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REVIEW

The untapped potential of urine shed bladder cancer exosomes: biomarkers, signaling, and therapeutics

Robert H. Blackwell[1*], Carrie A. Franzen[1], Robert C. Flanigan[1], Paul C. Kuo[2] and Gopal N. Gupta[1]

[1]Department of Urology and [2]Department of Surgery, Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, IL, USA

[*]Corresponding author: Robert Blackwell, MD, Department of Urology, Loyola University Medical Center, 2160 S. First Avenue, Fahey Center, Room 261, Maywood, IL 60153. Tel: 708-216-5100, Fax: 708-216-1699, Email: rblackwell@lumc.edu

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Abbreviations used: BSA, bovine serum albumin; EDIL-3, epidermal growth factor-like repeat and discoidin I-like domain-3; FACS, fluorescence activated cell sorting; HUVEC, human umbilical vein endothelial cells; mRNA, messenger ribonucleic acid; miRNA, micro RNA; MVB, multivesicular body; PCR, polymerase chain reaction; NTA, nanoparticle tracking analysis; PMSF, phenylmethylsulfonyl fluoride; siRNA, small interfering RNA; SEM, scanning electron microscopy MSC: mesenchymal stem cell; UC, ultracentrifugation

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ABSTRACT

Exosomes are small, extracellular vesicles ranging in size from 30-100 nm. Their contents originate from the cytoplasm of their host cell, including nucleic acids, intracellular and membrane-bound protein. Cancer cells have been found to produce exosomes at a significantly higher rate than normal cells, and function in cell-to-cell signaling, with surface protein interaction with recipient cells, as well as deposition of host cell RNA into the recipient cell. Bladder cancer exosomes have demonstrated the propensity to augment the aggressiveness of tumors by increasing tumor cell migration and angiogenesis, while systemically circulating exosomes in other malignancies have also been shown to establish a favorable niche for metastatic disease. With respect to bladder cancer, these exosomes are additionally shed into the urine and represent a potential source of urinary biomarkers to be utilized for a non-invasive diagnosis. Further, while exosomes promote aggressiveness of bladder tumors, there is also potential for therapeutic endeavors on the horizon, with loading of silencing RNA and chemotherapeutics being avenues under investigation. Here we review the current literature on exosomes in bladder cancer as biomarkers, their role in cell-to-cell signaling, and as potential delivery agents for modulatory agents and chemotherapeutics.

Keywords: biological markers, exosomes, RNAi, urinary bladder neoplasms

Introduction

Exosomes are small membrane vesicles secreted by both normal and cancer cells. These vesicles range in size from a diameter of 30-100 nm[1,2] and have been shown to originate from all of the epithelial cell types along the urinary tract, from the podocytes in the kidneys to the urothelium of the bladder [3] (**Fig. 1**). Initially thought to function only in the disposal of unnecessary cellular contents [4], exosomes have been found to have a significant biological role in cellular communication [2]. The mechanism for signal transmission includes both vesicle membrane protein interaction with the recipient cell membrane[5,6] and direct deposition of intravesical contents [eg, mRNA, microRNA (miRNA)] into the recipient intracellular space [7-9]. This is of particular importance in the setting of malignancy, as cancer cells have been demonstrated to produce higher proportions of exosomes than normal cells [10], augmenting their potential to increase local tumor invasiveness[11] and establishment of a premetastatic niche [12,13]. Exosomes have been isolated in a variety of bodily secretions (blood, urine, semen, saliva, ascites, breast milk, etc) [1,4,14], and have a lipid bilayer membrane enriched with cholesterol, sphingomyelin, and ceramide, providing them and their contents stability in numerous bodily fluids.

Despite their presence in nearly all obtainable bodily fluids, only urine remains as a readily available fluid that can be collected in large quantities in a non-invasive fashion [15,16]. As exosomes constitute approximately 3% of the total protein content in the urine [16,17], exosomes are of particular interest in biomarker identification and potential therapeutic application.

Exosome biogenesis and structure

Exosomes originate from within the endosomal network [2]. In brief, endocytosis of cellular plasma membranes produce endosomes with apical membrane proteins in their wall. These endosomes fuse into early endosomes, incorporating the apical membrane proteins into their membranes. These early endosomes undergo invagination of their plasma membrane, producing numerous, small internal vesicles containing

intracellular cytosolic contents (eg, protein, mRNA, miRNA). This later structure is termed a multivesicular body (MVB), or late endosome. Through fusion of the MVB to the cellular apical plasma membrane, the internal vesicles are released into the urinary space [2,3].

Given their small size (30-100 nm diameter[1,2]), exosomes have an internal volume of approximately 4.2-380 yoctoliters (10^{-24} liters), approximately the volume of a eukaryotic ribosome [18]. This volume allows for intravesicular contents of approximately ≤ 100 proteins or ≤ 10,000 net nucleotides of nucleic acid [18]. These intravesicle contents include mRNA and miRNA, and studies have demonstrated these contents are significantly different than their parental RNA content, suggesting preferential sorting [18-20], the mechanism of which has yet to be resolved [1]. Exosome membranes contain a variety of host cell and endocytotic localizing proteins given their origin from the endosomal pathway [18]. Membrane surface proteins markers that identify exosomes include those associated with MVB biogenesis (tumor susceptibility gene (TSG101) and apoptosis-linked gene-2 interacting protein X (ALIX)) [2,14,16,18], tetraspannins (CD9, CD63) [2,14,18], heat shock proteins (Hsc70, Hsp90) [1,14,18], and aquaporin 2 (AQP2) specifically for renal tubule epithelial cell-derived exosomes [21,22].

Exosomal membrane phospholipid composition is comparable to that of the parent cell, but with a high protein-to-lipid ratio [23]. There are regions of raft-associated lipids on their membrane (areas with a low protein-to-lipid ratio), that contribute to the increased concentration of sphingomyelin and cholesterol seen in exosomes. The sphingolipid ceramide is of functional significance, and concentrated in MVB intraluminal vesicles destined to become exosomes as opposed to lysosomes [24]. Other spingolipids may be of functional significance for exosome development along the endosomal pathway, including lyso-bis phosphatidic acid (LBPA), which is important in intraliposomal vesicle formation, as well as lysophosphatidylcholine (LPC), a molecule that is generated on the outer surface of MVB membranes, and accounts for the curvature necessary for the neck during intraluminal vesicle invagination [23].

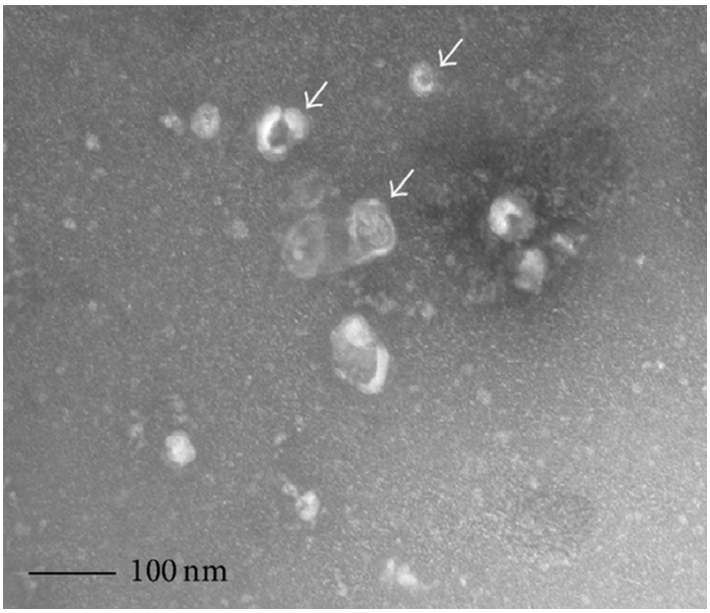

Figure 1. Transmission electron micrograph of bladder cancer exosomes. White arrows label exosomes. Used with permission [43].

Urinary exosome isolation

As patients with functional kidneys void several times per day, urine is an ideal, non-invasive source of biomarkers. Zhou, et al. investigated several factors important to the handling of urine specimens specific for exosomal analysis [16]. They found that timing of urine sample procurement (first or second morning void) did not change the exosomal protein recovery. Long term storage (7 months) of urine samples at -80°C resulted in improved protein recovery compared to -20°C. Also, the use of a protease inhibitor (a mixture of 1.67 ml NaN_3, 2.5 ml 10 mM PMSF, and 50 µl of 1 mM leupeptin per 50 ml urine) applied to the urine specimen upon collection preserved urinary exosome-associated proteins from degradation. Most clinically significant, they were also able to detect exosome-associated proteins at urine volumes as low as 10 ml. To our knowledge, a comparable study investigating urinary exosomal RNA recovery has yet to be performed.

The classical method of exosome isolation requires a series of centrifugations and ultracentrifugations (UC) to pellet debris and dead cells, followed by exosomes, respectively. This is a time-consuming and expensive technique which will likely need to be overcome for exosomal analysis to become clinically applicable on a large scale. Multiple techniques for exosomal isolation were compared by Alvarez and colleagues to assess ultimate protein, mRNA and miRNA yields [25]. These techniques included ultracentrifugation with/without a sucrose cushion or 0.22 µm filter (both used to increase purity), as well as nanomembrane ultrafiltration, and standard and modified protocols for an exosomal precipitation reagent ExoQuick-TC (System Biosciences; Mountain View, CA). Their results indicate that serial UC and the modified ExoQuick-TC protocol are the most applicable techniques for continued research and clinical application. In terms of total exosome yields, the modified ExoQuick-TC protocol had the highest yield for non-UC based techniques, while serial UC without sucrose cushion or microfiltration produced the highest yield for UC-based techniques. For protein analysis serial UC provided a higher level of purity. miRNA and mRNA quantification also demonstrated higher relative levels isolated via the modified ExoQuick-TC technique compared to serial UC. The 'in-hand' time, or person-time, required for the modified ExoQuick-TC protocol is much less than required for serial UC, at 70 vs. 140 minutes, however the overall turn-around time would still be greater for the precipitation protocol given an overnight incubation step that is not required for serial UC.

Immunoprecipitation represents another established technique to isolate exosomes. Following low speed centrifugation to eliminate any cellular contents or debris, a biotinylated characteristic exosomal protein antibody is incubated with the specimen, as has been previously described [26]. Once the exosomes are labelled, the specimen is incubated with antibody-bound magnetic dynabeads, allowing for exosomal isolation without the need for ultracentrifugation. Immunoprecipitation is an attractive option for exosomal isolation, although to our knowledge it has not been compared head-to-head with classical ultracentrifugation.

Confirmation of an exosomal product following isolation can be performed via a variety of techniques. Size determination can be performed by scanning electron microscopy (SEM) or nanoparticle tracking analysis (NTA), with an anticipated result of vesicles roughly 30-100 nm in size [27]. Exosome size estimates compared between these techniques indicate that NTA estimates larger sizes compared to SEM (approximately 110 nm vs. 30-50 nm) in comparable samples. These difference have been

attributed to the former assessing exosomes in solution, while SEM utilizes fixed and dehydrated samples. Quantification of the exosomal contents can be performed by the Bradford assay, a colorimetric assay for determination of total protein compared to bovine serum albumin (BSA) standards [28]. This provides a rough estimation of exosomal content for analysis and standardization for subsequent experiments. Western blotting or flow cytometry can confirm presence of characteristic exosomal protein markers (e.g., CD9, CD63, CD81, hsc70, etc) [28]. Flow cytometry can further purify specific populations via fluorescence activated cell sorting (FACS). An important consideration when utilizing flow cytometry to analyze/sort exosomes is the small size of the exosomes and the limitation on detecting vesicles of this size [29]. Assessment of urinary derived vesicles has demonstrated that detection occurs by two processes, single detection of large vesicles (larger than 300 nm) and swarm detection (detection of an event only when multiple vesicles simultaneous present to the laser and generate an event signal) of smaller vesicles. The caveat in exosome research is that swarm detection leads to a 1000-fold decrease in concentration estimation compared to the real concentration given the detection of groups of small vesicles as a single event, compared to larger vesicles [29]. Commercial antibody-coated bead kits are available for the binding of exosomes to facilitate FACS analysis and purification.

Overall, exosome isolation using serial UC, immunoprecipitation, or the modified ExoQuick-TC technique appear to be reasonable alternatives for future research and clinical application. Appropriate quantitation and assessment of purity is necessary to ensure accuracy and validity of study results.

Exosomes as biomarkers

The diagnosis of bladder cancer currently requires cystoscopy, an invasive procedure, and/or urine cytology, which is known for its low specificity, especially for low grade cancers [30,31]. Exosomes isolated from the urine have already demonstrated differential protein expression in the setting of acute kidney injury [32], lung cancer [15], prostate cancer [33-35], and represent an up-and-coming source of potential biomarker for non-invasive diagnosis of bladder cancer. ExoCarta represents a continually-updated database for reference of completed work characterizing exosomal protein, mRNA and lipids [36].

While exosome biomarker discovery remains in its infancy for bladder cancer, several studies exist demonstrating exciting avenues for future work. Perez, et al. investigated urinary exosomal RNA from bladder cancer and normal control patients using a whole transcriptome array [37]. Initial results found 4,102 transcripts, with 55% overlap between cancer and control patients. Fifteen gene targets were then selected based on differential expression between cancer and control patients for analysis with PCR, revealing two genes present only in cancer patients (GALNT1 and LASS2) and two genes present only in control patients (ARHGEF39 and FOXO3) (**Table 1**).

While studies of urine exosomal RNA expression are limited, several studies have investigated urinary exosomal protein expression [30,38,39] (**Table 2**). Welton and coworkers studied exosomal protein expression of HT1376 bladder cancer cells (a muscle invasive, high grade line) [30]. Initially 353 exosomal proteins were identified via mass spectrometry, of which the expression of 18 proteins of "biologic interest" were subsequently verified by Western blot and flow cytometry. These proteins were then assessed in patient samples via flow cytometry for four healthy controls and three bladder cancer patients. This analysis demonstrated an increase surface expression of several proteins in bladder cancer compared with control. These proteins have been implicated in several malignancies: MUC1 (colon, breast, pancreatic cancer) [40], β1 integrin (melanoma, breast, prostate cancer) [41], α6 integrin (melanoma, breast, prostate cancer) [41], CD36 (glioblastoma)[42], CD44, CD10 (bladder, leukemia, lymphoma), 5T4 (colorectal, gastric, ovarian), basigin (melanoma), and CD73 (breast, melanoma, bladder).

Table 1. Candidate bladder cancer exosomal RNA markers.

Gene symbol (reference)	Exosomal upregulation	Function
ARHGEF39 [37]	Normal cells	Activator of Rho GTPases, signal transduction
FOXO3 [37]	Normal cells	Transcription factor, trigger of apoptosis
GALNT1 [37]	Cancer cells	Initiate glycosylation in Golgi apparatus
LASS2 [37]	Cancer cells	Regulation of cell growth

Smalley, et al. performed mass spectrometry on urinary exosomes isolated from patients with bladder cancer (n = 4) and normal heathy controls (n = 5) and found 307 proteins expressed in all nine samples [39]. Differential expression was found in nine proteins, eight of which were enriched in bladder cancer, and one of which enriched in normal controls (**Table 2**).

Chen and colleagues compared urinary exosomal protein expression between bladder cancer and inguinal hernia patients [38]. A pooled comparison of urine from nine bladder cancer patients versus nine hernia patients evaluated differential expression of exosomal proteins via mass spectrometry, and demonstrated a total of 2,964 urinary microparticle proteins. Of these proteins, there was overlap with 282 of the 353 (80%) identified in Welton's study, indicating origin of the patient exosomal proteins from bladder cancer cells. Delving further, Chen explains that 168 of the 2,964 proteins had differential expression between hernia and cancer patients, of which 17 were also present in Welton's study and all were upregulated in bladder cancer patients.

Three of these markers identified by Chen are of particular interest, including HBA, HBB and TACSTD2. Each of these exosomal proteins was both upregulated in bladder cancer compared with hernia, as well as in bladder cancer when compared to exosomes isolated from patients in the setting of UTI and/or hematuria. As these clinical entities are frequent in the bladder cancer population and can increase the amount of protein in the urine 1000-fold [39], biomarkers that retain their ability to differentiate cancer from non-cancer in the setting of hematuria would be especially valuable. HBA and HBB are hemoglobin alpha 1 and beta, respectively, and obviously a component found in blood, limiting their utility as a bladder cancer biomarker. TACSTD2 is a cell-surface glycoprotein that is not present in blood, demonstrates minimal to absent expression on normal cells, and has been shown to be overexpressed in a variety of late stage malignancies including gastric, oral, pancreatic, colorectal, and ovarian carcinoma [38].

In each of the aforementioned studies of exosomal biomarkers, a critical weakness is the low patient numbers used in the analysis. As this is an emerging area of research in bladder cancer these studies should

guide future endeavors, with additional studies with larger, diverse patient populations warranted to confirm and expand upon these results.

Table 2. Candidate bladder cancer exosomal protein biomarkers.

Gene symbol (reference)	Fold difference (BC/NC)	Exosomal upregulation	Function
H4 [38]	79.31	Cancer cells	Histone H4, core component of nucleosome, transcription regulation
HBD [38]	50.74	Cancer cells	Hemoglobin delta, transport of oxygen
FINC [38]	45.76	Cancer cells	Involved in cell adhesion and migration
H2AY [38]	37.57	Cancer cells	Variant histone 2A, represses transcription in subset of nucleosomes
H33 [38]	31.64	Cancer cells	Variant histone H3, transcription regulation
HBA [38]	28.64	Cancer cells	Hemoglobin alpha, transport of oxygen
S10A8 [38]	20.92	Cancer cells	Regulation of inflammation/immune response, induces neutrophil chemotaxis
PPB1 [38]	18.74	Cancer cells	Alkaline phosphatase, dephosphorylation
S10A9 [38]	17.87	Cancer cells	Regulation of inflammation/immune response, induces neutrophil chemotaxis
SDC1 [38]	17.83	Cancer cells	Cell surface proteoglycan, links cytoskeleton to extracellular matrix
Resistin [39]	16	Cancer cells	Suppress insulin effect to uptake glucose in adipose cells
GTR1 [38]	15.2	Cancer cells	Glucose transporter, possibly constitutive uptake
RTN4 [38]	15.11	Cancer cells	Growth regulatory factor, negative regulator of axon-axon adhesion/growth
SPRR3 [38]	14.12	Cancer cells	Cross-linked envelope protein of keratinocytes
EGFR [38]	12.45	Cancer cells	Oncogene active in multiple malignancies
EPS8L2 [39]	11	Cancer cells	May play a role in membrane remodeling of actin cytoskeleton
GTPase Nras [39]	11	Cancer cells	Possesses intrinsic GTPase activity
MUC4 [39]	10	Cancer cells	Ability to promote tumor growth due to repression of apoptosis
TACSTD2 [38]	8.02	Cancer cells	Cell-surface receptor, transduces calcium signals
UAP56 [38]	7.99	Cancer cells	Involved in nuclear export of mRNA
AT1B3 [38]	6.97	Cancer cells	Maintaining electrochemical gradients of Na^+ and K^+ ions across plasma membrane
EPS8L1 [39]	6	Cancer cells	Substrate for epidermal growth factor receptor, function unknown
retinoic acid-induced protein 3 [39]	4	Cancer cells	May be involved differentiation and maintaining homeostasis of epithelial cells
alpha subunit of GsGTP binding protein [39]	3	Cancer cells	GTPase activity, G-protein coupled receptor signaling pathway
EH-domain-containing protein 4 [39]	3	Cancer cells	Role in early endosomal transport
Galectin-3-binding protein [39]	0.33	Normal cells	Promotes integrin-mediated cell adhesion. May promote host defenses against tumor cells.
5T4 (trophoblast glycoprotein) [30]	NR	Cancer cells	Antagonist of Wnt/β-catenin signaling pathway
CD10 (neprilysin) [30]	NR	Cancer cells	Zinc-dependent metalloprotease.
CD147 (Basigin) [30]	NR	Cancer cells	Extracellular matrix metalloproteinase inducer.
CD36 (thrombospondin) [30]	NR	Cancer cells	Platelet adhesion, cell adhesion
CD44 (Indian blood group) [30]	NR	Cancer cells	Cell-cell interaction, cell migration. Lymphocyte activation.
CD73 (5' nucleotidase) [30]	NR	Cancer cells	Conversion of AMP to adenosine
MUC1 [30]	NR	Cancer cells	Epithelial mucous barrier. ILK signaling pathway.
α6 Integrin [30]	NR	Cancer cells	Cell-cell and extracellular matrix interactions
β1 Integrin [30]	NR	Cancer cells	Cell-cell and extracellular matrix interactions

BC: Bladder cancer cells. NC: Normal urothelial cells. NR: Not reported

Exosomes in cancer progression

Exosomes transmit a cell-to-cell signal from their bladder cancer cells of origin to other cells, via surface protein interaction and deposition of host cell nucleic acids (**Fig. 2**). Our lab has previously demonstrated that, *in vitro*, bladder cancer exosomes isolated from SW780 cells are internalized by recipient cancer cells through interaction of heparin sulfate proteoglycan coreceptors and receptor-mediated endocytosis [43].

Beckham and colleagues recently published the first study to demonstrate that exosomes isolated from the urine of patients with high grade bladder cancer promote tumor progression by way of urothelial cell migration and angiogenesis [44]. In their study, exosomes were isolated from the urine of patients with high grade bladder cancer who were undergoing radical cystectomy, as well as healthy controls. Following isolation, these exosomes were applied to human umbilical vein endothelial cells (HUVEC) to assess tube formation (angiogenesis) as well as HUVEC and 5637 cells, *in vitro*, to assess migration in a wound assay. With application of cancer exosomes there was evidence of increased angiogenesis and cell migration, which were not present with application of exosomes from healthy controls. Profiling of bladder cancer cells lines and exosomes identified EGF-like repeat and discoidin I-like domain-3 (EDIL-3), a protein known to enhance angiogenesis and cell migration, as particularly abundant. When *in vitro* bladder cancer cell lines were transfected with a short hairpin RNA (shRNA) to EDIL-3, exosomes harvested from these cells had decreased EDIL-3 levels, and did not alter angiogenesis or cell migration in HUVEC or 5637 cell lines. In comparison, exosomes from bladder cancer cell lines transfected with scrambled shRNA continued to promote angiogenesis and cell migration. Overall, this study demonstrated that exosomes isolated from patients with high grade bladder cancer are capable of inducing an aggressive phenotype with characterized by tumor progression via angiogenesis and cell migration via an EDIL-3-mediated pathway.

Yang, et al. investigated the effects of exosomes isolated from the T24 muscle-invasive bladder cancer cell line on bladder cancer cell proliferation and viability [45]. They found that in a dose- and time-dependent manner, bladder cancer exosomes applied to grade II (5637) and grade III (T24) bladder cancer cells induced proliferation. Exosome treatment also decreased rates of apoptosis compared to control, with a rate decrease from 4.59% to 0.36% and 4.47% to 0.33% at 72 hours, for 5637 and T24 cells, respectively. On analysis of T24 cell mRNA and protein expression following exosome treatment, expression of anti-apoptotic genes Bcl-2 and cyclin D1 were increased, while expression of pro-apoptotic gene Bax was downregulated. Caspase-3 protein expression, also a pro-apoptotic gene, was also downregulated, while mRNA expression remained unchanged. There was also found to be an increased expression of in survival signaling pathways via increased expression of phosphorylated Akt and ERK1/2, similar to results of a study demonstrating increased tumor cell proliferation via activation of these pathways via gastric-cancer exosomes [46].

Exosomes in therapeutics

Aside from utilizing exosomes as biomarkers for diagnosis of bladder cancer, an exciting prospect is the use of exosomes as a delivery vector to effect target cell function. Exosomes provide a route for delivery of RNA into the cell, which is typically degraded in the blood by nucleases [47]. Our lab has already demonstrated that bladder cancer cells exposed to exosomes internalize them via receptor mediated endocytosis [43]. Intricate methods have been developed to localize self-derived exosomes (to reduce immunogenicity) from dendritic cells to the brain using a targeting moiety, allowing for the intravenous delivery of exosomes that are able to cross the blood-brain barrier and decrease target mRNA and protein expression in the brains of mice [48,49]. Similar techniques have been used to target exosomes packaged with miRNA to EGFR-expressing breast cancer cells in mice [50]. While these techniques are exciting for the treatment of systemic disease, in terms of treatment of bladder cancer there is an established precedent for intravesical delivery of therapeutic agents, allowing initial research and treatment to forgo the development of targeting moieties. While unintended effect on normal urothelial cells is a concern, it appears that multiple bladder cancer cell lines internalize labeled exosomes at a ≥ 50-fold higher rate than normal urothelial cells in culture [51], implying inherent cancer selectivity over normal cells. Using human embryonic kidney cell exosomes, our lab has electroporated and loaded PLK1 small interfering RNA (siRNA) into the exosomes, and used them as

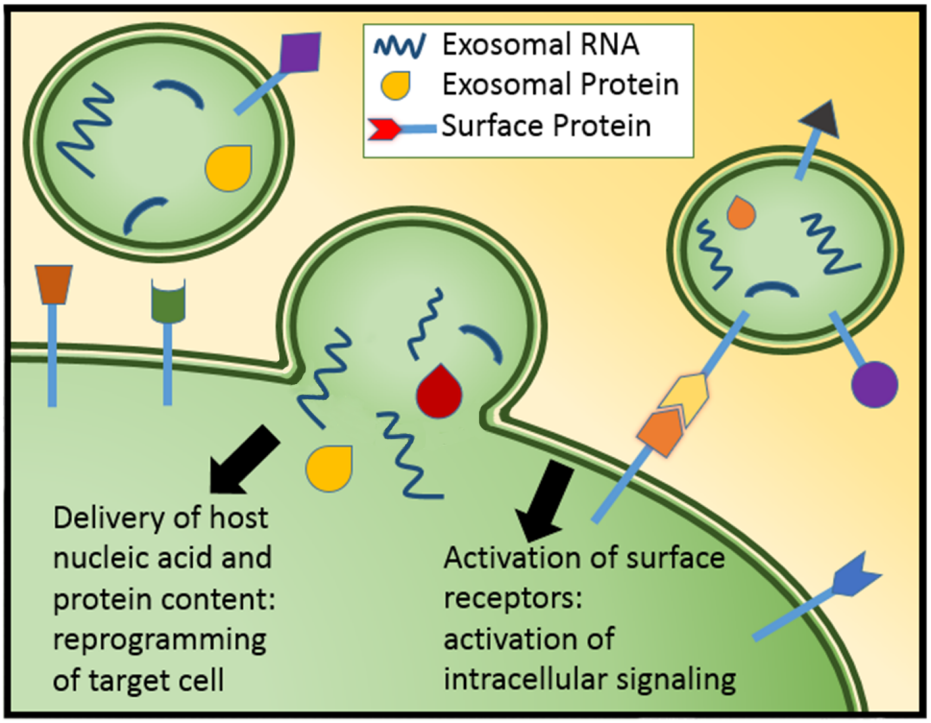

Figure 2. Schematic of exosomal-mediated cell-to-cell signaling. Exosomes transmit host-cell signals via fusion of the plasma membrane with deposition of host cell contents (i.e., cytosolic proteins and RNA) into the target cell. Intracellular signaling in the target cell may also be stimulated by plasma membrane surface protein-receptor interactions.

a delivery vector for the siRNA to bladder cancer cells *in vitro* [51]. UMUC3 bladder cancer cells treated with PLK1 siRNA-containing exosomes had a significant decrease in PLK1 RNA expression (approximately 40%) at 24 and 48 hours compared to UMUC3 cells treated with control scrambled siRNA-containing exosomes.

Another possible application of exosomes is loading with pharmacologic agents [52]. Work has already been done loading therapeutic nanoparticles into liposomes [53]. The goal of this method of treatment is to increase the concentration of drug to the tissue of interest, while minimizing toxic effects to normal tissues. Chemotherapeutic agent doxorubicin has been electroporated into exosomes previously engineered to target iRGD-expressing breast tumor cells [54]. When delivered systemically these exosomes concentrated preferentially in breast cancer tumor cells in a nude-mouse model, and demonstrated significantly decreased tumor growth over 21 days, compared to mice administered an equivalent systemic dose of doxorubicin. Loading exosomes with chemotherapeutic agents against bladder cancer may provide a method for significantly decreasing dosage requirements and adverse side effects in a patient population with pre-existing significant comorbidity.

With any application of biologic in therapeutics, a pure sample, free of contamination is necessary to truly assess the effect of the intervention and provide a safe treatment to recipient patients. Further, as stated earlier, exosomes can contain up to 10,000 net nucleotides of nucleic acids and 100 proteins [18], any of which may also effect recipient cell biology. Cell lines grown in culture with fetal bovine serum could potential incorporate extracellular proteins, while lines transfected via viral vectors could hypothetically transmit viral elements by means of their resultant exosomes. Stringent analysis and control measures are necessary to ensure any outcome from exosomes as therapeutics are the result of the intervention, and not from inherent properties of the exosomes or unanticipated alterations as a result of their production.

Future thoughts and conclusions

There are several hurdles between the current state of exosome research and clinical application. Classically, exosome isolation requires ultracentrifugation, which is time-consuming and expensive, limiting its clinical applicability. While the ExoQuick-TC technique provides less 'in-hand' time without the ultracentrifugation, the requirement of an overnight incubation still makes its use a timely endeavor. Regardless of isolation technique utilized, a pure sample is necessary for accurate exosomal mRNA and protein analysis. New, efficient, and quick techniques to isolate exosomes would advance this field both in terms of clinical applicability, as well as research and discovery.

Identification of markers specific to bladder cancer exosomes is also needed. Exosomes isolated from patient urine originate from multiple sources, including kidney, prostate, and possibly distant malignancies. This is of particular importance if urinary exosomes demonstrate proteomic changes in patients with non-urinary tract disease, as has been demonstrated already in kidney injury[32] and lung cancer[15]. Candidates for these bladder cancer exosome-specific markers are currently lacking. Perez's whole transcriptome array demonstrated only 4 genes (RHBDL3, LOC100130701, LOC100129952, HS.85445) that were present in all patient samples, cancer and control [37], however all of which are currently poorly characterized in human physiology.

The use of exosomes in therapeutics for bladder cancer is a field still in its infancy. Mesenchymal stem cells (MSC) can be utilized as a mass-producer of exosomes [55], which can be loaded with therapeutic contents via multiple techniques, including electroporation, MSC transfection, chemical-based transfection, or incubation of the target molecule [52]. While siRNA has already been loaded in exosomes and shown to effect bladder cancer cells [51], research into additional RNA targets and *in vivo* models should be performed. As a field that utilizes intravesical therapy regularly, investigation into packing our currently intravesical chemotherapeutic agents into exosomes would be a natural progression to attempt to improve oncologic results while minimizing dosage and side effect profile.

Urinary exosomes hold promise to play a prominent role in the future diagnosis and treatment of bladder cancer, with encouraging initial steps having been taken to identify potential exosomal biomarkers and intravesical therapeutic techniques.

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