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Brain Tumor Exosomes and Microvesicles: Pleiotropic Effects from Tiny Cellular Surrogates

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1. Introduction

Extracellular signaling is a necessity for tissue and organ system development, maintenance, adaptation and survival. From the biologic unit of the cell to the complete organizational structure that is the organism, cell-cell, cell-tissue, tissue-organ, and organ-system communication are essential to keep the organism functioning, homeostatic, and proliferative. Cell-derived extracellular vesicles such as exosomes and microvesicles are vital players in these forms of proximal and distal messaging, acting as discrete packets of information capable of altering recipient cell phenotypes, activities, and responses. These vesicles are part of the normal biologic repertoire of the organism, but are particularly exploited by neoplastic growths and cancers to change both the local and systemic environments to aid in tumor proliferation, invasion, and metastases, and in the defense of the tumor. This chapter will focus on exosomes and microvesicles from brain tumors, but similar stories may be told about such vesicles from almost any tumor type. Following this general introduction on exosomes and microvesicles we will discuss the experimentally determined and putative roles of exosomes in brain tumor biology, particularly in stress responses, metabolism, migration, immunology, and protection against chemotherapeutics.

2. Exosome/Microvesicle biosynthesis simplified

The interactions between cell surface components, extracellular matrix molecules, proteases and protease inhibitors, and “soluble” factors is complex (Weber, Bjerke, and Desimone ; Rozario and DeSimone ; Chirco et al. 2006; Gonzalo et al. ; Kessenbrock, Plaks, and Werb ; Stetler-Stevenson 2008; Hyytiainen, Penttinen, and Keski-Oja 2004; Tran, Griffith, and Wells 2004; Goldman 2004). However, the distinction between truly soluble factors and technically particulate forms of those factors becomes blurred in terms of exosomes and microvesicles (Peinado, Lavotshkin, and Lyden ; Camussi et al.). Exosomes and microvesicles are cell-derived, bilayer membrane-enclosed nanovesicles ranging from virus-sized entities (eg, exosomes, 30-100 nm diameter) to small bacteria-sized (eg, microvesicles, ~150 nm- 3000 nm

diameter) (Johnstone 2006) – ie, “fat balls”. These vesicles exist essentially anywhere there is a cell-liquid interface such as in blood, urine, semen, breast milk, bronchoalveolar lavage fluid, synovial capsule fluid, saliva, ascites, amniotic fluid, cerebrospinal fluid, and *in vitro* spent culture media (Simpson et al. 2009). The obtuse vesicular nomenclature and lack of molecular definitions for these microparticles generates confusion (Mathivanan, Ji, and Simpson); in this chapter, for these cell-derived vesicles we will refer to exosomes as extracellularly-released components of the endocytic pathway, while microvesicles will refer to “shed” vesicles released directly from cell-surface membrane budding (Figure 1). Please note that in this work “exosome” does not refer to the intracellular multi-subunit RNA processing complex (Mitchell et al. 1997).

Due to the directional nature of the biogenesis of the two forms of vesicle formation, exosomes and microvesicles largely retain the original topography of the cell itself, making these vesicles akin to tiny cellular surrogates (Figure 2). The contents include selected membrane lipids such as gangliosides GM1 and GM3, sphingomyelin, lyso-*bis*-phosphatidic acid/*bis*-monoacylglycerol phosphate (LBPA/BMP), and cholesterol. Protein contents are also strikingly conserved across exosomes and microvesicles from various cell types, including protein components of lipid raft membrane structures, tetraspanins, numerous metabolic enzymes, heat shock proteins, structural proteins, and nucleic acid binding proteins (Simpson, Jensen, and Lim 2008). Appropriately, messenger and microRNAs are also present in exosomes (Taylor and Gercel-Taylor 2008; Valadi et al. 2007) including those from high grade gliomas (Skog et al. 2008). Despite the similarities amongst the vesicles,

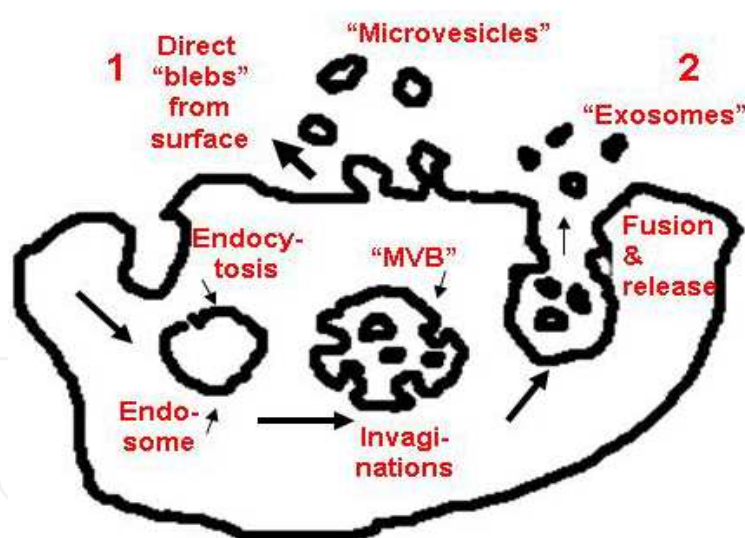


Fig. 1. Highly diagrammatic representation of the release of extracellular vesicles from a cell. Two basic mechanisms are depicted: 1) shedding or “blebbing” of vesicles directly from cell membrane surface, yielding microvesicles (sometimes called microparticles or “ectosomes”); 2) following the endocytic pathway, endosomal compartments develop invaginations that bud off into the endosomal lumen as small vesicles. The endosome is now called a multivesicular body (MVB). At this stage, the MVB may fuse with the lysosome for degradation and recycling of its contents. However, if the MVB fuses with the cell membrane, the vesicles within it are exocytically released as exosomes.

they retain sufficient cell-specific material to frequently allow identification of the cell type source of origin (Denzer et al. 2000; Bobryshev et al. 2001; Blanchard et al. 2002; Graner et al. 2009; Skog et al. 2008). Given that these durable vesicles, when released by tumors, circulate in blood and are found at particularly high levels in patients with cancer (Taylor and Gercel-Taylor 2008), exosomes and microvesicles are considered potential sources of biomarkers obtainable with minimally invasive procedures (Duijvesz et al. ; Krutovskikh and Herceg ; Simpson et al. 2009), including from urine (Dimov, Jankovic Velickovic, and Stefanovic 2009; Pisitkun, Johnstone, and Knepper 2006).

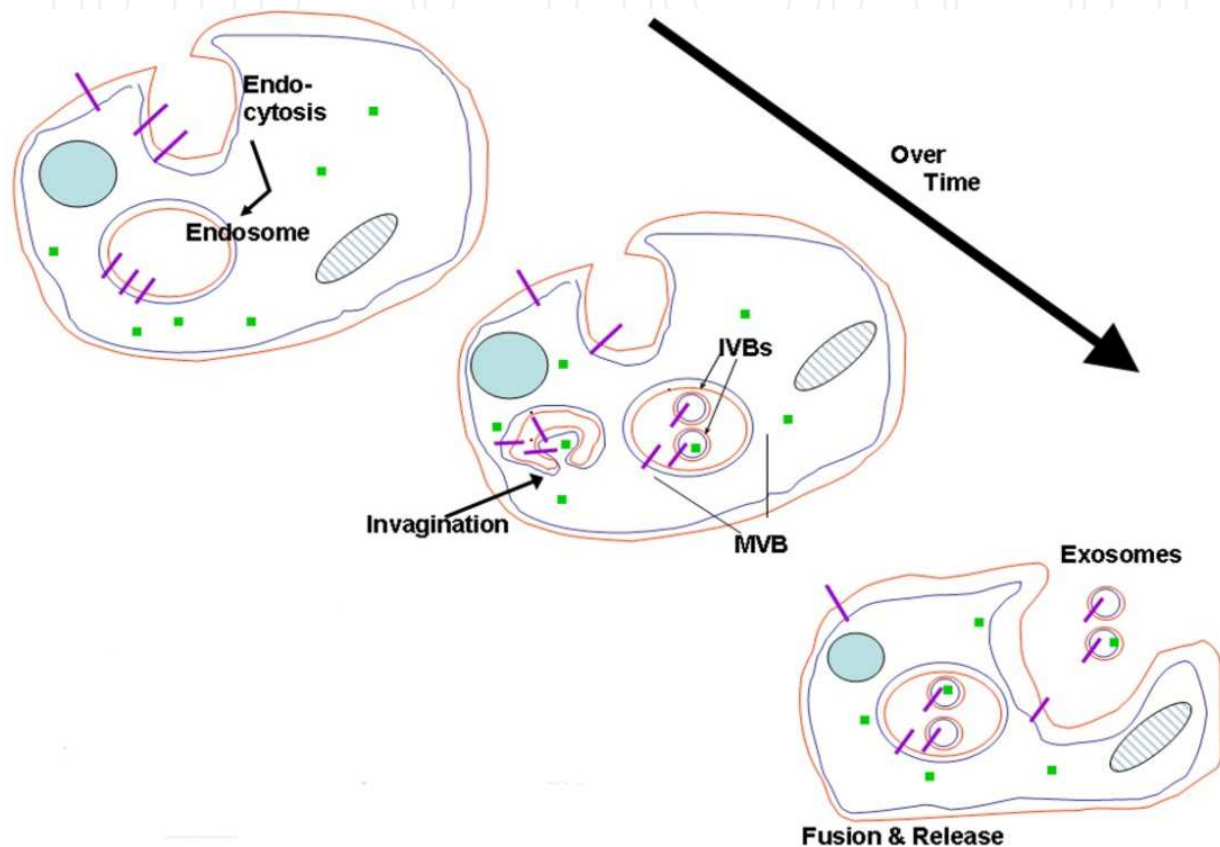


Fig. 2. The topography of exosomes mimics that of the cell of origin. Depicted is a highly diagrammatic cartoon of exosome formation showing the endocytosis of transmembrane proteins (purple lines) in the context of the outer lipid layer of the plasma membrane (shown in red) and the inner lipid layer facing the cytosol (shown in blue). During endosome formation, the cytoplasmic portion of the membrane protein remains in the cytosol; as the endosomes fuse to become more mature structures, invaginations occur, resulting in the pinching off of small vesicles into the luminal space of the endosome. These smaller vesicles may be referred to as intravesicular bodies ("IVBs") while the whole structure is denoted as a multi-vesicular body ("MVB"). The former intracellular domain of the transmembrane protein is now sequestered away from the cytosol; cytoplasmic contents also enter the IVB during formation (green dots). The typical fate of the MVB is fusion with a lysosome for content degradation and recycling. However, if the MVB instead fuses with the plasma membrane, the IVBs will be release extracellularly, and are now called "exosomes". Note that the membrane topography (red/blue positioning) and transmembrane protein orientation are the same as that of the original cell.

The mechanisms driving vesicle formation (at the level of invagination into the endosomal lumen and in the direct “blebbing” from the cell surface) have many of the same driving components, particularly the ESCRT (Endosomal Sorting Complex Required for Transport) machinery including ALIX/AIP1 (de Gassart et al. 2004) and PI3 kinase and related molecules (Stoorvogel et al. 2002). Lipid composition is also a key factor, with structure-altering, membrane-bending lipids playing key roles in the curvature-induced vesicular invaginations (Trajkovic et al. 2008; Subra et al. 2007; Laulagnier et al. 2005). The acidic pH of the endosomal lumen also enhances lipid bending away from the limiting membrane (Subra et al. 2007) and the low pH of the tumor microenvironment likely has similar effects on microvesicle budding and extracellular activity of the vesicles (Giusti et al. 2008), including exosomes (Parolini et al. 2009). As lipid moieties such as the phosphoinositides/phosphoinositol phosphates (PIPs) and proteins such as the Rab proteins are important in intracellular vesicular identification and trafficking (Perret et al. 2005), it is not surprising that these entities also play significant roles in extracellular vesicle formation (Ostrowski et al. ; Hsu et al.) Various membrane-fusion proteins of the SNARE families are also likely involved (Thery, Zitvogel, and Amigorena 2002). Thus, the intra- and extracellular environments, various lipid players, and numerous proteins involved in intracellular vesicle identity and function all play roles in the formation, intracellular targeting, and possibly extracellular targeting, of exosomes and microvesicles.

2.1 Basics of exosome/microvesicle cargo loading: filling up the fat balls

The sorting process/cargo loading of exosomes and microvesicles is still largely a mystery; whether certain proteins are destined or chosen to become vesicle content or are “trapped” into them is not clear. However, some fundamental principles seem to hold. One of the most common protein post-translational modifications that results in a vesicular destination is ubiquitination, frequently from E3 ubiquitin ligase activities; this may be regulated to some extent by deubiquitination complexes such as signalosomes (Liu et al. 2009). Curiously, cells that are the recipients of exosomes may also activate ubiquitin ligases as part of the downstream effects of extracellular vesicle interactions (Qu et al. 2009). There are also likely ubiquitination-independent mechanisms for vesicular assimilation of proteins as well (Gauvreau et al. 2009); these may include potentially ESCRT- or Rab-driven incorporation of proteins into a “frustrated” recycling pathway where the proteins are neither returned to the cell surface nor directed toward the lysosome (de Gassart et al. 2004). Curiously, lipid sorting into such compartments is a function of the length and/or saturation of their alkyl tails (Mukherjee, Soe, and Maxfield 1999). LBPA, an unconventional negatively-charged phospholipid, is also sequestered to late endosomes/MVBs with a potential structural role (Matsuo et al. 2004). Since LBPA is mostly enriched in internal membranes, whether it plays a role in plasma membrane dynamics is unclear. Membrane partitioning such as lipid raft formation is also a key player in exosome and microvesicle biogenesis and sorting (de Gassart et al. 2003; Calzolari et al. 2006; Staubach, Razawi, and Hanisch 2009; Del Conde et al. 2005). Proteins contained within those lipid rafts are thus sorted to exosomes and microvesicles and include GPI-linked proteins such as acetylcholinesterase (Graner et al. 2009), the NKG2D ligands MICA and MICB (Hedlund et al. 2009), Gce1 and CD73 (Muller et al. 2009), and CD55 and CD59 (Rabesandratana et al. 1998), etc. As mentioned above, the gangliosides GM1 and GM3 are also present in lipid membrane microdomains (Janich and Corbeil 2007) and in exosomes (de Gassart et al. 2003; Parolini et al. 2009).

Membrane proteins that are not necessarily typical constituents of lipid raft domains may shuttle into exosomes or membrane patches of microvesicles by aggregation, such as the transferrin receptor (de Gassart et al. 2004) or by “higher-order oligomerization” induced by antibody cross-linking at the cell surface, or by artificially acylation tagging of cytosolic proteins resulting in oligomerization and trafficking to released microvesicles (Fang et al. 2007). Further studies have shown that a variety of membrane anchors can target proteins to exosomes and microvesicles, but not all with the same efficiency (Shen et al.). A number of other membrane proteins are typically found in exosomes and microvesicles and are hallmarks of them. These include members of the tetraspanin network such as CD9, CD9P-1 TSPAN8, CD63, CD81, and CO-029 (Abache et al. 2007; Gesierich et al. 2006; Pols and Klumperman 2009; Rana et al.).

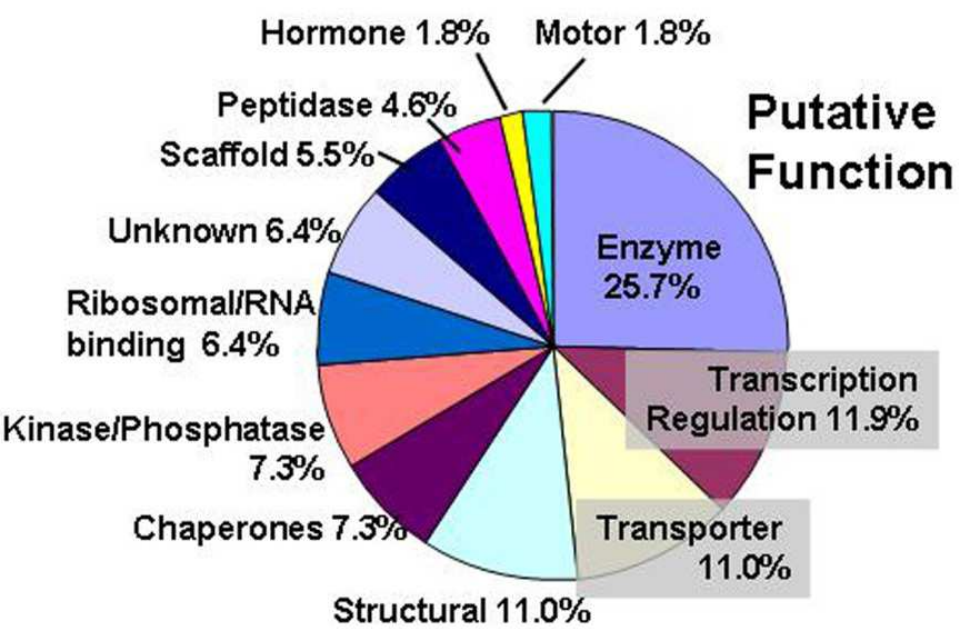


Fig. 3. Functional analysis of the proteomic content of exosomes/microvesicles derived from D283MED medulloblastoma cells. Over 130 proteins were identified by extraction of proteins from vesicles, separation of the proteins on SDS-PAGE, excision of the bands from BioSafe Coomassie Blue-stained gels, and tryptic digest of the excised bands. Peptides were separated and analyzed by LC-MS/MS, and peptides were mapped or identified by de novo sequencing using PROWL and Sequest algorithms. Proteins were categorized with Ingenuity Pathway Analysis software and by literature searches focusing on known functions; please note that many of the proteins identified were multifunctional, and this categorization represents the preponderance of evidence for function based on present literature.

Many cytosolic proteins (or at least many that are not widely recognized to have membrane associations) are exosome components; these include members of the heat shock protein (HSP) family, HSP/HSC70 and HSP90 in particular, and numerous metabolic enzymes. We have identified these proteins previously in proteomic studies of murine brain tumor exosomes and have cataloged and categorized them (Graner et al. 2009). We have performed further proteomic studies on human brain tumor exosomes (from the medulloblastoma cell

line D283MED) which confirm and extend the results of the murine studies. In particular, metabolic enzymes represent the largest class of exosome proteins (**Figure 3**). While their prevalence may reflect their cellular abundance, there is a clear enrichment of these proteins in exosomes/microvesicles.

2.2 Functions and activities of tumor exosomes/microvesicles: fun with fat balls

Originally regarded as a means of cellular content disposal, particularly if cells lack lysosome activity (Johnstone et al. 1987; Johnstone, Bianchini, and Teng 1989) (ie, “garbage bags”), exosomes and microvesicles have an extensive and increasing list of activities, roles, and functions locally and systemically when released by tumors into the extracellular environment. Here we will briefly overview some of those better-characterized activities, most of which are regarded as beneficial to the tumor in terms of growth, invasion, or protection.

2.2.1 Immunologic effects: floating fat balls as stimulators or suppressants?

Exosome/microvesicle studies in tumors were originally driven by the idea that as tiny, non-replicating cellular surrogates, these vesicles could be used as cell-free anti-cancer vaccines. The premise was that exosomes and microvesicles contained tumor-specific antigens (Andre et al. 2002; Cho et al. 2005) as a source of adaptive immune cell targeting. In addition, exosomes/microvesicles contain heat shock proteins, known “danger signals” that both carry peptide antigens themselves as well as provide a potent, adjuvant-like stimulus to the innate and adaptive immune systems, particularly at the level of antigen presenting cells (Graner and Bigner 2005, 2006).

2.2.1.1 Dendritic Cell Exosomes (“DEX”)

The initial works using exosome-based vaccines utilized exosomes derived from antigen-pulsed dendritic cells (DCs). DCs are perhaps the most effective “professional” antigen presenting cells known (Banchereau et al. 2000), and like all immune cells, one important means of extracellular communication for them is via the release of exosomes and microvesicles (Chaput et al. 2006). DCs process and present antigens to CD4+ and CD8+ T cells (“helper” cells and “cytotoxic or killer”/cytotoxic T lymphocyte/CTL cells, respectively), and if appropriately activated, will prime and stimulate the T cells for enhanced immune activities. From a clinical perspective, antigen-loaded and *ex vivo* activated DCs have been used in immunotherapy protocols as anti-cancer vaccines for a wide variety of tumor types and using an extraordinary range of antigen sources that are “pulsed” (applied to) the DCs (Palucka et al.). In general, *in vitro* (or *ex vivo*) DC production protocols are not standardized, require a great deal of hands-on time and effort, and the resulting cell preparations can be difficult to freeze and recover from thawing for repeated patient treatment (de Vries et al. 2005; Kalinski et al. 2009; Simon, Fonteneau, and Gregoire 2009). Exosomes and microvesicles from DCs (sometimes called “dexosomes” or “DEX”) can be harvested from the culture supernatants, stored frozen, and thawed without loss of stimulating activity. They can be injected into hosts as cell-free DC-type vaccines capable of stimulating anti-tumor responses. These DEX were the first exosome-based vaccines (Zitvogel et al. 1998); DEX are capable of transferring antigen-loaded major histocompatibility class I molecules (MHC I) to recipient DCs for priming of naïve T cells in the host (Andre et al. 2004). These DC-derived vesicles were produced in a large-scale GMP setting (Lamparski et al. 2002) and were used to treat patients with metastatic melanoma in

a clinical trial; the vaccines were safe, feasible, and produced one partial response with signs of specific immunity (Escudier et al. 2005). Another trial for patients with non-small cell lung cancer achieved similar results with some patients maintaining stable disease (Morse et al. 2005). As is the case for most immunotherapies, more work is needed on disabling the tumor-induced immune suppression and perhaps choosing patients with less tumor burden and lower stage, less advanced cancers to improve responses and outcomes. Nonetheless, the safety and feasibility of DEX as clinically-usable entities is evident; further advances in safe and tolerable immune stimulation, and the means to “suppress the suppression” of immunity driven by tumors are desperately needed.

2.2.1.2 Tumor-derived Exosomes (“TEX”)

Tumor-derived exosomes (sometimes call “TEX”) also have a history as vaccine material; as mentioned above, their antigen content and HSP-based adjuvant effects theoretically make them ideal cancer vaccines. This has proven true in a number of animal models of cancer (Altieri, Khan, and Tomasi 2004; Dai et al. 2005; Chen, Wang et al. 2006; Bu et al. 2006; Cho et al. 2009; Graner et al. 2009). One clinical trial performed in China using autologous tumor-derived (from ascites fluid of colorectal cancer patients) combined (or not) with the immune stimulant GM-CSF has been reported. One patient treated with both TEX and GM-CSF had stable disease, and another in that group had a partial response. In immune assays, delayed-type hypersensitivity (DTH) reactions were seen in patients immunized above a threshold amount, and selected patients had positive CTL assays (tetramer staining, cytotoxicity, interferon-gamma (IFN γ) release). No serious adverse events were reported (Dai et al. 2008). This trial seemed to bear out results from animal models. However, much of our current thought regarding TEX immunology is dominated by the pervading opinion that tumor-derived exosomes are purveyors of immune suppression thru increased regulatory T cell (Treg) activity, myeloid-derived suppressor cell activity, and suppression of activated T cells, natural killer (NK) cells, and inhibiting DC maturation (Taylor and Gercel-Taylor 2005; Liu et al. 2006; Valenti et al. 2007; Ichim et al. 2008; Xiang et al. 2009; Szajnik et al.). There is also evidence that TEX can prevent the binding of anti-tumor antibodies to their tumor-expressed target by essentially titrating out the antibodies, thus preventing antibody-dependent cellular cytotoxicity (ADCC) (Battke et al.). While a few of these studies have been performed in *in vivo* settings, most of the data are from human cells in *in vitro* assays. Thus, the complex interplay of *in vivo* and systemic immunity and immune suppression may be poorly represented in these assays, and our results in this area will be discussed later.

2.2.2 Coagulation and thrombosis: fat balls and clotting

Microvesicles, especially those derived from platelets, have long been known as procoagulant players in clot formation, particularly when the vesicles carry tissue factor (TF) (Freyssinet and Toti). There is an increasing awareness that microvesicles may also have roles in some of the thrombotic events that can occur in cancer (Aharon and Brenner 2009; Castellana, Kunzelmann, and Freyssinet 2009) indicating an interplay between microvesicles from other “normal” cell types such as platelets reacting with cancer cells. However, tumor-derived exosomes and microvesicles are also likely involved in this cellular cross-talk as well (Milsom et al. 2008), with the potential for coagulation to aid in delivery of growth factors or other stimuli to tumor cells. Tumor-derived microvesicles are believed to be partly responsible for Trousseau’s syndrome, a coagulopathy associated with the cancerous state

(Varki 2007); the involvement of tumor vesicles likely involves the presence of TF on their surfaces (Del Conde et al. 2007). These studies further indicate the systemic roles that tumor cell-derived vesicles may play in cancer growth, progression, and generation of other co-morbidities in cancer patients.

2.2.3 Signaling capacity: influential fat balls

Exosomes and microvesicles are carriers of protein and lipid molecules with direct and indirect signaling capacities and complexes (Record et al. ; Zumaquero et al.) including members of the EGFR family (Sanderson et al. 2008) including Her2/Neu/ErbB2 (EGFR2) (Ciravolo et al.) and the tumor-specific mutant EGFRvIII (Graner et al. 2009; Al-Nedawi et al. 2008). Importantly, both EGFR and EGFRvIII may actually pass from exosomes to recipient cells, leading to activation of those pathways with phenotypic changes induced in the recipient cells (Al-Nedawi et al. 2009). In addition, tumor exosomes and microvesicles also carry active EGFR ligands such as amphiregulin (Higginbotham et al.), thus indicating that the vesicles can carry not only the signal transducer, but the signal as well.

Much of the signaling in cancer cells is devoted to proliferative drive. Exosomes and microvesicles are known to drive proliferation of tumor cells that are exposed to the vesicles (Koga et al. 2005; Skog et al. 2008); we have seen similar proliferation when pediatric and adult brain tumor cells are exposed to their endogenous exosomes (data not shown). Exosome-driven proliferation has been associated with the presence of the anti-apoptotic protein survivin in tumor exosomes (Khan et al.), and in a potential recruitment phenomenon, tumor microvesicles can enhance endothelial cell proliferation (Hong et al. 2009) which presumably may promote tumor vascularization. The mechanisms behind these proliferative responses are not clear, although we have evidence that exosomes increase overall glycolytic metabolism of recipient cells (see below) which may be either a cause for cell growth or a means to abet it. To further complicate the issue, there is at least one report in the literature of tumor exosomes/microvesicles inducing apoptosis in the recipient tumor cells (Ristorcelli et al. 2008), so it is possible that exosome effects may be cell-type or cell context specific.

2.2.4 RNA transport: fat balls as diplomatic satchels

As noted above (Figure 3) exosome/microvesicle proteomics revealed a relatively high percentage of RNA binding proteins typically present in exosomes. Since the biogenesis of exosomes and microvesicles intersects with some pathways of intercellular transmission of viruses, particularly at the levels of endosome (de Gassart et al. 2004; Mercer, Schelhaas, and Helenius) (also, see below), researchers began asking if exosomes and microvesicles themselves might have actual viral qualities, such as the ability to transport and transfer RNA molecules to recipient cells. This was first answered by Valadi et al (Valadi et al. 2007) when they demonstrated that exosomes did indeed possess both messenger and micro RNAs (mRNAs, miRNAs). Encapsulated in the vesicles, these RNAs were highly resistant to degradation by RNases; furthermore, they demonstrated that mRNAs from one cell type could be passaged to another cell type, even of a different species (ie, mouse to human) resulting in translation of at least some of those messages (ie, humans cells now making mouse proteins). The numbers of mRNAs in exosomes/microvesicles and the genes represented by them are impressive (Mathivanan, Ji, and Simpson). One study involving brain tumor exosomes/microvesicles found that essentially the entire mRNA “expressome” is present in the population of collected vesicles, but there was significant enrichment for

certain mRNAs in exosomes/microvesicles that did not mirror the mRNA expression distributions from cells of origin (Skog et al. 2008). This type of horizontal transmission of epigenetic materials exists with normal cell types (Ogawa et al.) and even with viable tissues in culture (Aliotta et al.) as well as with tumor cells communicating with normal cells (Hong et al. 2009).

microRNAs are recently-discovered small (22-25 nucleotide) non-coding RNAs that have potent regulatory potential (Carrington and Ambros 2003). miRNAs act as silencing RNAs, binding in an antisense fashion to regions of the 3' untranslated regions (3' UTRs) of mRNAs, resulting in either destabilization and degradation of the message, or prevention of translation; the net effect is translational repression of the message (Nelson et al. 2003). Following the discovery of miRNAs in exosomes (Valadi et al. 2007) there has been an explosion in the study of miRNAs in exosomes/microvesicles (for example, (Taylor and Gercel-Taylor 2008; Hunter et al. 2008; Rosell, Wei, and Taron 2009; Luo et al. 2009; Kosaka et al. ; Wang et al.)). There has been a particular emphasis on using circulating miRNAs (ie, those present in patient blood/serum) as disease biomarkers, presumably in the form of circulating exosomes/microvesicles (Rabinowits et al. 2009; Michael et al. ; De Smaele, Ferretti, and Gulino ; Kosaka, Iguchi, and Ochiya ; Wittmann and Jack ; Corcoran et al.). Thus, messenger and micro RNAs, when delivered to cells in the context of exosomes and microvesicles, have the potential to almost act virally by passaging epigenetic materials that could alter the phenotypes of the recipient cells. This represents an extraordinary form of extracellular communication, with vesicles as either mechanisms for delivery of RNAs, or perhaps as reservoirs to rapidly dispose of the RNA messages or regulatory activities. As biomarkers, the major advantage of such "circulating" RNAs over proteins or other subjects of molecular diagnostics is the ability to amplify the nucleic acid signal using PCR-based techniques, providing both increased signal and high specificity for the same biomarker, and relatively cheaply compared to some of the mass spectrometry or NMR methods needed for protein and metabolite markers.

2.2.5 Passage of pathogens / pathogenic entities: fat balls gone bad

As alluded above, the association of virus internalization, component packaging, and release in association with vesicles of the MVB and endosomal complexes or membrane lipid raft regions (Calistri et al. 2009) has prompted study of relationships between viruses and exosomes/microvesicles. For Human Immunodeficiency Virus (HIV), it has been proposed that the virus may hijack the system and egress from the cells via the plasma membrane microvesicle pathway (Booth et al. 2006) and from the exosome pathway (Nguyen et al. 2003), leading to the "Trojan exosome" hypothesis (Gould, Booth, and Hildreth 2003). However, this concept remains controversial (Coren, Shatzer, and Ott 2008). Epstein-Barr Virus (EBV) has been another well-studied virus in the exosome/microvesicle field, where it was first noticed that the EBV protein LMP1 appears in exosomes from EBV-infected cells (Flanagan, Middeldorp, and Sculley 2003; Keryer-Bibens et al. 2006). The presence of LMP1 and the immunomodulatory protein galectin-9 in exosomes derived from infected cells are implicated in immune suppression of NK and T cells, particularly affecting the inflammatory Th1-type responses (Dukers et al. 2000; Klibi et al. 2009). Hepatitis B and C viruses (HBV, HCV) are embedded in the etiology of hepatocellular carcinomas; envelope proteins from these viruses appear in exosomes from infected cells, and HBV requires MVBs for production of enveloped virions while subviral particles are released differently (Masciopinto et al. 2004; Watanabe et al. 2007). Human Herpes Virus 6 (HHV-6), an

immunosuppressive, neurotropic virus, also has associations with hematologic and CNS malignancies (Ogata 2009; Saddawi-Konefka and Crawford). As with other viruses, mature virions in infected cells were found in MVB-like compartments and released in exosome-like vesicles. It is not known if these viron-containing exosomes are infectious, but the implications of infection and immune suppression aiding tumor take and progression are obvious. Finally, Cytomegalovirus (CMV) has become an increasingly interesting associate in high grade gliomas (Cobbs et al. 2002; Mitchell et al. 2008; Dziurzynski et al.). Exosomes from CMV infected cells contain CMV antigens (eg, gB) that are capable of stimulating CD4⁺ memory T cells (Walker, Maier, and Pober 2009), and while CMV DNA was amplified from glioblastoma (GBM) patient peripheral blood in one study (Mitchell et al. 2008), no CMV DNA was detected in another similar (but smaller) study (Lehrer et al.). However, neither of these studies used patient serum exosomes as target materials. In a proteomic study of murine SMA-560 brain tumor cell-derived exosomes, we identified the gag polyprotein pr65 and its precursor (Graner et al. 2009); these proteins originate from an endogenous murine retrovirus implicated in the progression of murine melanoma by subversion of immune surveillance (Mangeney et al. 2005). Thus, there remain many implications of viruses, virions, or viral particles/pieces in circulating tumor exosomes either as immune targets (potential antigens) or as further purveyors of immune suppression.

The roles of exosomes/microvesicles in bacterial diseases are less well studied, but there have been significant works in the mycobacter area. Exosomes from macrophage infected with *Mycobacterium avium* display antigens such as glycolipids from that pathogen. These exosomes provide pro-inflammatory signals to resting macrophage (Bhatnagar and Schorey 2007), as is true of exosomes from a number of pathogenically infected macrophage both *in vitro* and *in vivo* (Bhatnagar et al. 2007). However, while exosomes from *Mycobacterium tuberculosis*-infected macrophage contain proteomically-identified TB proteins/antigens (Giri et al.), exosomes from MTB-infected macrophage can actually suppress activation of naïve macrophage (Singh et al.). While the similarities, particularly immune relationships, between cancer and MTB infection have not gone unnoticed (Broxmeyer 2004; Schorey and Bhatnagar 2008; Cocito and Maes 1998), the roles of exosomes and microvesicles in these diseases likely remains complex.

Another intriguing (and perhaps frightening) role played by exosomes and microvesicles is in the neurodegenerative disease arena. Experimentally, prion diseases such as scrapie have been shown to be transmissible from infected cells (those with the prion protein, PrP, misfolded into the scrapie form, PrP^{Sc}) to uninfected cells by application of exosomes from the infected cells to the uninfected cells. PrP^{Sc} was found in the exosomes from the infected cells, and these cells initiated scrapie when injected into mice (Fevrier et al. 2004). PrP is a GPI-linked protein (Miesbauer et al.), and as mentioned above, membrane proteins with this lipid anchor are frequently sorted into lipid rafts that become part of the IVBs within MVBs, and eventually become exosomes. It is uncertain whether PrP converts to PrP^{Sc} in/on exosomes (Fevrier et al. 2005) but abnormal folding makes it a candidate for ubiquitination and thus sorting into MVBs/IVBs. Blood is considered the most common bodily fluid of transmission, but another study showed that at least the naturally occurring PrP protein is present in pseudo-afferent lymph fluid, and is preferentially enriched in CSF from sheep (Vella et al. 2008). Thus, there may be a neuronal reservoir for PrP (and PrP^{Sc} or other transmissible infectious prion forms) in exosomes found in CSF. For another neurodegenerative disease, Alzheimer's disease (AD), the etiology is believed to be related to the cleavage of cell surface amyloid precursor protein APP by β - and γ -secretases,

resulting in formation of a peptide called A β . Rajendran et al (Rajendran et al. 2006) found that from cells transfected with expression vectors for APP, they could find A β peptides in the extracellular medium in the context of exosomes. The cells processed APP to A β endosomally, with A β ending up in MVBs and extracellularly with exosomes. In immunoelectron microscopy sections of brains from afflicted patients, AD plaques contained exosome-associated proteins (eg, ALIX), further suggesting the role of MVBs and the potential for exosome involvement in the disease. Exosomes from cells expressing APP were found to contain a number of the secretases necessary for processing of APP into its secreted form, as well as the different C-terminal forms of cleaved APP, as well as A β itself (Sharples et al. 2008). γ -secretase inhibition resulted in less A β accumulation in exosomes, suggesting a potential therapeutic approach, but also suggesting the biomarker potential of A β in exosomes to diagnose AD. Thus, not only can pathogenic organisms utilize the exosome/microvesicle pathways for infectious transmission, disease “particles” may utilize them as well to passage from infected or diseased cells to unaffected cells, a novel form of transmission that does not require cell-to-cell contact.

2.2.6 Drug Efflux: fat balls as HazMat crews

In our proteomic analyses of medulloblastoma exosomes, transporter molecules constituted > 10% of the proteins identified (see **Figure 3**). Thus, it may not be surprising that exosomes/microvesicles may be exit vehicles for chemotherapeutic agents applied to cancer cells as a mechanism of resistance. A cisplatin-insensitive ovarian carcinoma cell subline was found to have greatly reduced lysosome size and activity compared to its cisplatin-sensitive original line, and that insensitive line produced more exosomes than the parent line (Safaei et al. 2005). Those exosomes contained a number of lysosomal markers as well as transport molecules. When the insensitive line was treated with cisplatin, 2.5 fold more drug was effluxed from the insensitive line than the sensitive one, and at least a portion of that was found in the insoluble exosome pellet from the spent culture medium. Chen et al (Chen, Posada et al. 2006) further implicated the endosomal/MVB pathway for egress of doxorubicin, demonstrating a role for VPS4a in MVB and exosome function and packaging of drug. There may also be a role for microvesicle-based drug efflux, since the plasma membrane plays a role in resistance to doxorubicin in the K562 leukemia cell line used in these studies (Chen et al. 2007). While exosomes and microvesicles may provide drug resistance mechanisms for tumor cells, one must also consider the possibility that in normal cells, exosomes/microvesicles may perform similar cellular detoxification roles to remove noxious small molecule compounds to protect normal cells, as well.

2.3 Summary

Exosomes and microvesicles are akin to miniature versions of the cell itself, possessing lipids, proteins, and even RNAs from the originating cell; some even refer to exosomes and microvesicles as “extracellular organelles”. These extracellular vesicles are intimately involved in the extracellular local and distal cross-talk between and amongst cell, tissues, and organs, and have a variety of functions associated with them. In particular, the relationships between cancer cells and associated “normal” cells, such as those of the endothelium, the microenvironment, and immune system, are all profoundly mutually influenced by these secreted vesicles in ways we are only slowly beginning to understand.

3. Brain tumor exosomes and microvesicles: fat balls on the brain

The primary literature on brain tumor extracellular vesicles is amazingly small (Graner, Cumming, and Bigner 2007; Al-Nedawi et al. 2008; Skog et al. 2008; Graner et al. 2009; Guescini, Genedani et al. ; Yang et al.), although one of the early microvesicle papers used C-6 rat glioma cells and called their vesicular entities “exosomes”, but by current standards the vesicles would be called microvesicles or microparticles (Trams et al. 1981; Johnstone 2006). Our group was arguably the first to identify *bona fide* brain tumor exosomes, despite some 20 years of previous study. Brain tumor exosomes and microvesicles appear structurally and proteomically like such vesicles from other cell/tumor types in culture, and from the sera of patients with diverse tumors (Graner, Cumming, and Bigner 2007; Graner et al. 2009; Al-Nedawi et al. 2008; Skog et al. 2008). One difference has been that exosomes from both adult gliomas and pediatric medulloblastomas have proven extremely resilient to lysis and extraction (Graner et al. 2009) (M Graner, unpublished; Johan Skog, personal communication), making protein analysis in particular rather difficult. Another interesting feature is the putative presence of mitochondrial DNA contained within (and perhaps on the surface of) glioma cell line extracellular vesicles (Guescini, Genedani et al.), which has also been seen in murine myoblast vesicles as reported by the same group (Guescini, Guidolin et al.) ; this finding runs counter to other groups’ findings (Valadi et al. 2007) and will likely be somewhat controversial. However, given the high glycolytic capacity of gliomas and the distressed and non-uniform appearance and activities of their mitochondria (Oudard et al. 1996; Oudard et al. 1997; Ordys et al. ; Santandreu et al. 2008), relationships between exosomes and mitochondria in cancer cells could potentially be an area of fruitful and energetic study. In the next sections we will discuss the broad range signaling abilities, metabolic effects, and immunological properties of brain tumor exosomes/microvesicles, as well as the exosome-induced migratory and protective phenotypes cells experience upon exposure to exosomes.

3.1 Brain tumor exosomes drive various activities in recipient cells: fat balls as game-changers

High grade gliomas such as glioblastomas (GBMs) are extremely heterogeneous with respect to cellular appearances and phenotypes, chromosomal abnormalities, gene and protein expression, etc, but almost universally share a lack of response to treatment (Mischel, Cloughesy, and Nelson 2004) whether the treatment is chemical, radiologic, or immunologic. Another commonality amongst GBMs is their extraordinary invasive potential that inevitably leads to tumor recurrence and patient demise (Hoelzinger, Demuth, and Berens 2007). Below we will describe mechanisms by which exosomes and microvesicles may contribute to these most nefarious features of high grade gliomas.

3.1.1 Brain tumor exosomes and EGFR signaling: fat balls carry the mail

The epidermal growth factor receptor (EGFR) has a long history as a major player and therapeutic target in brain tumors and many other tumors as well (Hatanpaa et al. ; Ye, Gao, and Cai ; Brandes et al. 2008; Vivanco and Mellinghoff ; Yarden 2001). Perhaps the most prominent mutation in EGFR in gliomas is the variant (either genomically mutated/encoded or resulting from a splice-site mutation and alternate usage) that eliminates exons 2-7 to yield “delta 2-7 EGFR” or “EGFR variant III” (EGFRvIII), reviewed in (Wikstrand et al. 1998; Pedersen et al. 2001; Sonabend, Dana, and Lesniak 2007). This mutant EGFR does not necessarily need to dimerize to signal (Chu et al. 1997) , does not bind ligand but is nonetheless

constitutively active and is capable of inducing tumor growth in otherwise non-tumorigenic cells in animal models (Batra et al. 1995). EGFRvIII is expressed in 25%-67% of all GBMs (Pelloski et al. 2007; Heimberger et al. 2005) and is generally considered a marker of poorer prognosis. The receptor is heterogeneously expressed in human GBMs (generally pockets of expression) compared to the more uniform presence of the wild type EGFR (EGFRwt) (Nishikawa et al. 2004). Coupled with animal model studies, it is suspected that EGFRvIII results in both autocrine signaling loops that lead to enhance EGFRwt signaling (Ramnarain et al. 2006) and paracrine mechanisms driving increased proliferation of the bulk of the tumor mass (which expresses EGFRwt), thus maintaining tumor cell expression heterogeneity (Inda et al.). While one usually invokes soluble extracellularly-released signaling factors for these mechanisms, the work of Al-Nedawi (Al-Nedawi et al. 2008) indicates that tumor-derived microvesicles could carry and transfer EGFRvIII from cells that express the mutant receptor to cells that do not. This group transfected U373 cells to express the mutated EGFRvIII (these glioma cells normally express neither EGFR nor EGFRvIII) and collected microvesicles from the transfectants that displayed EGFRvIII. Recipient U373 [EGFRvIII(-)] cells not only epigenetically “express” EGFRvIII following microvesicle receipt, but this also activates downstream signaling pathways associated with the presence of EGFRvIII including phosphorylation of ERK 1/2 and AKT, release of VEGF, increased Bcl-x expression with concurrent reduction in p27 expression, and enhanced soft agar colony formation. U373viii tumor-bearing mice also had EGFRvIII(+) microvesicles in their blood. We also found that murine glial tumor cells expressing EGFRvIII (SMA-560vIII) display the receptor on their exosome surfaces, and that patient serum exosomes/microvesicles (from 4 of 5 GBM patients) contained EGFRvIII, while all had EGFR in/on them (Graner et al. 2009). Skog et al (Skog et al. 2008) also found *EGFRvIII* mRNA transcripts in microvesicles in sera from 7 of 25 patients with GBMs; 14 of 30 patients had transcript-positive tumor biopsies (and curiously, one patient had *EGFRvIII*(+) serum microvesicles but the biopsy was negative). They also found that GBM cell line microvesicles contained angiogenic factors and could stimulate brain endothelial cells to form tubules. These cumulative results beg the question as to whether the autocrine/paracrine signaling mechanisms and resultant enhanced tumor growth and features of aggressiveness may be in part due to the passage and reception of exosomes and microvesicles from EGFRvIII-expressing cells affecting ostensibly EGFRvIII(-) cells to enhance, maintain, or extend aggressive tumorigenic phenotypes. Aside from EGFR and the vIII mutant, exosomes and microvesicles are reservoirs of other factors potentially involved in cell-cell signaling including L1-NCAM as we have shown. One of the highest scoring networks using Integrated Pathway Analysis from our proteomic efforts was “cell-to-cell signaling and interaction, cancer, hematologic system development and function” (Graner et al. 2009), implying that these vesicles have inherent signaling capacity or the ability to merge readily into signaling networks. Another important implication of the discovery of serum-borne tumor vesicles from patients with GBMs (and also, pediatric patients with medulloblastoma, data not shown) is that these vesicles escape the blood-brain barrier with potential for systemic effects, particularly involving immune function (Section 3.2).

3.1.2 Brain tumor exosomes and metabolomics: fat balls grease the wheels of glycolysis

Results from other brain tumor exosome/microvesicle experiments indicate that exogenously-added vesicles increase recipient tumor cell proliferation (Al-Nedawi et al. 2008; Skog et al. 2008). We have seen similar dose-dependent results in modified

clonogenicity assays using U87MG cells while adding differential quantities of exosomes to those cells (**Figure 4**).

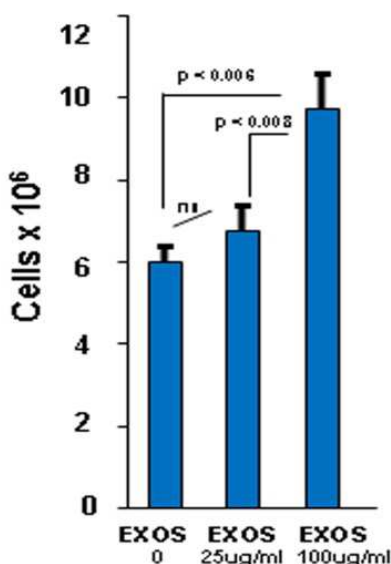


Fig. 4. Exogenous tumor exosomes increase tumor cell proliferation in a dose-dependent manner. U87 glioma cells were grown under standard conditions for 48 hrs in the presence of 0, 25, or 100 $\mu\text{g/ml}$ exosomes and counted on a hemocytometer. Higher concentrations of exosomes significantly increased cell proliferation.

Proliferation assays requiring redox readouts such as MTT/MTS assays in slower-growing cell lines pulsed with exosomes suggested that the proliferative effects may involve metabolic changes in the recipient cells, and our proteomics (Graner et al. 2009) (see also **Figure 3**) strongly suggested this also. We tested this by performing metabolomics experiments on U87MG cells grown in a stem cell-like medium with serum replacement; cells were treated (or not) with 25 $\mu\text{g/ml}$ exosomes for 44 hrs, and carbon flux was followed by incorporation of ^{13}C -labeled glucose as a metabolic tracer for an additional 4 hrs. Media and cells were extracted and analyzed by nuclear magnetic resonance/magnetic resonance spectroscopy (NMR/MRS), notably revealing very large increases in energy transfer compounds and glycolytic activity (**Figure 5**)

Decreased glutathione levels and increased lactate output are associated with transformation and metastasis in cell line models (Lu et al.); the lipid profile, with the exception of monounsaturated fatty acids (MUFA) and cholesterol, is remarkably stable. In a somewhat controversial finding in the literature, incubation of cells with oleic acid, a MUFA, promotes secretion of matrix metalloproteinase-9 (MMP9) and invasion of breast cancer cells (Soto-Guzman et al.). Whether there is an endogenous relationship between increased cellular MUFA production (as opposed to exogenous supplementation) and MMP activity/invasion remains to be seen, although it is possible that exosome lipid content may act in that supplementary fashion. Finally, the energy compounds and glycolysis profile, along with the soluble metabolite outputs, demonstrate that exosomes do indeed profoundly increase cellular metabolic activity in the form of glycolysis, in keeping with the Warburg effect (Ferreira). The mechanisms behind this need to be elucidated--is there increased enzymatic metabolic activity, or is there increased gene/protein expression of those enzymes, or are such enzymes actually delivered to the cells by exosomes (or any

combination of these scenarios)? From our proteomic studies we have identified more than half of the enzymes in both the “preparatory” and “pay-off” phases of glycolysis (Graner et al. 2009) (and data not shown), along with a variety of other enzymes in other metabolic cycles. Thus, it is not inconceivable that exosomes themselves directly provide, or at least contribute to, some of the glycolytic enzyme repertoire that leads to enhanced glycolysis in tumor cells pulsed with exosomes. The general renewed interest in tumor metabolism (Cairns, Harris, and Mak) will hopefully prompt more research into the area of exosome metabolic effects, both downstream and perhaps even within the exosomes themselves.

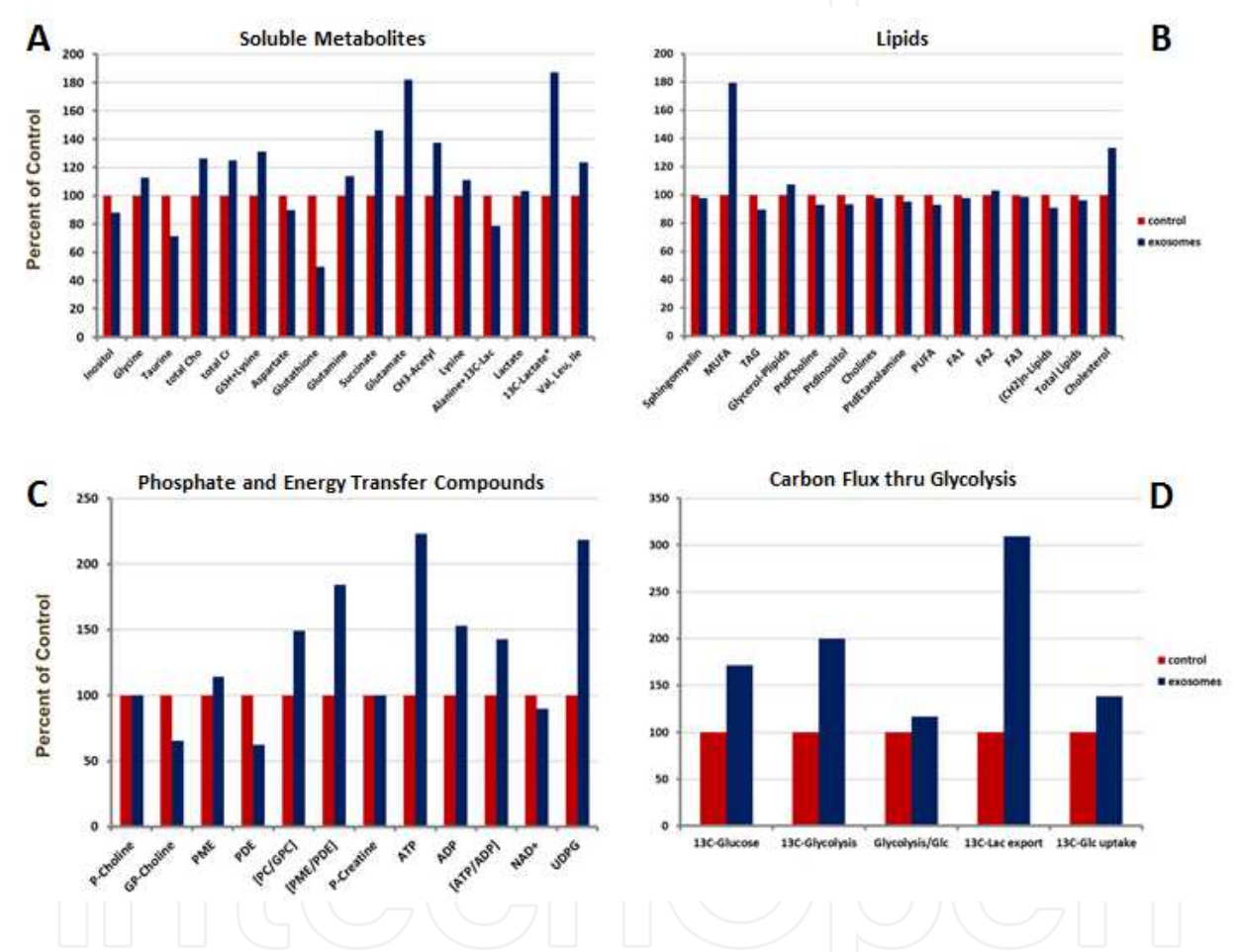


Fig. 5. U87MG exosomes applied to U87 recipient cells results in increased glycolytic activity. U87 cells were incubated with 25 $\mu\text{g}/\text{ml}$ U87 exosomes for 44 hrs prior to changing the medium into medium containing ^{13}C -labeled glucose for 4 hrs (blue bars). Control cells were grown without the addition of exosomes (red bars). Cells and media were harvested and extracted for soluble and lipid-based compounds, and analyzed by NMR/MRS for quantification and determination of carbon flux. **A** shows levels of soluble metabolites compared to control (untreated) U87MG cells; **B** shows lipid profile changes following exosome pulsing; **C** shows phosphate/energy compound changes during exosome exposure, and **D** shows changes in the glycolytic profile of U87 cells following incubation with exosomes.

3.2 Brain tumor exosomes and immunology: fat balls vacillate between vaccines and vacuums

As mentioned above (Section 2.2.1) tumor exosomes have gone from being seen as perfect vaccine material to their current status as infamous purveyors of immune suppression. Also as mentioned, most of those immune suppression studies have been performed in tissue culture settings that may not reflect the complexity of the mammalian immune system. Aside from our previous work demonstrating that murine brain tumor exosomes were potent prophylactic vaccines and drivers of antibody production (Graner et al. 2009), there has been only one other very recent study involving brain tumor exosomes and immunology (Bu et al.). That report determined that exosomes from culture supernatant of primary gliomas effectively provided antigens to autologous dendritic cells, which in turn could generate activated CD8⁺ T cells with potent cytotoxicity against autologous tumors cells *in vitro*. We pointed out in our publication (Graner et al. 2009) that there are a number of putative and known antigens in or on the glioma exosomes we utilized including the aforementioned EGFRvIII and glycoprotein nmb (GPNMB, also called osteoactivin, HGFIN, and DC-HIL). GPNMB is believed to be a *bona fide* antigen target in high grade gliomas (Kuan et al. 2006; Loging et al. 2000) and possibly in breast cancer (Rose et al.) as well as in melanoma (Tse et al. 2006). The exosomes also had HSP70 on their vesicular surfaces along with HSPs 60, 90, calreticulin, and protein disulfide isomerase, all known immune stimulators or “danger signals”, and capable of providing antigens to DCs. However, those exosomes also had HSP25 (human HSP27) on their surfaces, which is associated with lower immunogenicity of exosomes (Alexzander Asea, personal communication) and more aggressive tumors when cell surface localized (Bausero et al. 2004). Also we found transforming growth factor beta (TGF- β) with the exosomes, which is frequently associated with immune suppression (Flavell et al.); even the antigen GPNMB also has potential T cell inhibition activities (Tomihari et al.). Consequently, there seems to be a range of immune stimulatory and potentially immune suppressive moieties in/on the same population of exosomes. Also, quantity may be an issue. Our data suggest that tumor cell-derived exosomes at (relatively) low concentrations may inhibit interferon-gamma (IFN γ) release from mitogen-activated T cells, while at higher concentrations exosomes increase IFN γ release over control (mitogen-stimulated) T cells (Figure 6). Based on these data, context and quantity may be extremely relevant in the roles played by tumor exosomes in the double-edged sword of immunity. One may imagine that if peripherally activated (eg, from a draining lymph node) circulating T cells encounter low concentrations of tumor exosomes also in the periphery, circulating in blood, those T cells may face the suppressive features of the exosomes. However, if the T cells are in the vicinity of high tumor exosome concentrations (tumor under stress, or in the context of a bolus of exosomes in the form of a vaccine following a priming protocol), this may switch the T cells to an even further activated phenotype. While that ostensibly should lead to better anti-tumor immunity, it may also lead to activation induced cell death (AICD) of the T cells.

We found that tumor exosomes as antigen sources drive effective antibody production in short duration immunization protocols and with no adjuvant (Graner et al. 2009) (M Graner, unpublished) in mice. The significance of this is unclear; while antibody output is generally associated with Th2-type immune responses (ie, more regulatory than inflammatory), we nonetheless found IFN γ -producing T cells following tumor exosome vaccination concurrent with significant antibody titers against the exosomes (Graner et al. 2009). Similar enhanced simultaneous T- and B-cell immune responses have been seen when immunizing mice with

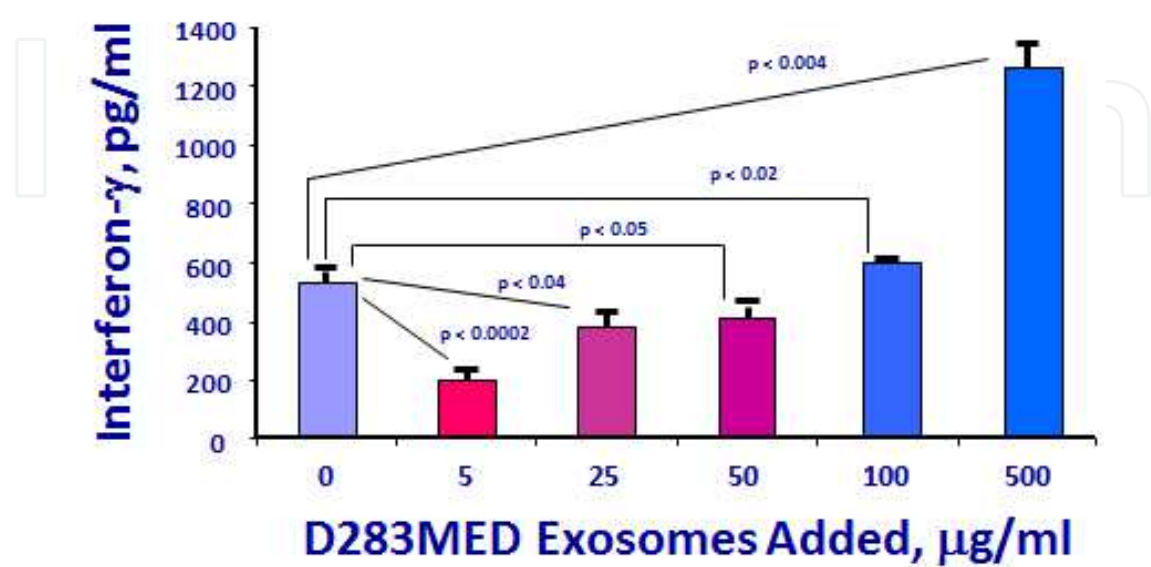


Fig. 6. Medulloblastoma exosomes differentially and dose-dependently alter interferon-gamma (IFN γ) release from mitogen activated human T cells. Peripheral blood mononuclear cells (PBMCs) were collected from a healthy donor and were stimulated with phytohemagglutinin (PHA) for 48 hrs (or not) in the presence of D283MED medulloblastoma exosomes harvested from tissue culture supernatants at the concentrations listed. After 48 hrs, media were harvested and IFN γ released from the cells was measured by specific ELISA. Statistical relationships (Student *t* tests) are shown.

heat shock protein vaccines (Li et al. 2008; Manjili et al. 2003). The relevant question here is if the antibodies have any biologic, anti-tumor activity. In the situation of true human, pre-established tumors, one may think of tumor-released exosomes acting as “auto-immunizations” against tumor antigens. However, it may be that the tumor “sheds” antigens via exosomes/microvesicles, driving futile immune responses against “decoy” circulating antigens. This may have 2 benefits for the tumor—1) driving antibody responses may serve to maintain a Th2 cytokine environment, which is suboptimal for effective cellular anti-tumor immune responses; and 2) free-floating, released exosomes may titrate out potentially “dangerous” antibodies generated against tumor antigens, thus preventing those antibodies from ever reaching the solid tumor mass. Concepts similar to this are cited above (Battke et al.). As further evidence that tumors may “offer up” exosome-bound antigens we show here that antibodies in serum from a patient with GBM show reactivity with the autologous tumor lysate on a Western blot but far more intense reactivity against the autologous serum exosomes (**Figure 7**)

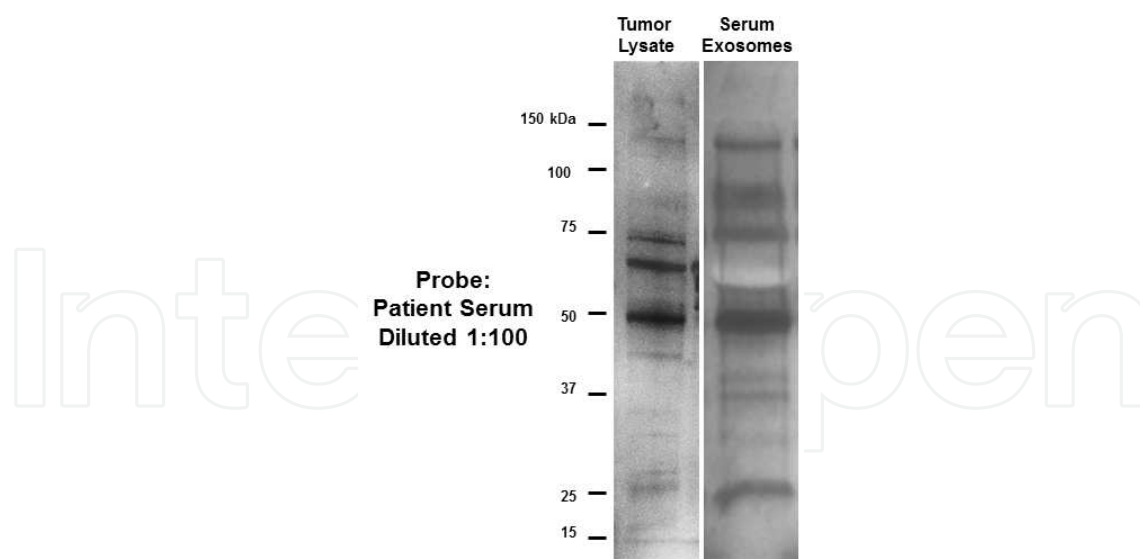


Fig. 7. Patient serum antibodies react with autologous tumor lysate and serum exosomes/microvesicles. A patient with GBM had blood drawn at the time of tumor resection. Exosomes/microvesicles were harvested from the serum; the remainder of the serum was diluted 1:100 and used to probe reducing, denaturing gels/blots of tumor lysate and lysed serum exosomes (25 μ g loaded per lane). Secondary antibody was biotinylated anti-human IgA/G/M, and tertiary reagent was streptavidin HRP. ECL developing agent was used, and images captured on a FluroChem Imager. Molecular weight markers are shown to the left.

We postulate that vaccination with exosomes, particularly in relatively naïve animals, may prompt effective antibody (and T cell) generation, but in situations where the tumor has had time to “self-vaccinate” by releasing tumor exosomes, the antibody repertoire may reflect immune editing and therefore actually benefit the tumor via “ironic immunity”. This concept bears further study, and such investigations are ongoing.

Thus complex interplays between stimulation, suppression, and “decoy allocation” that tie the tumor to the host immune response are further complicated by the potential roles played by exosomes. With antigens and heat shock protein danger signals, exosomes may be good vaccines; however with TGF β content and a host of other suppressive features, tumor-released exosomes may ultimately skew the host immune response into a position that benefits the tumor by decoy antigen release and stealth regulatory cytokine environment.

3.3 Brain tumor exosomes/microvesicles and tumor migration: attractive fat balls blaze the trail

Perhaps the most distressing activity performed by high grade gliomas is their ability to migrate and invade the parenchyma with devastating consequences of bilateral presentation if the tumor crosses the corpus callosum, with the possibility of disastrous leptomeningeal dissemination. The modulation of the extracellular environment, and the ability to proximally and distally signal extracellularly are critical factors in this manifestation of the glioma phenotype (Nakada et al. 2007; Berens and Giese 1999). The cells may migrate out of the range of focused external beam radiation therapy, thus limiting its usefulness, and this migration is a clear danger in the multifocal recurrences more and more frequently seen following treatment with bevacizumab (Rahman et al. ; Iwamoto et al. 2009; de Groot et al. ;

Verhoeff et al. 2009). Obviously, glioma invasion makes truly complete surgical resections impossible. Exosomes and microvesicles, as nanovesicles released into the extracellular environment, seem like perfect vehicles for extracellular remodeling, possessing proteases such as insulin degrading enzyme (IDE) (Tamboli et al.), MMPs 1 and 14 (Medina and Ghahary ; Hakulinen et al. 2008) and ADAMs 10 and 17 (Mathews et al. ; Stoeck et al. 2006). Exosomal Rabs (Hendrix et al.), HSP90 and plasminogen (McCready et al.), and tetraspanins (Rana et al.) have been implicated in tumor cell migration/invasion as well. Recently it was shown that L1-NCAM (CD171) and its migration-inducing cleavage product were found in exosomes of brain tumor cell lines (Yang et al.), and exosomes were partially involved in an animal model of tumor metastasis by preparation of the pre-metastatic niche (Jung et al. 2009). Amidst this background we have determined that glioma exosomes (from U87MG cells, as well as D283MED cells—not shown) can promote tumor cell migration across a plastic membrane at higher levels than that promoted by FBS (**Figure 8**)

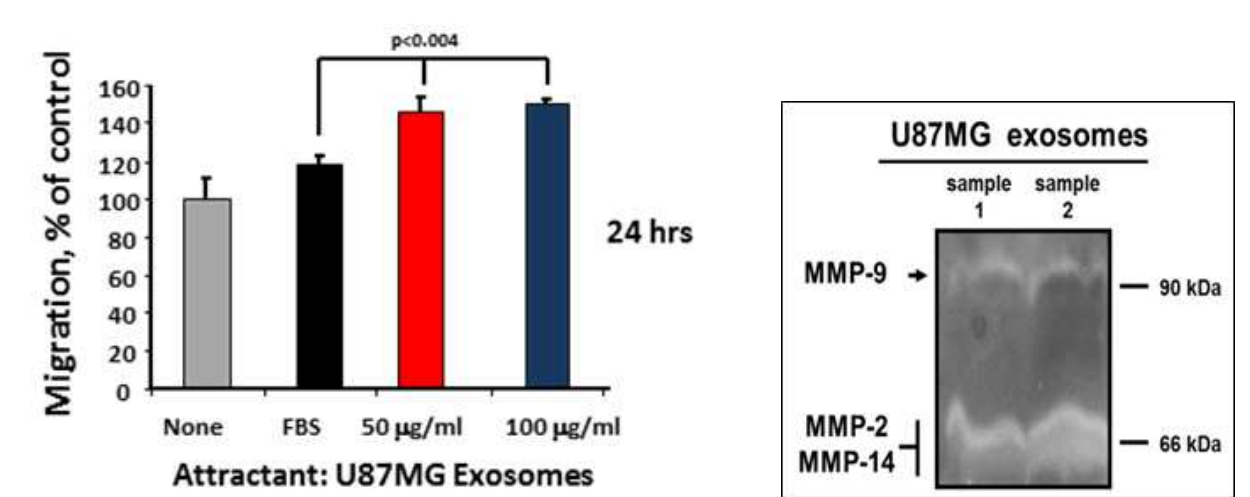


Fig. 8. Glioma exosomes promote tumor cell migration in Boyden chamber assays and possess intrinsic matrix metalloproteinase activities. **Left** U87MG cells were placed on the top chamber of an 8 µm pore-size plastic insert in wells; the lower chambers contained serum-free media, fetal bovine serum (FBS, positive control) or increasing quantities of U87 exosomes. Cells migrated for 24 hrs; the inserts were removed, the tops washed, and the bottoms were stained with crystal violet, and 5 random fields of duplicate wells were counted (avg, SD, and *t* test results shown). **Right** U87 exosomes were separated in a zymogram (gelatin) gel; MMP activation was induced, and cleared areas in the gel correspond to gelatinase activities of appropriate molecular weights for MMPs 2, 9, and 14 (confirmed by Western blotting, data not shown).

The Boyden chamber/migration assay is largely an attraction assay; the complexity of exosomes/microvesicles will make it interesting to determine what factors in or on these vesicles promote this migration and potentially could serve as attractants over a larger distance, akin to leaving “bread crumbs” of exosomes for cells to track and follow while migrating or metastasizing. The MMP activities suggest that exosomes will be able to also carve paths and remodel the extracellular matrix to allow for cells to migrate (either tumor cells out of the main mass, or even endothelial cells in for angiogenic events). These preliminary data indicate that brain tumor exosomes can both blaze paths and provide

attraction for glioma migration. As this is an important area of therapeutic attention, exosome biology may play a critical role in our attempts to regain local control of high grade gliomas following surgery, thus improving recurrent-free survival.

3.4 Brain tumor exosomes/microvesicles and chemoresistance: fat balls provide protection

High grade gliomas are extremely chemoresistant (Lu and Shervington 2008; Sarkaria et al. 2008). As mentioned above (**Section 2.2.6**), exosomes have transporter molecules among their proteomic content, and we have also shown that MRP3 is in glioma exosomes (Graner et al. 2009). These may provide mechanisms for drug export, literally packaging the chemotherapeutic agent in exosomes/microvesicles. As shown above (**Section 3.1.2**), exosomes may also accelerate metabolic activity which could lead to increased breakdown or chemical modification of the drug compound. While these mechanisms are undoubtedly important in glioma chemoresistance (along with the natural protection of the blood-brain barrier), no one has ever demonstrated an impact of exosomes on cellular resistance to drugs in neuro-oncology. Since all of this discussion is currently theoretical, we have first tested this putative protective property of exogenously-added exosomes and have discovered that glioma exosomes can indeed provide protection or enhance resistance to temozolomide (**Figure 9**). In fact, even low concentrations of tumor exosomes fully complemented cell growth back to normal despite the presence of the drug (which is the chemotherapy of choice for high grade gliomas and is a component of the current standard of care for these tumors) (Stupp et al. 2005).

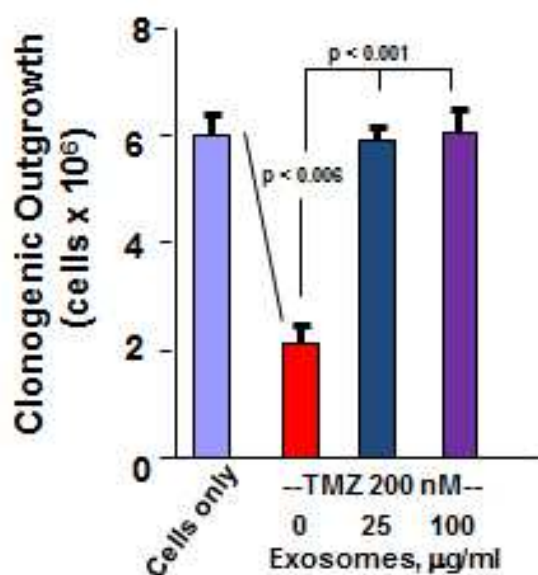


Fig. 9. Exogenously added tumor exosomes protect glioma cells from cytotoxic chemotherapy. U87MG cells were either left untreated ("Cells only") or were subjected to treatment with temozolomide/Temodar® ("TMZ") at 200 nM concentrations for 48 hrs. Exosomes at the concentrations listed were also added (or not), and cell growth was quantified in a modified clonogenicity assay as describe for **Figure 4**. Exosomes provide significant protection against the chemotherapy agent, and return cell growth to normal (+ exosomes, not significant compared to cells only).

Exosomes, by currently unknown mechanisms, provide some means of chemoresistance to the therapeutic drug of choice as the standard of care for high grade gliomas. However, this does strongly suggest that discovering this underlying mechanism may be a critical point for improving therapeutic efficacy of our drug treatments. This may go back to the cellular mechanisms for generating exosomes; however, as this is a process common apparently almost all cells, it is unclear if there are particular aspects that are tumor-specific. Iero et al (Iero et al. 2008) have suggested that a number of pharmacologic agents may be able to interfere with exosome release, but so far this does not appear to have made an impact in most of our current chemotherapy regimens.

3.5 Summary

Exosomes and microvesicles from brain tumor cells affect signaling capacities, alter metabolism, impinge on immunity, and may promote two of the most nefarious characteristics of high grade gliomas (and medulloblastomas)—their abilities to migrate and invade normal tissue, and their inherent resistance to treatment. Obviously, there is a tremendous space for research in this area as well as a great need to understand these complex vesicles and their interactions with the tumor, the tumor microenvironment, the normal tissues, and the potential systemic effects as the tumor attempts to control its surroundings locally and distally.

4. Conclusions

Exosomes and microvesicles serve as unique and profoundly important extracellular signaling vehicles both locally in the area of release, as well as at potentially great distances via the circulatory system. Despite some 20 years of research efforts devoted to these “fat balls”, we are finding more and more fascinating features about them almost constantly. With intense study of the biochemical compositions of the vesicles (proteomics, ribonomics, lipidomics) we are learning more about the similarities, as well as the curious differences, between exosomes from different cellular sources. We are also developing much better understandings of the biology of vesicular formation and release—something that many viruses and pathogens have long exploited. All of this knowledge complements the ongoing work to understand our ever-increasing—and increasingly fascinating—knowledge of the biologic impacts that exosomes and microvesicles have on recipient cells, and on micro- and macroenvironments. From the perspective of neuro-oncology, there is a wealth of information waiting to be mined, particularly in the areas of immunology, proximal and distal cell signaling, microenvironment alteration for migration or protection, and how tumor cells may use the physiology of exosomes as a means of therapeutic resistance. The almost viral quality of exosomes and microvesicles as carriers of messenger and microRNAs is one example of a vastly open region ripe for progress, and it almost certainly intersects with many other areas of research. As studies of exosomes and microvesicles become accepted as legitimate science and gain mainstream appreciation, we should see further advances in the research that leads to translational applications of this knowledge. Interdisciplinary approaches will be required as expertise in genetics, proteomics, metabolomics, lipid biochemistry, and microparticle/nanovesicle technologies all have a place in these endeavors. For patients facing the dismal prognoses of brain tumors, new clinical applications resulting from more basic and translational research cannot come fast enough.

5. Acknowledgements

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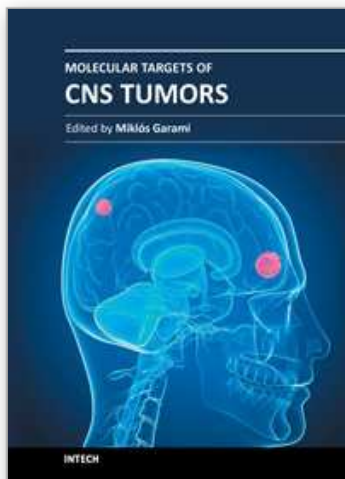
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