and recover after lyophilization

Resistance to proteinase K detergent has been shown due to exosome membrane content of the small heat shock protein, $\alpha\beta$ -crystallin, that can become susceptible in the presence of Triton X-100 [123]. Accordingly, we note that there is the ability of exosomes to function in intercellular communication and pathological conditions of diseases not anticipated in prior studies with cells nor a capability of various investigator generated artificial nano particles attempting to imitate properties of natural exosomes. Importantly, not only do the milk exosomes survive by resisting digestion, but are able to subsequently cross the intestinal barrier [125],[126],[127],[128]. This strong resistance to

harsh conditions of some exosomes seems analogous to extremophiles, that are organisms able to survive in extreme environments, such as intense heat, high acidity, and high pressure, not previously recognized as able to sustain life [129]. These resistance properties allow for storage at -20° C for years [130], and allow for lyophilization [131]. These unusual storage resistance properties have great practical significance since they mean that therapeutic exosomes become readily available in a wide variety of and even relatively primitive medical environments.

Tissue hypoxia is another prominent harsh condition that natural exosomes can resist

Further resistance to other harsh conditions, that are postulated to have come from abilities gained in the harsh primordial ancient conditions include: resistance to hyperoxia [132], but most outstandingly extracellular vesicle resistance to, and preference for, acting under distinct tissue hypoxic conditions [133],[134], as found in destructive microenvironments, particularly as in and around highly metabolic cancers; such as: breast [135],[136], ovary [137],[138],[139],[140], prostate, [141],[142],[143], and esophagus [144], in which generally the hypoxia induces cancer cell exosome miRNAs and other pro tumor functional exosome carried molecules and the further the altered hardy membranes of such exosomes, render them increased resistant to the hypoxia microenvironment.

These unusual properties may mean that hypoxia resistant MSC exosomes may have an important role in the treatment of hypoxic diseases [145]; such as in myocardial infarction [146],[147],[148], pulmonary hypertension [149],[150], thrombotic diseases [151]. and CNS stroke [152],[153]. An additional area in which hypoxia increases the potency of exosomes, is in treatment with mesenchymal stem cell (MSC)-derived exosomes that are in general healing and trophic in a variety of tissue inflammatory pathologies; particularly acting favorably on the injured vasculature [154],[155],[156],[157],[158],[159],[160]; with a promise of treating neonatal hypoxic conditions [161], prevention of hyperoxia-induced lung injury [162].

Summary pertaining to current exosomes as evolved from primitive ancient nano vesicles

According to these experimental derived and hypothesized ideas, the family of EV emerged before cells and had many valuable properties that have carried on to the present time. With subsequent

emergence of eukaryotic cells, mitochondria were retained as intracellular bacteria for energy metabolism and can release their own OMV used for intracellular communications [163],[164],[165]. and extracellular activation via inducing inflammatory cytokine proteins release [166],[167] and regulation of anti-bacterial functions [168]. In an analogous fashion, EV like exosomes probably were evolutionarily retained for their unique ability to transfer genetic instructions between cells, and further for their ability to serve these essential functions by their unique ability to resist harsh extracellular conditions, not at all possible for later developed and much more complex and susceptible eukaryotic cells themselves.

As noted, this discussion of exosome primordial origins is based on current sophisticated experiments and on added hypotheses. It is meant to emphasize the long biologic evolution of exosomes over billions of years of Darwinian experimentation via natural selection of optimal properties to achieve physiological nano vesicle exosomes. This compares to the hubris of one off efforts towards a quick fix of some current investigators who imagine artificial therapeutic nano vesicles for human uses that are deficient in many essential biologic properties generated by progressive steps in evolution over billions of years

Exosome resistance to harsh conditions may allow intracellular survival in phagolysosomes after target cell uptake, that is likely not a property of artificial nano particles.

As we noted previously, the resistance of exosome subsets in dietary milk to the strong digestive mixture of gastric enzymes in high acidity relates to their preserved actions in the frequent low pH of the cancer microenvironment. There is similar resistance at other sites of profound inflammation or necrosis and the correlative finding that in vitro production of active exosomes can be optimal at pH = 3-4 [169]. Indeed, acting in the special low pH tissue microenvironment in cancer is thought to be a key factor in participation of exosomes in malignancies, that artificial exosome-like nano particles have no ability to resist.

To this point, after exosomes are intracellularly taken up in targeted cells by phagocytosis [170] or micro-pinocytosis [171], we present a new idea. It is postulated that among a variety of intracellular pathways for the up take of exosomes and their contained bioactive variable miRNAs some exosome subpopulations may resist the very low pH and digestive enzymes present in phagolysosomes, as they similarly do in the stomach. As a consequence, studies employing confocal laser scanning of fluorescence antibody microscopy have shown many times that surprisingly, labeled

exosomes are frequently noted as seemingly intact in phagolysosomes of the targeted cells after uptake, [172],[173],[174],[175],[176],[177], in some instances phagolysosome delivered exosomes were confirmed as still functional by subsequent actions [178],[179],[180],[181],[182], [183],[184],[185],[186] in the particularly targeted cells [187],[188],[189],[190], [191],[192],[193],[194],[195]. Particularly instructive has been the recent very thorough recent study employing the most sensitive intracellular visualization technique technique of single molecule based super resolution microscopy [196]. This enabled following breast cancer-derived exosomes with two different fluorescent membrane markers (red) and simultaneously a phagolysosome enzyme (blue) in normal cell recipients. This clearly demonstrated the stable presence of the exosomes in the phagolysosome simultaneously, by dual-color imaging. Thus, this particular exosome subpopulation, postulated to be activated with unusual resistant membrane properties, can persist in phagolysosomes to possibly provide entirely distinct new mechanisms of intracellular release and translation of functional cargo like miRNAs to the acceptor cell over time, to then uniquely affect intracellular functions in targeted cells enumerated above. Observed slow drifting diffusion in local cytoplasmic microenvironments after uptake of exosomes is consistent with such an alternate intracellular release mechanisms.

Of course, it cannot yet be determined if the visualized exosomes are a subpopulation not able to release their contents, while companion transferred exosomes that already released their contents have dissolved and thus cannot be seen. The occurrence of this potential protected low dose exosome intracellular release that is over time may pertain to some unusual findings that biologic function can be transferred by very few exosomes, that seem to contain minute amounts of the relevant miRNA beyond currently accepted low level limits for function [40],[197]-[198],[199], but alternatively undergoing slow release and diffusion from exosomes intracellularly into the cytoplasmic microenvironment after uptake of exosomes is consistent with such an alternate intracellular release mechanisms [200]. However, it is unclear whether their contents had already been discharged or if they were injured and unable to do so. This area beyond current techniques should become greatly clarified by new methodologies, like ACRISPR-Cas9-based reporter system for single-cell detection of extracellular vesicle-mediated functional transfer of RNA [201].

DEVELOPMENT AND POTENTIAL USE OF ARTIFICIAL ENGINEERED LIPOSOME NANO PARTICLES AS SUBSTITUTES FOR EXOSOMES

The hubris of employing artificial nanovesicles instead of exosomes to therapeutically deliver short regulatory RNAs

There have been continued failures to treat via RNA interference with artificial nano particles despite original high hopes. The discovery of RNA interference (RNAi) led to new potential means of treatment via gene regulation and was touted as having enormous clinical potential for treating various disorders. It was anticipated that intercellular delivery of artificial nanoparticles carrying siRNA or miRNA, possibly via viral vectors, would prove to be easily achievable. Despite this original optimism and much work and money consumed, many relatively unsolvable problems developed: leading to diverse current justified doubts about whether large scale efforts to create artificial nanoparticles to deliver genetic therapies should go on.

However, inappropriately and sadly, in the face of *prodigious* numbers of failures, there continues to be significant funding by pharma and NIH, to support ever increasingly complex proposed ideas to deal with the problems of using the artificial non-physiologic nano particles, to “build a better mouse trap.” Continuous uninformed plans usually result in striking futuristic proposal diagrams of conceived particles with placed appendages. These are ceaselessly aimed at developing artificial nanoparticles that turn out to not be very clinically useful for in vivo delivery of small RNAs and other desired contents, and thus as before, continue to be inferior to natural EV, such as exosomes, that need far less adjustments for particular natural in vivo use.

The foremost problem of these failures has been the challenge of delivery to targets. This should have been anticipated. It was not, instead, early high-profile publications created expectations that this challenge could easily be overcome for RNAi. *This was hubris*; especially regarding quick promises with attempted delivery by artificial nanoparticles. In no way could these mimic the superior properties of natural physiologic EV like exosomes, that likely developed over billions of years of progressive trial and error adjustments of evolutionary natural selection to achieve the fittest near optimal design and capabilities. This compares to quick and superficially conceived artificial nanoparticle formulations that lacked effectiveness in vivo; despite in vitro findings. Additionally the non physiologic artificial preparations had new unwanted, but really considering their foreign nature, expected incompatibilities. Some were new toxicities; setting investigators to apply new artificial alterations to overcome these. Thus, there often is applied artificial adjustments of artificial concepts; i.e. further hubris. It is not realized that each adjustment brings new side effects deal with.

Despite these continued failures, the literature still is as crowded with proposed artificial EV variations favorably judged by appealing diagrams rather than biocompatibility. Scores of FDA approved artificial therapeutic nanoparticles have been developed with few reaching realistic clinical use and effectiveness. A liposome based artificial nanoparticle was first developed nearly 25 years ago, but is still struggling, as have many liposome-based exosome imitations [202],[203],[204], even those with polyethylene glycol coating (called stealth liposomes) to increase duration in vivo [205]. **Despite** these increasing doubts, and amid the growing published skepticism of others, there persist enduring enthusiasts [206]. However, they are amid growing published doubts of others, who note that there has been improved safety rather than increased efficacy; especially due to the lack of significant alteration induced benefits in the course of late-stage solid tumors [205],[206]. The waning of interest in liposome-based artificial exosomes is happening amid growing findings in the mesenchymal stromal cell (MSC) therapy field that their produced exosomes are of comparable value. This has resulted in many adult stem cell therapy companies turning toward exosome production, because they are easier to store, administer, and there is a reduced potential of toxicity and unnecessary unnatural complexity [207].

Both siRNA and miRNA have advantages for delivery via exosomes

Regarding therapeutic exosome delivery of these short RNAs to achieve gene silencing, there are different advantages for each. First, miRNAs can be naturally present in exosomes and thus need no transfection. Second, a given miRNA can be associated with the subpopulation of activated exosomes, again obviating transfections [40],[91],[92]. Third, the ability of miRNA to act in the targeted cell to affect multiple mRNAs does not necessarily produce total non-specificity. Instead there may be a broad action of oligo clonal specificity. This may be appropriate when multiple mRNAs are involved. Forth, as such there is no requirement to determine which particular mRNA is crucial for gene silencing obtained with particular miRNAs.

Regarding delivery of siRNAs, a weakness is the need for transfection of the producing cell or the exosomes. Transfecting the cell introduces the possible untoward effects of a virus that could be oncogenic. Further, chemical transfection of the exosomes [208] may have problems [209]. Therefore, this may not be very suitable for clinical use. New commercial reagents have arisen enabling transfection of exosomes with RNAs. Exo-Fect is such a nucleic acid transfer agent that enables the transfection of nucleic acids directly into isolated exosomes. The transfected siRNA, mRNA, or miRNA, can then be delivered into target cells by these altered exosomes. More common

current approaches are to transfect the exosome producing cell or the producing animal [210],[211] with a marker like green fluorescent protein genetically fused with an exosome marker like tetraspanins. This enables following the exosomes that enter the target cells [212]. Finally, there is simply linking a lipid dye to the exosome membrane or better yet the producing cell and thus not the exosomes that might result in injury of the exosomes that might affect transfers [213],[214].

Using available methods of loading exogenous siRNAs, an obvious great potential advantage of this exosome delivery is the precision of affecting a single mRNA based on gene sequence. Further, there is the potentially useful availability of having activity due to both the sense sequence (miRNA-5', miRNA-5p;) usually active, and the reverse anti-sense sequence carrier chain (miRNA-3', miRNA-3p) that also can be the selected strand to interact with RISC to mediate regulatory activity on the target gene mRNA in vertebrate mammalian systems [215],[216],[217],[218].

On the road to a fruitless goal to develop liposome-like exosomes

The still persisting original artificial candidate first developed decades ago is Abraxane. This is not a vesicle but just an albumin bound to the anti-cancer drug paclitaxel. It is a good example of a biologically uninformed struggle to achieve artificiality. It first received FDA approval as a nano therapeutic in 2005 for the treatment of breast cancer that was resistant to chemotherapy. This formulation led to enhanced activity and reduced paclitaxel toxicity, and seemed to act in disease considered refractory to therapy, with the conventional taxanes alone. However, despite administering paclitaxel in this manner, serious toxic side effects continued; precluding use in patients. Further, there were new side effects thought due to the immune sensitizing properties of lipophilic vehicles inducing new hypersensitivity reactions to constituents of these particles in patients; who now needed pretreatment with corticosteroids, plus H1 and H2 antagonists [219]. Subsequently developed artificial nano particles still have had a high incidence of hypersensitivity reactions [220],[221]. In contrast, such a problem is unknown for exosome therapies that in fact produce few if any significant side effects [222],[223]; except bacterial contamination of mis-handled preparative cultures [224].

Thus, the next step was making liposome vesicles to encapsulate anti-cancer drugs and eventually RNAi to then achieve artificial nano vesicles. However, such foreign particles encounter multiple non-specific host defense systems aimed at recognition, neutralization, and elimination of invading foreign particles. Therefore, as would be expected, the reticulo endothelial system (RES) consisting of highly

phagocytic macrophages associated with the vasculature, was the main site of liposome accumulation and clearance following systemic administration. This led from these "first-generation liposomes," to development of "second-generation liposomes". These had longer-circulation time by modulating the lipid composition and including in the membranes the synthetic polymer poly-ethylene glycol (PEG) that was shown to reduce RES uptake; leading to calling them "stealth liposomes" with claims of increased bioavailability in a few tested tumor systems [225],[226]. It was said that this brought high target efficiency and activity for the liposome encapsulated anti-cancer agents. However, despite some protection from the RES and PEGylation caused loss of their long circulating properties due to enhanced blood clearance [227],[228]. It was shown subsequently that this was due to the immunizing capacity of these artificial nano-particles, due to expected development of specific IgM antibodies [229]. This is typical of the engineered nano particle field where the creators have little experience with biology or immunology that can thwart their fancy plans [230],[231].

This untoward aspect is familiar to immunologists as involving B-1a IgM producing thymic independent B cells, that differ from conventional helper T cell-dependent IgG-producing B-2 B cells [232]. These B1 B cells produce an Ag-specific natural background antibody repertoire, targeting self Ag, such as phosphatidylcholine and other lipids frequently contained in liposomes, to subsequently then prime B-2 IgG-producing cells [233], to account for the significant immunogenicity of PEGylated liposomes [234],[235]. Further, this led to classical complement activation due to these IgM Ab coated liposomes, and also exosome surface alterations leading to activating the alternate complement pathway [236],[237]. These are all disasters for the immunologically unaware. *Thus, not only was there lack of positives achieved, but creation of negatives with these human bio engineer conceived artificial nano particles!!!*

There has been more than 50 years of considerable research, yielding a plethora of positive preclinical results of liposome encapsulation of agents, including those mediating RNAi. However, there has been little actual clinical translation for liposomes mediating RNAi therapy [238]. This has been attributed to face saving issues of pharmaceutical manufacture, government regulations pertaining to quality assurance and cost, as well as intellectual property [238],[239]. Instead, the major impediment has been poor realization of typical negative effects of the immune and reticuloendothelial systems on artificial nanoparticles, ruling out human in vivo clinical uses and furthermore, and most importantly, major inefficiency in solid tumor penetration [240]. *Overall, the clinical benefit of 'improved' nanoformulations has been very limited so that enthusiasm for artificial*

nanoparticle delivery of RNAi to patients has waned. This is largely because the clinical benefits have been reductions in toxicity rather than improvements in efficacy.

Still, still, workers and reviewers in the field talk of many adjustments to liposome based therapy to achieve more useful results even after so many years of trying [238],[239],[240],[241],[242], while simultaneously ignoring the elephant in the room; i.e. the superior therapeutic activities of natural EV such as exosomes. *Therefore, in sum, after spending much time and billions of dollars attempting to create such human designed and produced artificial nano particles to deliver RNAi therapeutic agents like miRNAs and siRNAs, major funding agencies like the NIH and Big Pharma have had few practical results. It seems that at best the most perfectly developed liposome would be a cheap complex incompatible imitation of an exosome; like from a bargain store.*

All of these problems noted above are not among many properties of the totally natural physiologic EV like the whole exosome elephant that are impossible to imitate with artificial engineered nano particles. For exosomes, delivery and systemic in vivo alterations of specific cell targeting, of duration, uptake and intracellular processing can accompany effective small RNA intracellular release. The natural functional universality depends on many unique biologic characteristics that frankly are impossible to reproduce in artificial nano particles. This means that such human designed constructs always are fated to fail from the start in attempts in to imitate these numerous aspects.

Thus, it is a game that creators and proponents of artificial nano particles always will lose; trying to compete with a whole complex cellular organelle whose optimal designs have been achieved through endless alterations by trial and error for evolutionary optimal perfection over billions of years. Interestingly, in most papers on artificial nanoparticles the investigators that are trying to achieve human designed nano particle RNAi delivering agents, seem to not even be aware that they are attempting to imitate already available natural nanoparticle exosomes. *There never is a comparison of the artificial nano particle to natural physiologic exosomes. Taking all of the above into account, if this is not a supreme example of human hubris then what is?* Note that hubris is defined as excessive pride or over self-confidence; (arrogance, conceit, egotism, pomposity, superiority; in Greek tragedy): i.e. defiance of the Gods; here defiance of evolution).

Incomprehensibly, the last two multiple year reports of the NIH's NCI Alliance for Nanotechnology in Cancer, including therapies, amazingly did not mention exosomes [243],[244]. A program administrator, who is an author of these reports, said to me that the natural exosome nano-vesicles,

were "" for these purposes. This is great arrogance and consequent mis-use of public funds considering these natural physiologic nanoparticle exosomes have undergone multiple fine evolutionary adjustments by trials of Darwinian natural selection over eons. The hubris is that this is compared to just a few years of superficially conceived NCI and Big Pharma attempts to develop artificial nanoparticles for delivery of miRNA and siRNA, that as expected has had many failures, with the result that so very few have been FDA approved nor clinically useful [245], [246]. *Therefore, things have come to the suggestion that these artificial approaches may be abandoned [247],[248].*

Artificial engineered nanoparticles carrying RNAs, to mediate RNAi are an expected failure

Regarding the literature on artificial nano particles, there is a vast potential, in various fields, with many avenues to explore that papers have accumulated and continue to increase weekly. There are never-ending claims about some great newly engineered nanoparticle able to deliver small therapeutic RNAs; often especially fashioned to target cancer. But none of this has proven to be clinically successful [247]. A very thorough meta-analysis on delivery concerning efficacy of nanoparticles for cancer-targeting quantitates the numerous failures [240]. Targeting and delivery are important because systemically administered nanoparticle carriers cannot function if they do not access the diseased cells and tissues at a sufficiently high effective dosage.

Nanoparticles, once injected into the body, face both physical and biological barriers (for example, diffusion, flow and shear forces, aggregation, protein adsorption, phagocytic sequestration and renal clearance) that affect the percentage of administered nanoparticles reaching target diseased tissue and cells. In comparison, natural exosomes suffer none of these deficiencies. Exosomes have inherent semi-specific surface receptivity of the targeted cells controlled by the producing cell as important carrier effects facilitating delivery and further carrier effects that influence intracellular pathways to enhance delivery of bio active pertinent selected RNAs that artificial nano particles could never replicate; it's a "no brainer". Overlooking these biologic properties needed for delivery and function suggests a complete absence of a sense and knowledge of the complexity of biology by the creators of artificial nano particles for RNA delivery.

The meta-analysis mentioned was of publications over the 10 years 2006-2016 [240]. It shows that the efficiency of nanoparticle delivery to tumors has not improved at all over this period. There were over two hundred papers to work with, but only about half had enough time points to be useful. An overall summary was that only about 0.7% of a systemic dose of artificial nanoparticles actually

reaches tumor tissue, This may be an overestimate since they could not determine if the nanoparticles were actually targeting the malignant cells, or just going into the tumor matrix. Further, typical nanoparticle loadings based on mouse studies, extrapolate to unfeasible human doses of 90 to 200 mL of nanoparticle suspension, which is an unlikely clinical strategy. Even synthesizing the nanoparticles on that scale would currently be a challenge. This review showed that there is a gulf between reality and peer reviewed science pertaining to biological barriers that nanoparticles were supposed to cross but does not exist.

Further there are severe bioengineering constraints of this approach with thus much wasted effort and money. Even if this technology could be developed, the dollars cost of delivery would be a huge barrier. If the price of current cancer drugs are expensive, added artificial nano cancer delivery would only be chosen for wealthy patients, not those of lower socioeconomic classes. Additionally, toxicity of artificial nano particles could be a massive issue that is rarely discussed [249],[250],[251].

The analysis of delivery includes pertinent questions about how nanoparticles get out of blood vessels or extravasate into tumor tissue. The dominant view is that this happens through leaks in the tumor vasculature due to gaps between endothelial cells; a characteristic of cancers. However, this may be mistaken, and other processes need to be investigated; considering that trans endothelial cell pores are difficult to demonstrate.

In contrast, exosome experiments clearly show inflammation augmented and even trans endothelial cell passage into the tissues in this physiological case like passage across the blood brain barrier [252],[253]; and other natural barriers especially with engineered targeting peptides on their surface [254]. Natural exosomes additionally can have nutritive effects on vascular regeneration [255],[256]. In contrast, no engineered artificial nano particle has been demonstrated able to do such innate profitable interactions with vessels these without local disease induced vascular permeability, and then poorly [257]; requiring further engineering attempts that generally have not been very successful [258],[259]. Note that the mechanisms and pathways for nanoparticle transport into tumors are indeed very important but poorly known. If the extravasation is mediated primarily by the transcellular route, exosomes are natural nanoparticles that can actively utilize this transport pathway and can be designed further. At present, the nanotechnology community has not thoroughly investigated this transport mechanism. Instead, a heavy emphasis has been placed on studying nanoparticle transport through intercellular gaps via the artificially induced enhanced permeability mechanisms [260],[261]; an approach that in comparison has thus far yielded poor efficiency of delivery for artificial nano particles.

Findings true only under limited experimental conditions, frequently are inflated and overblown with futuristic rhetoric

An additional problem has been wanting to get results fast once ideas are seen with great promise. However, this rarely can happen because of the myriad of barriers between testing and usable material, with engineered nanoparticles compared to natural exosomes. Often, the money goes to the low-hanging fruit which isn't as advanced but is hoped to deliver much sooner, but it nearly always does not. Findings in this area, that may be true only under limited experimental conditions, frequently are inflated and overblown with futuristic rhetoric by the media. Such rhetoric may be necessary to attract the public's attention for more funding, but such statements create unintended side effects. Researchers may be forced to create fiction-like stories for funding, instead of proposing solutions to real problems.

Thus, the next time a breathless press release comes out touting a new artificial particle revision; touting that finally they deliver much more than previously achieved, be very doubtful. Then, as the hype recedes, and the real problems become more apparent, there can be much recognition of the superiority of natural exosomes compared to artificial nano particles. Considering the years of failures, trying to finally achieve properties at the level of natural exosomes seems really to be "beating a dead horse". This seems to be an incurable disease of blindness that such commercial developers possess. Thus, still, in the face of mounting clinically obtained repeated evidence of failure, researchers and companies continue to pursue nanotechnology constructs that ignore these facts. They do this to the peril of their investors, and to the detriment of their most important stakeholder, the cancer patients whose lives depend on the clinical success of these products [262],[263].

Pertinent is the report of a worker in the field for many years who spent almost 5 years working for a nanoparticle company. They employed a system based on stabilized micelles that was reasonably simple, but there were characteristically unsolved issues about particle behavior in vivo. However, company management did not encourage taking a closer look. His strong impression was that the entire field of nanoparticle-based drug delivery is plagued by wishful thinking, sales pitch and shoddy biology. To paraphrase an old joke about advertising, it could be concluded that half of nanoparticle literature is rubbish and it is not even clear which half. In contrast, nature is already full of natural extracellular nanoparticles, some like exosomes, that can attack cancer or be bioengineered to really do so.

Perhaps one should not judge the potential of technology based limitations of unintelligent people looking for a miracle in their imagined artificial nano particles, the hope that they will see the light and turn to natural nano particles like exosomes or analogous also natural bacterial bio engineered OMV to be for far better physiological alternatives. In artificial nanomedicine, there is said to have been low quality of researchers, poor discussions, simplistic conclusions, fabrication of results to make them more appealing for newspapers and funders, vanity, ignorance, and outright hubris. There have been failures of the scientific community; particularly funding agencies, companies and scientific journals. Many of the papers on marvelous nanoparticles are simply pieces of low scientific value, low intellectual quality and very poor insight and seriousness that has created a field of ignorance that unfortunately impedes a proper development of a truly challenging and potentially very clinically useful discipline. Instead, the field should work to improve and make more specific exosomes as starting material derived from the great industry of nature's Darwin's factory of progressive improvements to perfection over three billion years.

The inadequacy of artificial nano particles has led to emergence of more complicated agents and that are engineered to interfere with host responses

The natural antagonism of host systems to artificial particles, poor delivery, lack of effectiveness, and potential toxicity of artificial nano particles has become evident. Realization of the insolubility of problems with artificial nano particles has caused abandonment of highly venture capital funded start-up companies that attempted to make effective artificial exosomes. Also, there has been dissolution of significant divisions set up by Big Pharma that were planned to accomplish the false goal of artificial delivery of RNAi [245],[246]. However, undaunted after thousands of discouraging papers and growth of never ending problems, there continues to be sought increasingly artificial engineered and chemically modified artificial nano vesicles aimed at substituting for natural biologic exosomes, as well as co-treatments to alter host responses interfering with the hoped for superior properties of the artificial nano particles. This undoubtedly is an example of "throwing good money after bad."

These recognized problems, have led to current proposals for further engineering of already engineered artificial particles that can be viewed as overengineering of artificiality. In view of the detailed meta analysis showing no significant penetration of solid tumors [240], much new over engineering is aimed particularly at vesicle penetration of solid cancers by developing multistage size-switching systems that could maintain relatively large initial sizes during blood circulation, but then switching to small particles once accumulating at tumor site for deep penetration and effective tumor distribution [264],[265]. Numerous stimuli are to be utilized to trigger particular aspects of the tumor

microenvironment like linking accompanying enzymes [266],[267] [268],[269] [270], and improved design [271], like co administration of the cancer vessel homing peptide iRGD with multistage responsive nanoparticles [272], or construction of pH-responsive nanocarriers for accommodating to in the highly acidic local environmental of highly metabolic cancers [273]. However, there is no proof that these complex alterations address the overall problems, since solid tumor penetration is the last of a series of steps in the host that produce incompatibility of the artificial nano vesicles.

A related approach of this persistent attempt to avoid of multiple host mechanisms, and remarkable avoidance of the advantages of natural exosomes, is to alter the local tumor microenvironment to cause greater receptivity to artificial therapeutic nano particles, treating with complex, multi-action nonsteroidal anti-inflammatory drugs [274]. However, such further engineering, like giving functional aspects [275] to already over engineered artificial particles, increases the foreignness and incompatibility problems of these constructs; by undoubtedly introducing new problems in a patient already being treated with multiple agents for cancer. More complex attempts that we consider misguided and wasteful are introduction of entirely new artificial nano-particles like superparamagnetic iron oxide particles [276]. Similarly, there is particle-linked light-induced apoptosis activatable agents for targeted cancer therapy [277]. The most way out suggestion is the introduction of Fullerene-based delivery nanoparticles [278]. However, alas, fullerene particles can induce inflammatory reactions themselves [279]; probably due to Ab and complement activations; indeed antibody responses to Fullerenes were documented 20 years ago [280].

At this point it is incredible how much work was done (about 60,000 total papers up to 2018) and billions of dollars spent in the face of the continued demonstration of the inadequacy of artificial nano particle therapies. I am amazed that there are several journals, and perhaps societies, dedicated to this moribund subject. *Finally, in this context, it was stunning to read a recent evaluation of the field that mentions with some great surprise that there actually are “natural liposomes that are called exosomes”.* This is an example of the biologic ignorance; especially about natural physiologic exosomes and of losing track of things when focused on the artificial among the investigators that are liposome centric. In reality, the liposomes were first developed to be better than, or at least as good as natural exosomes. Exosomes were readily dispensed by pharma as considered too complex and diverse; and were estimated to be difficult for passing the FDA. In reality, papers of the artificial field should be said to be about liposomes imitating exosomes [281],[282]. Agonal efforts to stick to liposomes have been attempts to fashion exosome-like liposomes composed of the unusual lipids

found in just one kind of exosomes, compared to standard liposomes. Experiments showed small differences favoring the hybrids [281].

In summary here, after 30 years, many billions of dollars and wasted careers of many promising talented scientists, the artificial nano particles have been a huge and complete failure. It seems prudent to now turn the considerable engineering skills and developed nano biotechniques from “beating a dead horse” to instead focus on engineering natural physiologic exosomes. This is beginning to happen, recognizing these extracellular vesicles as natural, safe and efficient delivery of drugs and RNAs to influence gene expression [283],[284],[285],[286],[287], and capable of being engineered without loss of their natural abilities [288],[289],[290],[291].

To start, exosomes have none of the generic problems of the artificial nano particles and will require lighter engineering to perform specific therapeutic tasks; certainly not needing alteration of poor receptivity of the host that they is the opposite for natural exosomes. Further, exosomes transferring miRNA mediated epigenetic functional alterations truly allows these physiologic nano particles to enter the world of precision medicine. This compares to the one-size-fits-all approach of artificial “Johnny One Note” engineered nanoparticles, in which treatment is for the average person, without consideration for the differences between individuals. Precision medicine applies more to exosomes because of their given advantageous inherent biologic variability and plasticity that is appropriate to, for instance the variability and plasticity of cancer and autoimmune diseases. This facilitates fashioning them to the individual patients and their specific variety of a disease. Further, it is said that no two patients with systemic lupus or allergic asthma are the same. This is true and emphasized by the mixed actions the usual drug therapies applied to these complex diseases, and certainly applies to cancers as well. This recommended nanomedicine treatment evolution from artificial to natural nano particles is a “no brainer”. However, some times investigators and administrators and investors can not give up on what they have been doing for years and can be reluctant to move to new things. For this, I point out the famous quote of Albert Einstein that “insanity (for here maybe irrationality), is doing the same thing over and over again and expecting a different result.

For nano vesicle therapies, natural physiologic exosomes or altered natural exosomes is the best way forward

Many investigators still are erroneously propelled to create artificial nano particles to replace exosomes, that we stress are naturally derived from progressive optimizing adjustments over billions of years of evolution. This weakness of artificiality is recognized as a false idea by many others who

have found that more meaningful results result from focused use of natural exosomes, or altered natural exosomes [292],[293],[294]. Finally, there is a vastly growing new field employing, so called exosome mimetic nano particles. These consist of exosome-like nanoparticles with outside membranes from various cell types like lymphocytes or platelets, and also even exosome membranes themselves [295]. These cleverly constructed more sophisticated artificial nano particles have been used for delivery of drugs in cancers [296],[297],[298],[299],[300] and for metastases [301], as well as RNAi in autoimmune colitis [302],[303].

Overall therefore, no artificial nanoparticles have been developed that have the natural essential valuable properties of exosomes; namely:

- 1). As natural, exosomes provide effective avoidance of uptake by the reticuloendothelial system [304],[305].
- 2). Natural ability uptake into targeted cells via exosome surface signatures of ligands that bind surface receptors on targeted acceptor cells [306],[307].
- 3). Therapeutic exosomes can have a long *in vivo* duration of action for days after systemic administration of a single physiological dose [91],[92].
- 4). Exosomes are able to resist the harsh stomach milieu of strong acidity plus digestive enzymes [91],[99],[100],[101] and importantly likely in phagolysosomes after cellular uptake. Additionally, resisting harsh tissue condition of hypoxia. Together, these enable exosome function in the combined acidic and hypoxic milieu of cancers in the tissues [133],[134],[136],[141].
- 5). Thus, there is little resulting renal excretion of nucleotides and amino acids from digested unprotected exosome RNAs and proteins, compared to the far greater *in vivo* lability to *in vivo* digestion of artificial nano particles.
- 6). Exosomes have a natural ability to cross the normal *unperturbed* blood brain vascular barrier [252],[253],[254], that enables systemic treatment of brain cancers and CNS inflammatory disorders [262],[263], and similarly also cross the blood cerebral fluid barrier [264].
- 7). This includes the revolutionary ability to administer exosomes via the nasal route, that then traffic

into the brain for the treatment of: neuro inflammatory conditions [263], spinal cord injury [265], prevention of brain damage from epilepsy [267]; interestingly via a nasal spray [267], and even attempts to treat a mouse model of autism [268].

8). Accordingly, a recent meta-analysis of all publications on artificial nano particles designed to treat cancers found there was ability to penetrate solid tumors in only 0.7% of instances; likely a nonspecific effect [240], where exosome resistance attributes mean that it is the opposite considering that they likely derive from ancient vesicles that arose from the noxious primordial seas near the origin of life.

9). Compared the many possible toxicities with artificial engineered nanoparticles, exosome treatments induce, few toxicities. Occasionally their toxic responses are due to bacterial contaminated exosome cultures in substandard laboratories with effects due to the organisms and their released LPS. Recently (December 6, 2019), there was an FDA Public Safety Notification and warning per specific local companies about making “Exosome Products” causing serious adverse events in Nebraska patients treated with unapproved products marketed as containing exosomes.

10). Exosomes have diverse biologic carrier properties beyond mere transfers of RNAs or proteins that are aided by these properties, and by definition cannot to be present in artificial nano particles. These consist of surface signatures for specific binding and subsequent targeted cell surface activation, then on to postulated generic carrier intracellular biologic effects influencing subsequent RNA intracellular delivery and resulting alterations in the function of the acceptor cells.

11). Additionally, there are further carrier effects on intracellular pathways postulated to specifically augment biologic actions of minute amounts of transferred RNAs. In two instances testing proper dose responses, mere nano moles [91] of a given miRNA and even femtomoles [40] were transferred by exosomes as the limiting dilution doses that were able to mediate in vivo functional biologic effects.

12). Importantly questioned, but probably an essential property of exosomes is their great variability and the likely huge number of exosome subsets [270]. This variability is firstly due to a myriad of surface phenotype combinations and other descriptive properties like size and secondly the great diversity of carried agents transferred that actually is a positive attribute. This is not possible with artificial nano particles that are purposely engineered to identically transfer a single substance to

produce a selected given effect; perhaps to more easily meet FDA standards than to achieve imitation of normal physiology. For artificial nano particles this essentially is the creation of a single note played by one instrument in what is in reality is a complex orchestra of effects; whereas exosomes are much more than a bag containing a single agent.

There has been notable early progress regarding therapeutic use of native natural exosomes. These span eleven areas:

- 1). Unique dual antigen *and* gene specificity of immune T cell [40],[78];[79] or B cell [40],[90],[91],[92]-derived suppressive exosomes. These “activated exosome” subsets are easily coated with Ab free light chains to achieve chosen exquisite Ag-specific specificity for binding Ag on the surface of particular acceptor cells, and seem to be the special subset that can associate with selected chosen functional miRNA, resulting in unprecedented dual antigen specific transfers of particular functional genetic modifications [40],[90],[91],[92].,
- 2). Demonstration that systemic [256],[273] or even local nasal [268],[274],[275] administration enables exosomes loaded with anti-inflammatory molecules to easily cross the blood brain barrier to act in the central nervous system to inhibit several models of local inflammatory diseases.
- 3). Ease of RNA loading of exosome by multiple means [276] and demonstration that mere gentle sonication allows for a large increase in loading of an anti-cancer drug [277].
- 4). Ability to be genetically induced for surface expression of ligands like those for growth factors [273],[278].[279], enabling targeting of cancer cells dependent on such factors, or
- 5). Applying specific surface peptides to exosomes enabling binding to specific organ receptors to deliver chosen siRNAs [256],[273],[280],[281] and can also,
- 6). Deliver synthetic DNAs [282],[283],[284], and now most interestingly deliver CRISPR/Cas9 [285],[286],
- 7). *Ex vivo* generation of dendritic cells exposed to whole antigens or dendritic cell-derived exosomes pulsed with specific Ag peptides enables development of specific peptide/MHC surface complexes to

generate exosomes that are nano-mini-APC to act as strong vaccines [287],[288] that are useful against cancer [289],[290] or infections [291],[292].

8). There is a reduced tumor burden by delivery of chosen miRNA-modified exosomes in cancers [293],[294], and there are several unique therapeutic approaches to cancer using modified exosomes [295]

9). Exosomes are easily constructed for therapy of genetic abnormalities [296], like in Huntington's disease [297], and therapy of autoimmune models [298], like arthritis [299],[300] for which exosomes are treated *ex vivo* to carry relevant inhibitory cytokines [301],[302], or express surface ligands altering immune responses [303],[304],[305],[306].

10). Finally, there is a vast literature indicting the treatment efficacy and safety of MSC, recently realized to be due to their released exosomes, as we have shown in spinal cord injury where effects were due to these exosomes targeting healing M2-type macrophages [186].

Potential problems of exosome therapeutics.

The unusual biological nature of exosomes makes it possible to produce general therapy due to genetic mismatch between human donors and patients. There also appears to be toleration of exosome therapy from different species like in cows' milk, and even across kingdoms; i.e. from plants. If there is a need for personal autologous nano vesicles, these can be obtained from syngeneic EBV B cell lines, since nearly all people are infected and immortal autologous EBV lines are easy to generate in vitro to produce of autologous exosomes. There are many other RNAs and proteins in the exosomes that are theoretically capable of mediating off target effects, although this has not been a problem in any system studied thus far. The difficulties of host detection and elimination of the artificial exosome-like liposomes via the RES does not apply. Firstly, the mechanisms of the Ab surface binding and miRNA association have to be determined. Further, if recurrent pulsed therapy is needed, our recent discovery of *effective oral therapy* can be employed [91],[92].

CONCLUSIONS

Exosomes are natural physiological nano vesicles with functions across all species and likely involvement in all intercellular interactions, that principally transfer of non coding RNAs to influence

specifically targeted cells by altering their DNA expression epigenetically for change in cell functions. As likely derived from antecedent ancient vesicles arising near the origin of life in the primordial seas they have great resistance to various of noxious environments like the acid/enzymes of the stomach, in intracellular phagolysosomes, and also the hypoxic microenvironment of cancers or other of necrotic tissues. This seems due in part to the unusual membrane lipid composition of an exosome subset that allows binding of Ab light chains and association with functional miRNAs.

Therapy with exosomes as intracellular organelles has expected complexity. This is viewed as an advantage since there are many ways to profitably use native natural exosomes and their targeted pathways to achieve modifications that can optimize functional effects of targeted cells, that notably have so far yielded quite amazing results. Treating with homogenous artificial engineered nanoparticles is old thinking from prior concepts developed for therapies based on thinking of exosomes as drugs when they are complex biologics more like cells, but really are intracellular organelles. As such they are therapeutically more like corticosteroids with multiple actions compared to more focused specific histamine receptor antagonists with a single action. Thus, for native exosomes, their complexity actually is a natural therapeutic advantage. Exosome treatments promise an easily achieved far greater therapeutic index compared to the myriad of problems faced by artificial nanoparticles; that includes new toxicities, biologic insufficiencies and general ineffectiveness; some seemingly insoluble.

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Conflicts of Interest

None

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