Reviewer 2: Minor revision

Dear Reviewer:

Thank you for your revisions and suggestions which add value to the manuscript. Please find below line by line answers to your queries. In the revised document, your revisions are highlighted in BLUE for your convenience.

This mini-review is on the whole a well-formulated contribution of current interest. There are some aspects that should be considered by the author, they are:

- 1. Historical background of exosomes. Historical aspects are often a ticklish task to deal with. There is no international consensus about the definition (and nomenclature) of extracellular vesicles (EVs) among which exosomes are prominent. Also, a majority of reports, so far, on exosomes are carried out on exosomes delivered by various cells grown in vitro while only a minority describes exosomes isolated from physiological media (body fluids). Hence, as given in a review by Marian Aalberts et al, Reproduction, 147 (2014)1-14, EVs (exosomes) in seminal plasma and prostatic fluid were first reported in the 1970s. Subsequently these vesicles were found to be exocytosed by the epithelial cells of the prostate gland and therefore formally could be denoted exosomes (at that time the generality of vesicle release from cells was not known) and the vesicles were denoted “Prostasomes” as given in the first review of 1985 on prostasomes/exosomes (Biochim Biophys Acta 822 (1985) 203-218). Another recognizing characteristic of exosomes is their unprecedentedly high membrane cholesterol/phospholipid ratio yielding high molecular ordering (Biochim Biophys Acta 984 (1989) 167-173).

Answer: Page 4, line 3 under Exosomes biogenesis in cancer. References # 19-22 added.

Historically however in the late 70s, extracellular vesicles have been isolated from physiological media such as seminal plasma and prostatic fluid, hence called “prostasomes” after the organ of origin ie. prostate (Aalberts, Ronquist 1977). Prostasomes have been studied for their dual role in normal reproduction as well as malignant prostate growth (Ronquist 2004, Sahlen 2004).

- 2. The author discusses meritoriously the role of various forms of RNA in exosomes but should mention Argonaute proteins, which are known to bind miRNAs and transport them to recipient cells, e.g. vesicles in blood plasma, associated with Argonaute2, the key effector protein of a miRNA-mediated gene silencing mechanism (Proc Natl Acad Sci U S A 108 (2011)5003-5007).

Answer: Page 11, bottom of the page. Reference # 83-86, 33 added.

However, Argonaute2 (Ago2) complexes carry a population of circulating miRs independent of exosomes in human plasma and the circulating Ago2 complexes have been suggested as another mechanism responsible for the stability of plasma miRs. This information is important for the development of biomarker approaches based on analysis of circulating miRs. (Arroyo 2011).

A consensus needs to be reach on dependable isolation methods for exosome biomarker research (Van der Meel). Reproducible protocols that obtain the purest exosome fractions for downstream RNA profiling with lack of contaminating Ago2-complexes would meet the standards of clinical care (Van Deun, Mestagh, Kalra 2013).
3. The author should also briefly discuss the role of exosomes in multidrug (chemotherapy) resistance (MDR). One of the mechanisms leading to the MDR phenotype is the activation of efflux proteins belonging to the ATP-binding cassette (ABC) transporter superfamily. P-glycoprotein and the multidrug resistant protein 1 (MRP 1) are members of this family with a putative role in cancer chemoresistance. There is an interesting recent report on MRP 1 localization in lipid raft domains and prostasomes in prostate cancer cell lines (OncoTargets and Therapy 7 (2014) 2215-2225).

Answer:
Page 12, first line under ExoDrug

Ideal cancer therapeutics should exhibit interference with tumor growth and invasiveness as well as circumvene multidrug resistance (MDR) in order to obtain remission and effectiveness in cancer immune surveillance to prevent relapse.

Page 13, line 6. Reference # 94,95 added.

One of the main causes of disease relapse in cancer patients is phenotypic changes in the tumor that results in multidrug resistance (MRD). Exosome-mediated communication of drug resistance among tumor cells and between tumor cells and microenvironment has been suggested as a mechanism of MRD (Corcoran 2012, Wang 2014) and exosomal blockages of drug resistance transfer could result in improved disease-free survival.

Minor points:

- Page 4, exosomes biogenesis in cancer. The second sentence (lines 3-5) is unclear to me. The first formation results in a so called “early endosome” (by invagination of the plasma membrane) and the second inward budding (on multiple loci of the endosomal membrane) gives rise to the intraluminal vesicles (forming the multivesicular body, MVB) that, after fusion between the membrane surrounding the MVB and the plasma membrane, are released as exosomes extracellularly.

Answer: Changes made page 4, line 7 under Exosomes biogenesis in cancer

Exosome biogenesis is a two step process with formation of endosomes by invagination of the cell membrane with trapping of extracellular material intraluminally. Consequently, a second inward budding with trapping of a portion of the cell’s cytoplasm on multiple loci of the endosomal membrane, gives rise to intraluminal vesicles (forming the multivesicular body, MVB) that, after fusion between the membrane surrounding the MVB and the cell surface plasma membrane, are released as exosomes extracellularly.

- The distinction between the findings of the Nobel laureates and the exosomes is not clearly expressed. These laureates studied the mechanism of release of solitary (free) molecules (extracellularly, either in an endocrinological system or a neurotransmitter system) confined within intracellular membrane-surrounded secretory granules (cf.: Douglas´ work in the early 1970s) and how the granules reached the plasma membrane and the fusion event between the two membranes resulting in the secretion of the granules´ molecular content (hormones or neurotransmitters). In case of exosomes, membrane surrounded vesicles were released
extracellularly to reach (with their cargo) the target cells for delivery of a complex assembly of bioactive molecules.

Answer: The reviewers’ point is accurate and a good observation. Rothman emphasizes the principle of membrane fusion in the cell in his Nobel Lecture (Ref#19). Membrane fusion is what governs exosome formation and activity as well.

- Page 5, first line. The word “shedding” is not properly used in this context. Here it is understood “from the plasma membrane” meaning the formation of so called “ectosomes” that should be distinguished from exosomes.

Answer: Change made: page 5, line 7

- Page 9, next last line. What is onco-exosomes? Does the author mean “oncosomes”?

Answer: Page 10, line 10 from bottom. Reference # 73 added.

The oncogenic cargo of cancer-related exosomes contains bioactive molecules including DNA, mRNA and miRs (hence these cancer-related exosomes are often referred to as oncosomes) (Principe). Noncoding transcripts such as miRs are part of the exosome repertoire by which oncosomes assist…