

Cellular endocytosis and gene delivery

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**Abstract**

Endocytosis is the process by which cells take up macromolecules from the surrounding medium. The best characterized process is the so-called clathrin-dependent endocytosis, although much is also currently known about clathrin-independent endocytic processes such as those involving caveolae and lipid rafts.

An understanding of endocytosis and the cellular trafficking which occurs thereafter has a great deal of relevance to current molecular medicine. Gene therapy, which is presently being investigated for its therapeutic potential in treating immunodeficiency and metabolic diseases, cancer, and heart disease, employs a variety of viral and non-viral vectors which can be delivered to the target cells of the body and are subsequently endocytosed and disassembled.

Via a variety of vectors, genes can be delivered to organs *in vivo* or cells *ex vivo*. Various routes by which such vectors can be delivered have been investigated. The mechanisms by which vectors such as adenoviruses, adeno-associated viruses, retroviruses and liposomes enter the cell are being increasingly focused upon as the effort to increase the efficiency of gene therapy continues. This review focuses on mechanisms of endocytosis and how they relate to the internal trafficking of viral and non-viral vectors in gene therapy.

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## Cellular Endocytosis

Cells are able to take up macromolecules from the surrounding medium by endocytosis. In this process, the material to be internalized is surrounded by an area of plasma membrane, which then buds off inside the cell to form a vesicle containing the ingested material. The best-characterized form of this process is receptor-mediated endocytosis, which provides a mechanism for the selective uptake of specific macromolecules. The macromolecules to be internalized first bind to specific cell surface receptors. These receptors are concentrated in specialized regions of the plasma membrane, called clathrin-coated pits (1),(2) which can be internalized to form clathrin coated vesicles (CCV). There is evidence that cells also possess clathrin-independent endocytosis pathways. One pathway of clathrin-independent endocytosis involves the uptake of molecules in small invaginations of the plasma membrane called caveolae, which have been implicated in cell signaling and a variety of transport processes, including endocytosis (3).

The CCV have been extensively studied and their characteristics are well described. They comprise a clathrin coat which is linked to cargo-bearing receptors via a heterotetrameric adaptin complex. The adaptin complex is comprised of HA2 or AP-2 subunits which in turn is made up of 100 kDa  $\alpha$  and  $\beta$  adaptins together with smaller  $\mu$ 2 (50 kDa) and  $\sigma$ 2 (17 kDa) subunits.  $\alpha$ -adaptin and  $\beta$ -adaptin both contain clathrin-binding sites.

Signals for the incorporation of cargo receptors into plasma-membrane-derived CCV have been defined based upon examination of their protein sequences. The best

characterized portion of the protein is the YXXØ motif where Ø represents a bulky hydrophobic amino acid (4). Such signals are found in both constitutively endocytosed receptors (e.g. transferrin receptor) and those such as the EGF receptor, which are only endocytosed following ligand binding. In the latter case, ligand binding causes phosphorylation of the receptor and a conformational change that results in exposure of the internalization signal. The  $\mu$ 2 adaptin subunit has been shown to interact with these tyrosine-based internalization signals. An alternative internalization signal, dileucine, does not interact with the  $\mu$ 2 chain in vitro, although there is evidence indicating an interaction with AP-2 (5). An exception to the model of AP-2-mediated recruitment of receptors to CCV is provided by G-protein-coupled receptors. In this instance the activated proteins are recruited by b-arrestin, which is believed to function as a specialized adaptor protein as it also binds to clathrin (6).

The process of CCV formation is generally agreed to be cytosol- and ATP-dependent with the additional requirement for multiple GTP-binding proteins of which the best characterized is dynamin (7). As well as binding directly to a-adaptin, dynamin binds to proteins that contain Src homology 3 (SH3) domains through interactions with its proline rich C-terminus (8). The growth-factor-receptor-binding protein Grb2 also binds dynamin via an SH3 domain and activates its GTPase activity in a synergistic manner with phosphoinositides (9). This requirement for proteins such as dynamin is apparent upon disruption of EGF-receptor recruitment of Grb2. Indeed, upon inhibition of Grb2 binding via microinjection of a peptide corresponding to its SH2 domain, receptor endocytosis is impaired (10). Recent work has also shown that the F-actin cytoskeleton is required for

the formation and internalization of clathrin coated pits. Reflectively, via fluorescence microscopy, disruption of F-actin dynamics has been shown to significantly impede clathrin-coated mediated endocytosis (11).

In contrast, caveolae typically appear as rounded plasma membrane invaginations of 50-80 nm in diameter on electron microscopy sections. Their composition, appearance and function are cell-type dependent. In endothelial cells, caveolae can be more constricted at the mouth, or they may contain a diaphragm that restricts diffusion (12). In muscle cells, caveolae are often observed in the form of composite clusters or linear rows of multiple flask-shaped units involved in the formation of T-tubules (13). Although caveolae do not show an electron-dense layer on their cytosolic surface in thin-section electron micrographs, they do have a protein 'coat' composed primarily of a protein called caveolin-1 (or caveolin-3 in muscle cells) (14).

Caveolins are integral membrane proteins of 21 kDa. They have an unusual topology in that the cytosolic N- and C-terminal domains are cytosolic connected by a hydrophobic sequence that is buried in the membrane but does not span the bilayer (15). Caveolins are palmitoylated in the C-terminal segment, they can be phosphorylated on tyrosine residues, they bind cholesterol, and they form dimers and higher oligomers (16), (17),(15).

Caveolins are essential for the formation and stability of caveolae: in their absence no caveolae are observed, and, when expressed in cells that lack caveolae, they induce

caveolar formation (18). During endocytosis of caveolae, caveolin-1 moves along with the vesicles into the cytosol with no visible remnant left in the plasma membrane (19). Recent data indicate that the presence of caveolin-1 may actually slow down the endocytic process via caveolae (20). This implies that the role of caveolin may be to stabilize caveolae and thus to counteract an underlying raft-dependent endocytic process. The finding that certain ligands internalize via a lipid raft-dependent but clathrin-independent mechanism in cells that lack caveolae has led to the postulation that lipid rafts can internalize independently of caveolae (21). In this context, it is significant that caveolin knockout mice survive relatively well, although their cells do not have detectable caveolae (3). More recently, caveolin has been shown to be not only involved in endocytosis, but in cellular processes such as calcium signaling, homeostasis and cholesterol transport. Hence, these domains may actually have a multitude of significant functions within the cell and may serve as key regulatory domains (22), (23).

In addition to caveolins, caveolae are known to contain dynamin (24), the same GTPase that is involved in the formation of clathrin-coated vesicles described previously.

Dynamin has been localized to the neck of flask-shaped caveolar indentations, and is therefore likely involved in pinching off the caveolar vesicle via a mechanism similar to its role in coated vesicle fission (25).

In recent years evidence has been accumulating that in addition to caveolae, several other clathrin-independent endocytosis pathways exist and that they may have a more important role in cell function than previously suspected. In particular, much effort is

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focused on understanding membrane lipid rafts and microdomains (26). Rafts have been defined as dynamic assemblies of cholesterol and glycosphingolipids that are present in the lateral plane of membranes. The hypothesis that lipid rafts harbor a functional role in membrane transport was initially based on two observations. Firstly, glycosphingolipids were found to be preferentially targeted to the apical pole of polarized cells (27).

Furthermore, glycosphingolipids were shown to be part of detergent-resistant membranes (DRMs) that also contained apically targeted GPI-anchored proteins (28). Thus, the hallmarks of the raft hypothesis are the spontaneous partitioning of lipids and proteins in discrete membrane domains (microdomains), a behavior based on their intrinsic physico-chemical characteristics and that these microdomains, along with their associated proteins can be recovered as detergent-resistant complexes by biochemical flotation experiments.

Recently, two membrane proteins, flotillin 1 and flotillin 2, have been found to coassemble to form microdomains with many of the predicted properties of lipid rafts (29). While much remains unknown about the role of these proteins in endocytosis, the microdomains formed upon their interaction have been shown to invaginate and form vesicles via clathrin and caveolae independent mechanisms (30, 31).

Furthermore, Cheng et al demonstrated that several different clathrin-independent mechanisms of endocytosis occur in Chinese hamster ovary cells and that each has a distinct need for sphingolipids. Indeed, depletion of glycosphingolipids, a subgroup of sphingolipids, was found to differentially affect and decrease caveolar, RhoA and Cdc42 dependant endocytosis (4, 32). Despite the fact that by some estimates, as much as 50% of the overall endocytic activity of the cell (33) occurs via such clathrin-independent

mechanisms, the lack of well-defined systems has prevented the detailed identification and understanding of the precise function of these alternate pathways despite the surge in interest in identifying the roles of such 'raft constituents' in endocytic platforms (34).

Phagocytosis and macropinocytosis are forms of endocytosis in which larger molecules are engulfed into the cell. Large molecules such as pathogens, dust particles, and cell debris can be ingested by specialized immune cells, upon which they are encapsulated by a vesicle termed a phagosome. Phagosomes mature to fuse with lysosomes, ultimately becoming vacuoles within which the engulfed pathogen is degraded. Single proteins and fluid particles can be taken up via macropinocytosis, which involves the invagination of the plasma membrane and trafficking to the endosomes. Ultimately, these processes are more non-specific and involve larger particles than the aforementioned clathrin- and caveolae-dependant endocytosis pathways (35).

Following their internalization, endocytosis vesicles rapidly fuse with endosomes. Once again, the best studied pathway is that of the CCV. The delineation of the various components of the pathway in the cytosol and the regulation of the steering of different molecules to their respective targets is not well understood (1). The most accepted model is internalization followed by the formation of early endosomes, late endosomes and lysosomes (36). This classification reflects a definitions of endocytic compartments based on pulse or pulse–chase protocols and on the presence of marker proteins associated with these respective compartments.

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The early endosome is the major sorting station on the endocytic pathway. From this organelle, material can be directed towards the pathway of recycling to the plasma membrane (e.g. transferrin), to later endocytic compartments and to regulated secretory vesicles (37). Studies on transferrin-receptor recycling have defined a tubular 'recycling endosome' into which transferrin receptors enter subsequent to sorting from late-endosome-directed material (12). This recycling endosome is typically less acidic (pH 6.4–6.5) than the sorting endosome (pH 6.0) and exhibits a pericentriolar localization (38).

Late endosomes represent the point on the endocytic pathway where the cation-independent (ci-) mannose 6-phosphate receptor (M6PR) is most concentrated. Degradative enzymes are active in this compartment, but are more concentrated in lysosomes (39). The M6PR protein has been found to be concentrated on the internal vesicles of late endosomes, while lysosomal membrane proteins are on the external membrane (40). Transfer of material between late endosomes and lysosomes appears to be a direct fusion event which results in a transient hybrid organelle (41). Lysosomes are also multivesicular, but completely lack M6PR.

Among the proteins that are present in the various compartments of the endocytic pathway are several small GTPases. These proteins have been particularly scrutinized because of their usefulness as molecular markers for various types of endosomes. Rab5, one such GTPase, regulates the rate of clathrin-dependent endocytosis at the plasma membrane (42) and could be used as a marker for early sorting endosomes in addition or

in conjunction with the transferrin receptor. Rab11 localizes to a pericentriolar subpopulation of transferrin-labelled endosomes (recycling endosomes) (43).

Thus, the process of endocytosis is a complex process that has yet to be completely defined despite significant progress in our understanding of its various components. The various endocytic pathways, the interactions between the numerous proteins that are involved in the process and, above all, the mechanisms that direct cargo selection, attachment and transport to the various organelles of the cell have yet to be characterized.

As various medications enter the cell and are processed via these endocytic pathways, an increased understanding of endocytosis and the cellular trafficking which occurs thereafter has a great deal of relevance to current molecular medicine. Indeed, gene therapy, which is presently being investigated for its therapeutic potential in treating immunodeficiency and metabolic diseases, cancer, and heart disease, employs a variety of viral and non-viral vectors which can be delivered to the target cells of the body and are subsequently endocytosed and disassembled. The mechanisms by which vectors such as adenoviruses, adeno-associated viruses, retroviruses and liposomes enter the cell are being increasingly focused upon as the effort to increase the efficiency and safety of gene therapy continues.

The endocytic route by which a molecule, virus or drug complex enters the cell is highly dependant upon its size, charge and composition, as well as upon the cell type into which it is entering. For example, while specialized immune cells may non-specifically take up

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molecules such as the remnants of other cells which have undergone apoptosis via phagocytosis, specific complementary sequences are needed by hormones to enter the target epithelial cells via receptor-mediated endocytosis. Furthermore, while approximately 100 nm is the optimal size for the uptake of a molecule via the numerous types of clathrin-mediated endocytosis, particles of 500 nm in size are typically endocytosed via caveolae-dependant endocytosis (44).

As the endocytic route by which a drug is able to be taken up can influence which cell type it targets, its levels of degradation subsequent to cell entry, and the efficiency by which it can enter the cell, suitable vectors must be found depending upon the purpose of the medication being administered. For example, drugs can be encapsulated in large liposomes and coated with opsins in order to target them for phagocytic uptake, while others can be complexed with a peptide ligand for receptors overexpressed by tumor cells. Such allows for manipulation of the various endocytic pathways to achieve specificity and efficiency.

### **Viral vectors and their pathways into the cell**

Adenoviruses carry double stranded DNA and upon infection, introduce their DNA into the host cell. Unlike adeno-associated viruses, the genetic material of adenoviruses is not incorporated into the genome of the target cell. Thus, while the viral genes are transcribed, they are not replicated upon cell division and they will not be passed on to daughter cells. Despite the fact that any adenoviral gene therapy treatment would be

transient, such viruses are nevertheless being considered for their role as vectors for therapeutic genes to help treat infarcted areas of the heart, tumors, and other diseased target tissues.

Adenoviruses enter target cells via receptor-mediated endocytosis. Penton and fiber proteins of the capsid of the virus interact with the coxsackievirus-adenovirus receptor cell surface protein to initiate cell binding (45),(46). These receptors are then localized to clathrin-coated pits via an NPXY motif of  $\beta_3$  and  $\beta_5$  integrin subunits of the cell.

Dynamin, a cytosolic GTPase, mediates the endocytosis of the virus into the cell by facilitating the constriction and budding off of the clathrin-coated pits. Proceeding endocytosis, the proteins of the viral capsid are shed and the pH of the viral endosome decreases as a result of proton pumps. At pH 6.0, the virus is able to escape from the vesicle and enter the cytosol. The capsid proteins of the virus are trafficked to the nucleus by microtubules and further dismantled. While the majority of the proteins of the capsid remain at the periphery of the nucleus, the viral DNA is extruded through the nuclear pores (47).

Adeno-associated viruses (AAV) are also being considered in the search for efficient gene therapy vectors due to their non-pathogenic properties. In contrast to adenoviruses, adeno-associated viruses carry single stranded DNA which is inserted into the genome of the host cell. These viruses enter host cells via a mechanism very similar to that of adenoviruses. Heparan sulfate proteoglycan is the primary receptor to which adeno-associated viruses attach, while fibroblast growth factor receptor 1 and  $\alpha_v\beta_5$  integrin co-

receptors facilitate the entry of the virus into the cell. Adeno-associated viruses enter the cell through the internalization of clathrin-coated pits and like adenoviruses, escape endosomal degradation via acidification of the late endosome. Adeno-associated viruses are trafficked through the cytoplasm and uncoated in the nucleus. Their single stranded genome is converted to double stranded DNA and subsequently inserted into chromosome 19 (48). The clinical relevance of such work was demonstrated by Spencer et al who successfully delivered a lysosome enzyme across the blood brain barrier of mice via a lentivirus delivery system targeting the receptors of the central nervous system. Such work is extremely significant in that this area is difficult to target under normal circumstances due to the fact that proteins are unable to cross this physiological barrier, yet the delivery of such macromolecules may be necessary for the treatment of many neurological disorders (49).

These two virus types remain among the most commonly used and studies gene therapy vectors. Although issues with these vectors remain, namely the immune response against the viruses, transient expression, and sub-optimal gene delivery efficiency due to lack of the respective primary receptors on target cells, some success has been observed in clinical and pre-clinical trials in which adenoviruses and adeno-associated viruses have been used. For example, Wohlfahrt et al demonstrated that glioblastoma tumors could be effectively killed in mice via injection of the tumor cells with an adenovirus vector expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (50). Furthermore, adenoviral vectors harboring genes for vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are currently being used in clinical trials

aimed at treating ischemia (51, 52). However, such trials have had limited success thus far, possibly due to the brief expression of the encoded angiogenic genes following delivery of the virus. Clinical trials in varying stages which involve adenoviral vectors are also being used to treat cystic fibrosis, hemophilia, arthritis and heart disease (53),(54). In several studies, cystic fibrosis transmembrane regulator cDNA was delivered to the epithelial cells of the lumen of the airways leading to the lungs. These trials served as a significant base which aided in establishing optimal dosages of the vector and currently, further trials similar in nature are now being conducted (54).

Retroviruses are another category of viruses that are used as vectors in gene therapy and several features of retroviral gene therapy such as long term expression and absence of immunogenicity are very attractive (55). However, in clinical trials of gene therapy for X-linked severe combined immunodeficiency there have been reports of leukemia associated with treatment (56-58) and this has led many researchers to look for alternatives. Another category of viral vectors which are related to retroviruses and which are gaining increased attention because of potential better safety and efficiency are lentiviruses (55, 59).

#### **Non-viral Vectors and Naked DNA**

Small molecules such as liposomes have also been focused upon in the effort to find efficient vectors for gene therapy. Liposomes are synthetic phospholipid vesicles that enclose an inner aqueous compartment into which drugs or other large molecules can be

placed. As such large molecules would normally be unable to cross the lipid bilayer of the cell, carriers such as liposomes are essential in order for their successful delivery.

These carriers have been found to enter cells via receptor-mediated endocytosis via the clathrin-coated pit pathway as well as via direct fusion with the membrane (60),(61).

While conventional liposomes are ultimately cleared from the bloodstream by the reticulo-endothelial system or, upon endocytosis, are significantly degraded by lysosomes, new approaches to increase the delivery efficiency and prevent degradation of the enclosed molecules are currently being investigated (62). For example, the usage of pH-sensitive liposomes, in which the low pH of the endosomal compartment results in the destabilization of the endosomal membrane and the subsequent liberation of the enclosed drug or molecule, has been found to be more efficient than that of conventional liposomes (63).

In a recent study, vitamin-A coupled liposomes were used to deliver small interfering RNA (siRNA) to hepatic stellate cells of rats in order to examine the efficacy of this treatment on liver cirrhosis(64). Hepatic stellate cells, when activated, produce and secrete the collagen which can accumulate and lead to the development of cirrhosis. As hepatic stellate cells take up circulating vitamin A via endocytosis, liposomes coupled to this molecule were used to deliver siRNA targeting gsp46, a chaperone protein required for the secretion of collagen from these cells. It was found that the liposomes containing the siRNA were in fact taken up by the hepatic cells and successfully suppressed the secretion of collagen. Hence, this strategy remains extremely promising with respect to

clinical applications, as it resulted in prolonged survival and the near complete resolution of the liver fibrosis that had been induced in the rats of this study.

Furthermore, immunoliposomes, which are liposomes linked to antibody fragments, have been constructed and tested on a variety of tumor models. As many types of tumor cells overexpress certain growth factor receptors, immunoliposomes offer a way by which such cells can be specifically targeted and the chemotherapeutic drugs within efficiently delivered. For example, Park et al generated anti-HER2 immunoliposomes which contained the chemotherapeutic agent doxorubicin. As it was demonstrated that these constructs were in fact internalized and showed potent tumorigenic properties, similar carriers are being considered for clinical trials (60).

The delivery of naked DNA encoding various proteins and peptides has also been attempted. Plasmid DNA, which consists of a promoter and a gene of interest, is known to be taken up by the cell in a variety of mechanisms. Seternes et al recently showed that pDNA is taken up by endocardial endothelial cells of Atlantic cod via receptor-mediated endocytosis. Scavenger receptors, which bind to negatively charged macromolecules, were shown to mediate the endocytosis in this study (65). While problems with this type of vector exist, namely rapid degradation and transient expression, clinical trials are currently being conducted and have had moderate success. Trials involving the treatment of limb ischemia via the injection of naked pDNA encoding for vascular endothelial growth factor are currently underway. Electroporation, the application of an electric field to the plasma membrane in order to increase its permeability, as well as the coupling of

naked DNA to a lipid carrier are methods by which the entry of the pDNA into the cell can be more efficiently accomplished (66). Indeed, clinical trials in which cDNA/liposome complexes were delivered to the respiratory epithelia of cystic fibrosis patients have resulted in transient gene expression for the CFTR protein. Current studies are focusing on a gene delivery approach that would lead to increased gene expression and a decreased immune response (67) as well as linking the vector to peptides that enhance the transfer into the cells (68), but that also lead to the specific targeting of certain cells (69) .

The use of nanoparticles, small spheres which can be complexed to molecules such as DNA or siRNA, have been used in clinical trials to target and deliver such cargo to tumor cells (70). Nanoparticle complexes can be fused to specific ligands which can target them to a particular cell type which expresses the respective receptor, resulting in more efficient and specific uptake. In a mouse xenograft model, Li et al recently demonstrated that siRNA, which is under other circumstances quickly degraded in physiological conditions, can be encapsulated and protected within nanoparticles fused with the anisamide ligand for sigma receptors expressed by lung cancer cells. Via this system, nanoparticles more efficiently gained cellular entry and anti-EGFR siRNA was able to induce tumor cell growth inhibition and apoptosis [(71)].

Naturally-occurring cell penetrating peptides (CPPs) are also currently being considered for the promotion of efficient cellular uptake of macromolecules such as siRNA, DNA, peptides and liposomes. Specifically, an 11-amino acid peptide derived from the Human

immunodeficiency virus type I (HIV-1) Tat protein, has been found to be a potent facilitator of cellular uptake. While the method of endocytic uptake taken by the Tat peptide varies according to the size and nature of the cargo, clathrin-independent (72), clathrin-depedant (73), and macripinocytic pathways (74) have all been implicated in its internalization. Given the wide array of cargo molecules and efficiency of entry obtained via usage of these peptides, they too have become a tool for the cellular delivery required for gene regulation.

The use of these peptides and manipulation of a specific endocytic pathway, macropinocytosis, was demonstrated in an elegant study conducted by Nishimura et al (75). After searching a phage-display library, a CPP containing both a lymph-node homing sequence, as well as a cell penetrating motif was identified. The peptide was fused to a proapoptotic molecule and used to selectively target leukemia and lymphoma derived cells via the macropinocytic pathway, which has recently been shown to play a major role in the uptake of arginine-rich CPPs fused to proteins (76).

The macropinocytic pathway, as well as the utilization of CPPs has proven to be important in viral vector gene therapy also. While adenoviral vectors are desirable due to their high titer propagation and ability to enter the cell regardless of its mitotic stage, they remain less efficient vectors for cells lacking the CAR, which serves as their primary receptor. However, fusion of Adenovirus with the Tat peptide results in higher efficiency of infection of non-CAR expressing adherent blood cells. The integral role of macropinocytosis in CPP/CAR-independent endocytosis was demonstrated in that the use

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of this fusion construct in conjunction with a macropinocytic inhibitor resulted in significantly decreased Tat-Adv-dependent gene expression (77).

Ultimately, each vector type is associated with its own set of strengths and caveats when used to manipulate a given endocytic pathway and deliver cargo to target cells. While viral vectors are extremely efficient in gaining entry to the cell and utilizing the endocytic pathway components to deliver their encapsulated cargo, the immunogenicity, specificity and safety issues associated with these vectors poses constraints on their usage in a clinical setting. However, while non-viral vectors such as nanoparticles and liposomes do not elicit an immune response, they also exhibit decreased levels of efficiency with regard to cellular uptake. As such, the incorporation of certain aspects of viral vectors into the designs of synthetic vectors may optimize these compounds such that efficient manipulation of the endocytic process can be achieved.

### **Conclusion**

The natural specificity of ligands and antibodies can be harnessed and utilized via their conjugation to synthetic compounds. Such specific targeting of diseased cells is crucial to the safety and efficiency of gene therapy and remains the first of several major obstacles impeding the usage of many of the currently designed vectors in a clinical setting.

Furthermore, as cargo needs to escape from the endocytic vesicles and proceed to the nucleus after cellular uptake, synthetic compounds need to harbor a mechanism that mimics that used by viral particles to facilitate release into the cytosol. Nuclear entry of

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non-viral vectors is also a significant barrier in non-mitotic cells. While viral vectors can actively transfer their genetic material through the pores of the nucleus, synthetic vectors are currently unable to efficiently deliver their cargo through an intact nuclear envelope.

The efficiency of viral vectors makes them attractive models upon which to base the synthesis of future compounds or, in contrast, foundations to modify such that toxicity and specificity can be optimized. For example, shielding, or coating, of viral surfaces with polyethylene glycol (PEG), a hydrophilic polymer, has been found to reduce the immunogenicity of such vectors (78). Furthermore, retargeting of viral vectors via conjugation to synthetic ligands after such shielding could induce novel and targeted specificities. Also, it has been demonstrated that endosomal release of synthetic compounds such as DNA polyplexes can be facilitated via the covalent linkage of PEI to melittin analogs (79).

The delivery of nucleic acids (as well as proteins and drugs) to damaged or diseased cells has significant therapeutic implications, assuming efficient and practical vectors can be found. Hence, the mechanisms by which these molecules are taken up and processed by cells is becoming increasingly important. Despite our progress in understanding its various components, endocytosis is a complex process, much of which remains to be clarified. The need to elucidate the various pathways and steps of endocytosis gains in significance, as the efforts to efficiently deliver and to prolong the expression of various genes continue. Ultimately, while a variety of cellular uptake and endocytic strategies can be manipulated in the effort to efficiently deliver genetic material to targeted cells,

clinical usage of any one of the existing viral or synthetic vectors remains limited.

Fusions of the existing vector types, which incorporate the desirable attributes of each, may provide the most efficient means by which to obtain optimized vectors which successfully and specifically traverse the endocytic pathways of human cells.

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Table. Viral vectors and endocytic pathways.

<b>Vector</b>	<b>Receptor</b>	<b>Endocytic Pathway</b>	<b>End Destination in Cytoplasm</b>	<b>Target Cellular Organelle</b>
Adenovirus	Coxsackievirus-Adenovirus Receptor Cell Surface Protein	Clathrin-Coated Pits	Microtubules (?) / Endosome	Nucleus
Adeno-Associated Virus	Heparan Sulfate Proteoglycan, $\alpha$ V $\beta$ 5, FGFR1	Clathrin-Coated Pits	Nuclear Pores (?) / Endosome	Nucleus
Retrovirus	Transmembrane (TVA950) or glycoposphatidylinositol (GPI)-anchored (TVA800)	Lipid-Raft Dependant Pathway, Clathrin-Coated Pits	Endosome	Nucleus
Liposomes		Clathrin-Coated Pits/Membrane fusion	Endosome	Nucleus

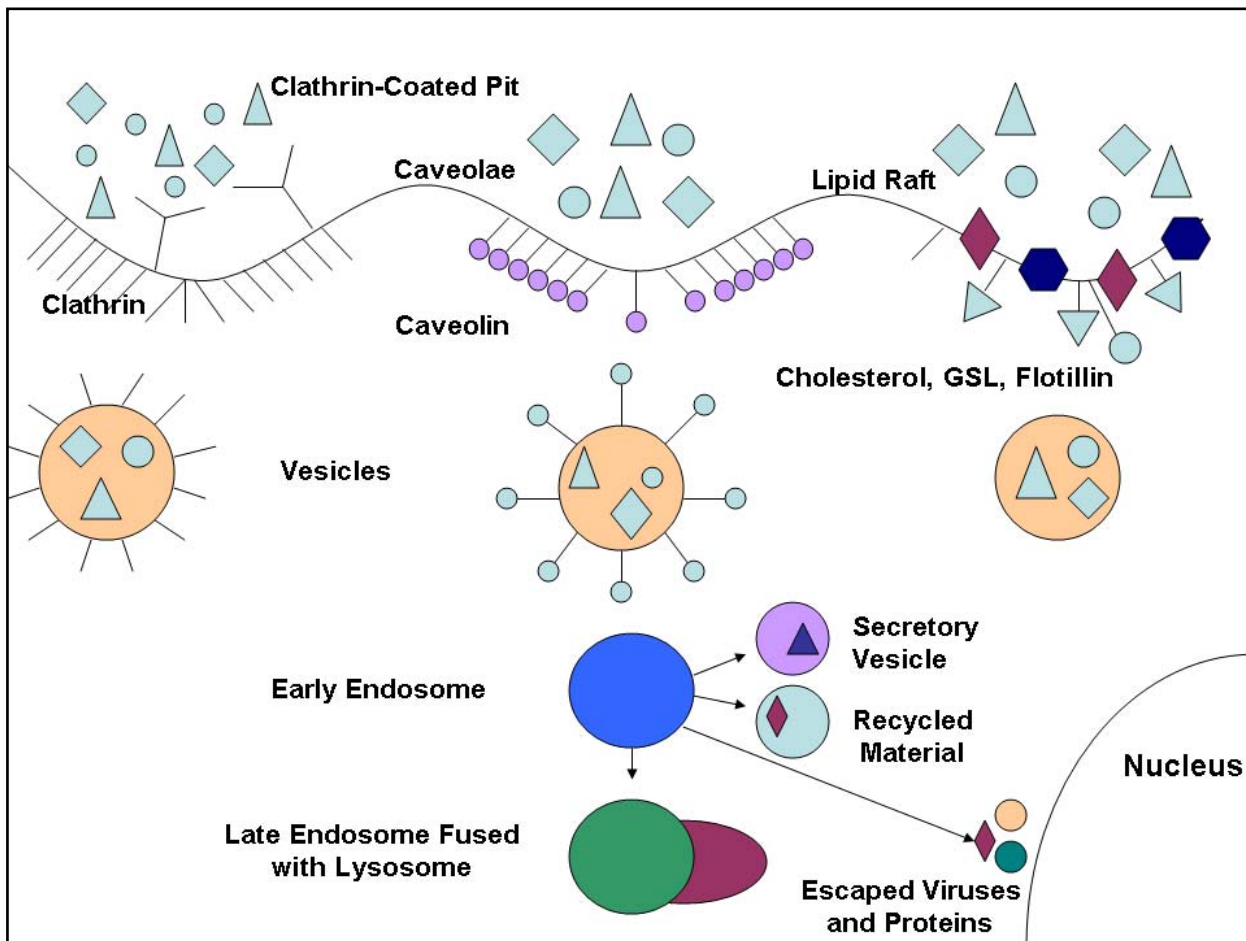
FGFR: fibroblast growth factor receptor

Figure legend

**The Endocytic Pathway in Gene Therapy.** Gene therapy vectors enter the cells via membrane structures which allow endocytosis to occur, such as clathrin-coated pits, caveolae, and lipid rafts. Clathrin coated pits are lined by clathrin, caveolae are lined by caveolin and lipid rafts are cell membrane domains that contain cholesterol, glycosphingolipids, and possibly the protein flotillin. Each structure invaginates to form a vesicle and subsequently, an early endosome. The early endosome sorts the endocytosed material into material which will be recycled back to the plasma membrane, material which will be secreted and material which will be degraded by fusion with the lysosome in later steps. Some viruses and proteins can escape lysosomal degradation via a decrease in the pH of the endosome. These particles/molecules can then be trafficked into the nucleus, where they can act to silence or activate various genes. GSL: glycosphingolipids.

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