

Cellular Uptake and Intracellular Trafficking of Oligonucleotides: Implications for Oligonucleotide Pharmacology

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One of the major constraints on the therapeutic use of oligonucleotides is inefficient delivery to their sites of action in the cytosol or nucleus. Recently it has become evident that the pathways of cellular uptake and intracellular trafficking of oligonucleotides can strongly influence their pharmacological actions. Here we provide background information on the basic processes of endocytosis and trafficking and then review recent literature on targeted delivery and subcellular trafficking of oligonucleotides in that context. A variety of approaches including molecular scale ligand-oligonucleotide conjugates, ligand-targeted nanocarriers, and the use of small molecules to enhance oligonucleotide effects are discussed.

Introduction

THE DELIVERY OF ANTISENSE and small interfering RNA (siRNA) oligonucleotides to their intracellular sites of action within tissues remains a major challenge for the oligonucleotide therapeutics field (Burnett and Rossi, 2012; Kole et al., 2012). Recent research has made it clear that the mechanisms of cellular uptake and subcellular trafficking of oligonucleotides play key roles in determining their biological effects (Varkouhi et al., 2011; Juliano et al., 2012a). Thus, this article will briefly examine basic aspects of endocytosis and intracellular trafficking and will then discuss studies connecting these processes to oligonucleotide pharmacology and therapy. In particular, we will examine the merits of certain receptor families and their trafficking pathways as targets for enhanced oligonucleotide delivery. We will also discuss the uptake and pharmacological effects of various receptor-targeted oligonucleotide conjugates or nanocomplexes. Much of the review will focus on cell-based studies, since meaningful investigations on oligonucleotide trafficking *in vivo* are rare; however, a few interesting examples will be mentioned. This review will examine studies with splice switching oligonucleotides (SSOs) as well as antisense (AS) and siRNA oligonucleotides.

Basic Essentials of Endocytosis and Trafficking

Both single and double stranded oligonucleotides usually enter cells by one of several endocytotic pathways (Doherty and McMahon, 2009; Howes et al., 2010; Varkouhi et al., 2011; Juliano et al., 2012a). Uptake via clathrin-coated pits is the

archetypal and best-studied route and many adaptor and accessory proteins for this pathway have been identified, for example, the key adapter protein AP-2. After pinching off of the coated vesicle by a dynamin mediated mechanism (Metten et al., 2009), the vesicle is uncoated under the influence of auxilin and heat shock protein 70 and is ready to begin its intracellular journey. Many physiologically significant macromolecules such as low-density lipoproteins and transferrin enter cells via the clathrin pathway and may be used as markers for this route. The caveolar pathway, involving relatively small lipid rich vesicles (<100 nanometers as compared to sub-micron sizes for other pathways) marked by hydrophobic hairpin proteins termed caveolins, has also elicited a great deal of interest (Lajoie and Nabi, 2010). Notably, the cytosolic faces of caveolae are decorated with many proteins involved in signal transduction (Sorkin and von Zastrow, 2009); however, the magnitude of the caveolar contribution to the internalization of large molecules is unclear. Cholera toxin is a widely used but imperfect marker for uptake via caveolae.

There are also several non-clathrin non-caveolin dependent routes of endocytosis that are garnering increased attention. For example, one pathway gives rise to high volume tube shaped endosomes that are enriched in glycerophosphatidylinositol (GPI)-proteins (such as the folate receptor, FR α) and that are thought to be particularly important for fluid-phase endocytosis. The acronym for this pathway is the clathrin and dynamin independent carriers/GPI-AP enriched early endosomal compartments pathway (Howes et al., 2010). This route can be marked using high molecular weight

dextran or other neutral polymers. Macropinocytosis describes a process whereby actinomyosin-driven cell protrusions pinch off large volumes of extracellular fluid that are then internalized in large vesicles; thus, this also represents an important route for fluid phase endocytosis (Kerr and Teasdale, 2009). There are several additional clathrin and caveolin independent pathways, but in most cases the mechanisms involved are only beginning to be delineated (Howes et al., 2010). Thus, in summary, we are currently aware of multiple pathways for endocytosis with more probably remaining to be discovered. This creates complexities but also opportunities for oligonucleotide pharmacology. For example, by using ligands that target antisense or siRNA to specific cell surface receptors, one can influence the initial route of internalization. This may have important implications for subsequent intracellular distribution and for the ultimate pharmacological effect of the oligonucleotide (Alam et al., 2010).

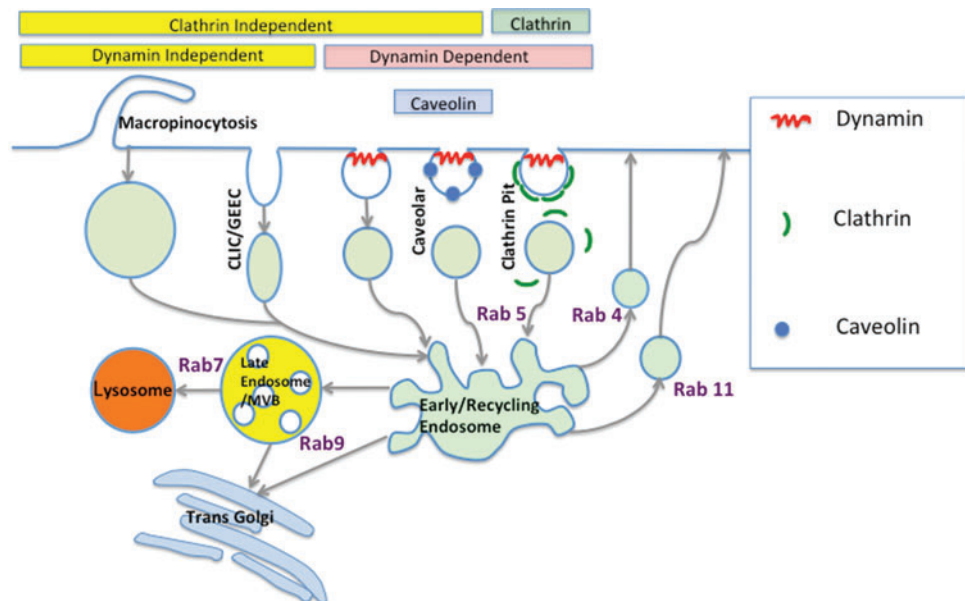
Initial uptake of oligonucleotide is followed by intracellular trafficking into a variety of endomembrane vesicular compartments including early/sorting endosomes, late endosomes/multivesicular bodies, lysosomes, and the Golgi complex (see Fig. 1). If a receptor was involved in the initial uptake, the receptor and its ligand are usually dissociated and the receptor can often recycle back to the cell surface (Hanyaloglu and von Zastrow, 2008; Xu et al., 2013). Intracellular trafficking is an extremely dynamic process that is regulated by a plethora of proteins and lipids that control the size, content and ultimate destination of vesicle membrane and contents. In the typical pathway of endocytosis, maturation of vesicles from early to late endosomes and thence to lysosomes is accompanied by dramatic changes in the protein and lipid composition of the endosome as well as by progressive reduction in pH (Pfeffer, 2007; Huotari and Helenius, 2011). Trafficking often involves a dynamic flux of small shuttle vesicles between larger endomembrane compartments (Spang, 2009; Hughson and Reinisch, 2010). During vesicular budding and fusion events, discontinuities in the lipid bilayer may occur thus potentially allowing for partial escape of vesicle contents (Deamer and Bramhall, 1986; de Gier, 1993;

Gurtovenko et al., 2010); this “leakage” to the cytosol may be an important facet of oligonucleotide pharmacology.

A variety of individual proteins or multiprotein complexes are involved in the regulation of intracellular trafficking. Many aspects of trafficking are controlled by members of the large Rab family of GTPases (Stenmark, 2009; Hutagalung and Novick, 2011); this includes vesicle coating or uncoating processes, linkages to the cytoskeleton for movement of vesicles, and membrane recognition and fusion events. Individual Rabs also serve as markers for particular compartments (Pfeffer, 2013); for example, Rab5 is characteristic of early/recycling endosomes, while Rab 7 demarcates late endosomes. The specific docking of different types of vesicles to each other involves multiprotein tethering complexes for recognition, while vesicular fusion events are controlled by soluble Nsf attachment protein receptor proteins that can cause membrane destabilization (Cai et al., 2007). Trafficking via late endosomes/multivesicular bodies to lysosomes is regulated by the multicomponent endosomal sorting complexes required for transport (Henne et al., 2011), which also contributes to the formation of exosomes that are released externally and can traffic between cells (Bobrie et al., 2011). The atypical retrograde trafficking pathway (Johannes and Wunder, 2011) that conveys materials from early endosomes to the Golgi rather than to lysosomes involves the retromer complex and its associated sorting nexins. Trafficking via this pathway can protect vesicle contents from lysosome-mediated degradation. Ubiquitin and its recognition proteins also play a key role in trafficking particularly for endosome components destined for proteolytic degradation for (Clague et al., 2012). Note that the multiplicity of proteins involved in intracellular trafficking could provide many opportunities to modulate these processes, possibly in ways that would enhance oligonucleotide effectiveness.

In summary, intracellular trafficking involves an extremely dynamic and highly orchestrated set of processes that deliver both membrane components and vesicular contents to their appropriate places within the cell. It is clear that altering the subcellular trafficking of molecules can have significant

FIG. 1. Routes of endocytosis and trafficking. This depicts several of the common pathways of endocytosis including the clathrin-coated pit/vesicle pathway, the caveolar pathway, macropinocytosis, and clathrin/caveolin-independent pathways. Several membrane bound compartments involved in intracellular trafficking are also shown. Key proteins including clathrin, caveolin, dynamin, and various Rab GTPases are shown. Color images available online at www.liebertpub.com/nat



therapeutic implications (Mossalam et al., 2010). Because of the complexity of intracellular traffic, its manipulation presents many challenges, but this complexity also offers many opportunities to intervene. Recently there has been much interest in the trafficking of oligonucleotides because of the potential pharmacological importance (Overhoff and Sczakiel 2005; Alam et al., 2008; Kortylewski et al., 2009; Alam et al., 2010; Ming et al., 2010; Koller et al., 2011; Ming et al., 2011; Varkouhi et al., 2011; Juliano et al., 2012a); we will discuss some of the key findings in more detail below.

Enhancing Receptor-Mediated Endocytosis of Oligonucleotides: Opportunities and Limitations

A tried and true method for enhancing cellular uptake of oligonucleotides is to link the nucleic acid to a ligand that binds with high affinity to a cell surface receptor. This tactic has been used for both molecular-scale, covalent ligand-oligonucleotide conjugates (Delevey et al., 2009; Juliano et al., 2012b) and for oligonucleotides complexed with nanoscale lipid- or polymer-based carriers (Li et al., 2013; Xu et al., 2013). Ideally this approach can provide both increased uptake and improved selectivity, particularly if the receptor chosen is differentially expressed on a specific cell type. In designing approaches for receptor-mediated delivery of oligonucleotides it is vital to be aware of the basic biology of the receptor involved. Here we will briefly outline some of the key characteristics of three major receptor families that have been used in the delivery of nucleic acids (see Fig. 2).

The integrin family

Integrins are heterodimeric cell surface proteins that are involved in cell-extracellular matrix (ECM) adhesion, organization of the cytoskeleton, and various signaling processes (Hynes, 2002; Schwartz, 2010). The integrin family in mam-

mals includes 18 alpha subunits and 8 beta subunits giving rise to 24 individual heterodimers (Margadant et al., 2011). Integrins ordinarily bind to large ECM proteins such as fibronectin and collagen, but the discovery that the short peptide arg-gly-asp (RGD) could bind to certain integrins (Pierschbacher and Ruoslahti, 1984) led to the development of many small molecule ligands for this receptor family. The cytoplasmic tails of integrins associate with proteins such as talin, filamin, and kindlin that then provide a mechanochemical linkage to the actin cytoskeleton.

In addition to their role in cell adhesion and cytoskeletal organization, integrins also participate in signal transduction. This has been divided into “inside out” and “outside in” signaling. Inside out signaling refers to the fact that an integrin can exist in different affinity states for its ECM ligands (Kim et al., 2011). These states correlate with the overall shape of the integrin and the spacing of the cytoplasmic α and β tails and are regulated by the binding of talin to the β tail. Outside in signaling refers to the fact that integrin engagement with ligand can both directly trigger signaling processes and also modulate signaling through other receptor systems (Streuli and Akhtar, 2009). The best example of the former is integrin-mediated activation of focal adhesion kinase, a cytosolic tyrosine kinase that can trigger a multiplicity of downstream signaling events (Parsons, 2003). An example of the latter is integrin-mediated enhancement of the mitogenic Erk/mitogen-activated protein (MAP) kinase pathway that lies downstream from receptor tyrosine kinases (RTKs) (Juliano et al., 2004). Integrin engagement modulates activation of the Erk kinase (Edin and Juliano, 2005) as well as its entry into the nucleus (Aplin et al., 2001).

It is well known that integrins are internalized and then usually recycle to the cell surface (Bretscher, 1992; Sczekan and Juliano, 1990; Caswell et al., 2009), although in some cases they may be ubiquitinated and degraded in lysosomes.

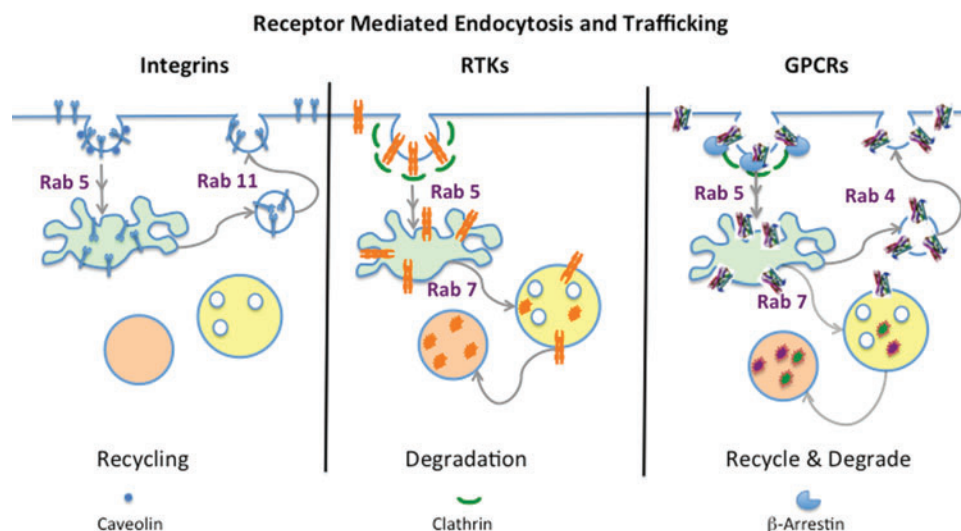


FIG. 2. Endocytosis and trafficking of receptors. The *left panel* illustrates the $\alpha 5 \beta 1$ integrin being internalized via a caveolar pathway to Rab 5 positive early endosomes and then returning to the cell surface via Rab 11 positive recycling vesicles. The *middle panel* illustrates epidermal growth factor receptor (EGFR) being internalized in clathrin coated vesicles, entering Rab 5 positive early endosomes, and then, due to previous ubiquitination, being destined for Rab 7 positive late endosomes and then lysosomes where the receptor is degraded. The *right panel* illustrates a G protein-coupled receptors (GPCR) being internalized in clathrin coated vesicles with the involvement of beta-arrestin and then returning from early endosomes to the cell surface via Rab 4 positive recycling vesicles. A portion of the GPCR may also traffic to lysosomes. The fate of the ligand is not illustrated in these diagrams. Color images available online at www.liebertpub.com/nat

Similar to the situation in signaling, there are interesting reciprocal effects on recycling between integrins and other receptors, especially the RTKs (Caswell et al., 2008; Wickstrom and Fassler, 2011). Internalization of certain integrins via clathrin-coated pits is thought to play an important role in focal adhesion disassembly. However, some integrins are internalized by clathrin-independent mechanisms; thus, $\alpha\nu\beta3$ and $\alpha5\beta1$ have been reported to internalize via caveolae (Karjalainen et al., 2008). After internalization and entry into Rab5 positive early endosomes, integrins can recycle to the cell surface via a Rab4 positive “short-loop” pathway (e.g., $\alpha\nu\beta3$) or via the Rab11 positive perinuclear recycling compartment (e.g., $\alpha5\beta1$) (Caswell et al., 2009).

Because they are structural as well as signaling proteins, integrins are usually expressed at quite high levels compared to other receptors, often at 10^5 copies per cell or more (Mulgrew et al., 2006; Szabo et al., 2012). This is one reason why integrins are attractive for designing delivery approaches. Even more importantly, although most cells express several members of the integrin family, in a number of cases there is strong differential expression of a particular integrin. The most extreme example is $\alpha IIb\beta3$ that is exclusively expressed in platelets or their precursors. Another example of wide interest is $\alpha\nu\beta3$, which is highly expressed in angiogenic vasculature and in certain types of tumors (Desgrosellier and Cheresch, 2010). Thus, various versions of cyclic RGDs that preferentially bind to $\alpha\nu\beta3$ have been pursued for delivery of nucleic acids as well as conventional drugs and imaging agents (Niu and Chen, 2011).

Broad interest in therapeutic possibilities involving integrins has led to the development of a variety of highly selective integrin ligands as well as information on how the ligands affect integrin structure and function (Shimaoka and Springer, 2003). Thus, a number of small molecule ligands exist for $\alpha IIb\beta3$, $\alpha\nu\beta3$, and $\alpha4\beta1$, and the $\alpha IIb\beta3$ drugs have found a role in the clinic in preventing thrombotic problems (Millard et al., 2011). Synthetic integrin ligands usually bind in the cleft between the α and β subunits, and virtually all are competitive inhibitors of the physiological ligands. Thus, another advantage of utilizing integrins for delivery strategies is the ready availability of a number of very selective, high affinity ligands.

In summary, integrins have some attractive features for selective delivery of oligonucleotides. They are often expressed at relatively high levels, they efficiently recycle to the cell surface, and high affinity ligands are available, at least for some integrins. Potential liabilities include the fact that some integrins are very broadly expressed (e.g., $\alpha5\beta1$, a fibronectin receptor), while high affinity ligands are not available for all members of the family.

The G protein-coupled receptor superfamily

Numbering several hundred, the G protein-coupled receptors (GPCRs) comprise the largest receptor family and its members play roles in a multiplicity of physiological functions (Armbruster and Roth, 2005). GPCRs vary greatly in primary sequence but share a common topology based on seven transmembrane helices. The classic mechanism of GPCR signaling involves a ligand activated conformational change in the receptor that alters its association with its bound heterotrimeric G protein partner, resulting in the ex-

change of GDP for GTP on the G protein α subunit and release of both the $G\alpha$ subunit and the $\beta\gamma$ subunit complex from the receptor (Cabrera-Vera et al., 2003). The G protein subunits can then interact with a variety of downstream effectors (Gilman, 1995). For various G protein subtypes, the effectors would include adenylate cyclase, phospholipase C β , and various ion channels. The GTP loading and activity of the $G\alpha$ subunit is negatively regulated by members of the regulators of G-protein signaling protein family (Kimple et al., 2011), thus restoring the inactive state.

GPCR signaling events are highly connected with the internalization and intracellular trafficking of the receptor (Drake et al., 2006; Hanyaloglu and von Zastrow, 2008). Internalization includes a process that de-sensitizes the receptor and reduces signaling via “classic” second messengers like cyclic AMP but can also include the recruitment of new signaling moieties with different downstream effects. Receptor internalization involves phosphorylation of the cytosolic tail by members of a family of GPCR kinases. This triggers the recruitment of the key adaptor protein β -arrestin that can then interact with the AP-2 protein allowing recruitment of the GPCR into clathrin-coated pits and endocytotic vesicles. The formation of a GPCR/arrestin complex on the cytoplasmic face of the endosome results in the formation a new G Protein-independent signaling complex that can recruit the c-Src tyrosine kinase as well as other proteins and project into mitogenic signaling pathways (Shukla et al., 2011). Thus, GPCRs have dual signaling mechanisms depending on their localization within the cell.

Internalization of activated, agonist bound GPCRs is primarily via the clathrin dependent pathway; however, certain GPCRs have been observed in association with lipid rafts or caveolae. In most instances, the internalization of GPCRs that are not liganded with an agonist is much more limited. After trafficking to Rab5 positive early endosomes GPCRs can undergo two distinct fates. One involves sorting to multivesicular bodies and thence to lysosomes for degradation, while the other involves returning to the cell surface via Rab4 or Rab11 positive recycling compartments (Hanyaloglu and von Zastrow, 2008).

GPCRs often display strong differential expression in various tissues or in disease states. One situation is the over-expression of certain GPCRs in various cancers (Li et al., 2005). For example, the gastrin releasing peptide receptor GRPR has been implicated in a number of carcinomas (Cornelio et al., 2007) and radiolabeled GRPR ligands have been successfully used for tumor imaging (Garrison et al., 2007). More directly to the point here, we have used a peptide ligand for GRPR to deliver oligonucleotides to GRPR-positive prostate tumor cells (Ming et al., 2010). One potential problem with utilization of GPCRs for delivery purposes is their relatively low abundance (Post et al., 1995; Houston et al., 2006). While expression levels differ dramatically between different GPCRs and in different tissue settings, in general they are lower than, for example, integrins. As a rather broad generalization, GPCRs are often expressed at 10^3 to 10^4 copies per cell (Levitzki et al., 1974).

Molecules that act on GPCRs comprise approximately 40% of all clinically utilized drugs while GPCRs may account for 28% of the “druggable genome” (Hopkins and Groom, 2002; Filmore, 2004). Thus there exists a huge armamentarium of GPCR ligands, some of which have made it to the clinic, while

many more have been abandoned and now lurk in chemical catalogs or in the storehouses of pharmaceutical companies. This provides the opportunity for development of a large variety of ligand–oligonucleotide conjugates or liganded oligonucleotide nanocarriers designed to enhance delivery via GPCR mediated endocytosis.

Thus with GPCRs there is a mixed picture in terms of delivery. High affinity ligands are often available for various individual GPCRs. However, these receptors are not as highly expressed as members of some other receptor families are. Additionally, the receptor recycling process is incomplete and a substantial fraction of receptor can be delivered to lysosomes and degraded, thus eventually reducing receptor expression on the cell surface.

The receptor tyrosine kinases

Members of this large family of receptors play key roles in mitogenesis and cell growth control and are activated by polypeptide ligands. Binding of the ligand to the RTK leads to receptor dimerization, autophosphorylation of the cytosolic domain, and tyrosine phosphorylation of bound partner proteins. This leads to the creation of multiprotein signal transduction hubs that can branch to activate several downstream pathways (Hynes and Lane, 2005; Lemmon and Schlessinger, 2010; Koch and Claesson-Welsh, 2012). A typical example of this is stimulation of the epidermal growth factor (EGF) receptor leading to activation of the Erk/MAP kinase mitogenic pathway as well as the AKT survival pathway.

The intracellular trafficking of RTKs is tightly linked to their functions (Parachoniak and Park, 2012). The epidermal growth factor receptor (EGFR) is one of the best-studied RTKs and can serve as an example. Thus the cytosolic tail of activated EGFR interacts with adapter proteins including AP-2 to cluster the receptor in clathrin-coated pits. After dynamin mediated scission of the coated vesicles from the membrane and release of the clathrin coat, the EGFR enters early endosomes and is then trafficked to late endosomes/multivesicular bodies and thence to lysosomes, where both the receptor and its ligand are degraded thus resulting in termination of signaling (Lemmon and Schlessinger, 2010). Interestingly, internalization of EGFR is required for signaling to one of its downstream pathways, the AKT kinase pro-survival pathway, but not for signaling to the Erk/MAP kinase pathway (Goh et al., 2010). Other instances of interplay between signaling and trafficking pathways of RTKs are also known (Miaczynska and Bar-Sagi, 2010). In comparison to integrins, RTKs tend to recycle to a lesser degree and are more likely to traffic to the lysosome for degradation; however, there are many variations for different members of the RTK family.

Differential expression of RTKs is found in a number of disease states, particularly cancer. For example, the overexpression of HER2 in certain forms of breast cancer is well known (Shepard et al., 2008). Another instance involves members of the Trk family of RTKs that are primarily expressed in neuronal or neuroepithelial tissues and that may have a role in neuroblastoma (Brodeur et al., 2009). The number of RTKs per cell can vary over a wide range. Thus, in one study the EGFR was present in various cell lines in amounts ranging from 10^3 to 10^6 copies per cell while in another study the VEGFR2 was present at about $0.5\text{--}1.0 \times 10^5$ copies per vascular endothelial cell (Imai et al., 1982; Napione

et al., 2012). Thus RTKs can be expressed at levels of thousands to hundreds of thousands of copies per cell; this is potentially a useful range in terms of utilizing RTKs for targeted delivery.

The physiological ligands for RTKs are all relatively large polypeptides and thus present problems in terms of their use in selective delivery. There are a variety of high affinity monoclonal reagents for the external domains of RTKs (Hynes and Lane, 2005; Krause and Van Etten, 2005). These can be used themselves as targeting reagents, converted to Fab fragments, or reconfigured as scFv reagents (Tohidkia et al., 2012). There are a number of examples of delivery of drugs, imaging agents, or nucleic acids with targeted nanoparticles using approaches involving antibodies to RTKs (Kirpotin et al., 2006; Liu et al., 2011; Hwang et al., 2012). However, because of the size and complexity of RTK ligands, opportunities for direct conjugation with oligonucleotides are limited.

As with the integrins, RTKs are often expressed at quite high levels and thus may be useful for targeted delivery of oligonucleotides. RTKs are primarily internalized via the “classic” clathrin pit pathway, enter low pH endosomal compartments, and ultimately traffic to lysosomes. Thus, RTKs may provide an effective means of delivery to these compartments; this may be augmented by using pH sensitive linkers. However, in contrast to some integrins that rapidly recycle, the cell surface display of RTKs must be restored by synthesis of new molecules. Therefore, strategies that involve multiple dosing may not be appropriate if RTKs are used for targeted delivery.

Perspective on receptor targeting

In the preceding paragraphs we have discussed some of the characteristics of integrins, GPCRs and RTKs that may have implications for their utilization in oligonucleotide delivery strategies. Obviously, there are other receptor families that may be of interest. However, many of the same considerations would apply in those cases. Thus, in designing strategies for receptor mediated oligonucleotide delivery several important aspects must be kept in mind. Perhaps most important is the abundance of the receptor. Although antisense and siRNA can be very potent, still it is important to have sufficient levels of receptor expression to be able to deliver significant amounts of material to the cell interior. Another key aspect is how actively the receptor internalizes and the degree to which it recycles. If ligand engagement shunts most of the receptor to lysosomal degradation, then it will not be possible to maintain delivery over an extended period whereas an actively recycling receptor will allow this. An essential issue is the chemistry of the receptor ligand. Will it be possible to couple the ligand to an oligonucleotide or to a nanocarrier without loss of ligand affinity or specificity? This can be a challenging problem as discussed at length elsewhere (Alam et al., 2013). Finally, one needs to realize that receptors are not just useful targets for drug delivery, but that they are also key elements of the cell’s regulatory system. When a ligand-conjugated oligonucleotide or nanocarrier binds to a cell surface receptor it will undoubtedly trigger an entire avalanche of signaling events. This aspect of targeted drug delivery has been largely ignored (our laboratory is equally guilty here) but needs to be addressed if receptor mediated delivery of both oligonucleotides and conventional drugs is to progress.

Cellular Uptake and Intracellular Trafficking of Oligonucleotides and Ligand–Oligonucleotide Conjugates

This section will examine current information on the uptake and intracellular trafficking of “free” oligonucleotides and of molecular-scale ligand-oligonucleotide conjugates, while the behavior of oligonucleotide nanocomplexes is discussed in another section. The overall biodistribution of various forms of “free” or “naked” oligonucleotides has been well studied (Juliano et al., 1999; Geary, 2009; Bennett and Swayze, 2010). Whereas uncharged oligonucleotides such as morpholino and peptide nucleic acid derivatives, as well as most forms of siRNA, are rapidly excreted via the kidney, phosphorothioate (PS) oligonucleotides bind more avidly to plasma proteins and cells and are thus retained in the body for longer periods. Certain cell types, particularly kidney proximal tubule cells and liver Kupffer cells, exhibit preferential uptake both for PS antisense compounds and for siRNA (Butler et al., 2000; Molitoris et al., 2009). Despite many studies on biodistribution to tissues, it is only recently that the subcellular trafficking of oligonucleotides and their molecular scale conjugates has been explored in some depth. For example, a study using PS oligonucleotides in transformed hepatocytes and in murine livers indicated that productive and non-productive intracellular pathways for delivery coexist, with the nonproductive pathway probably trafficking to lysosomes (Koller et al., 2011). Other studies in cell culture and in mouse models, have reported the so-called “gymnotic” uptake of free antisense oligonucleotides modified with locked nucleic acid moieties (Stein et al., 2010; Zhang et al., 2011).

Investigators have searched extensively for cell surface receptors for free oligonucleotides but much of this literature is controversial. Thus $\beta 2$ integrins (Benimetskaya et al., 1997) and scavenger receptors (Butler et al., 1997) have been suggested as possibilities, but some of the evidence is problematic (Butler et al., 2000). A putative oligonucleotide transmembrane transporter has been described (Hanss et al., 2002), but this work has not seen any follow-up. In lower organisms, a double stranded RNA transport protein termed SID-1 plays a key role in the cell to cell spread of RNA interference (Winston et al., 2007); however, although there is a mammalian homolog, reports of its role in siRNA uptake in mammalian cells have languished (Duxbury et al., 2005; Tsang et al., 2007; Wolfrum et al., 2007). The clearest example of receptors for oligonucleotides involves the toll-like receptor (TLR) family that is essential for the innate immune response (Robbins et al., 2009; Kawai and Akira, 2011). As a simplistic summary, TLR9 binds DNA containing CpG motifs, TLRs7/8 bind single stranded RNA, while TLR3 binds double stranded RNA. Although TLRs are sometimes found within endosomes rather than at the cell surface, they nonetheless seem to be able to assist in the uptake of oligonucleotides by cells.

Oligonucleotide conjugate chemistry

There is a growing literature on oligonucleotides conjugated to ligands designed to promote cellular uptake (Watts et al., 2008; Lonnberg, 2009; Marlin et al., 2010; Singh et al., 2010; Juliano et al., 2012b; Nguyen and Szoka, 2012). Conjugate groups can be incorporated into oligonucleotides by direct online synthesis on a DNA/RNA synthesizer or by

post-synthetic conjugation to reactive groups incorporated during automated oligonucleotide synthesis. A recent review gives a comprehensive overview of the developments in conjugate chemistry for oligonucleotide synthesis (Singh et al., 2010) and compares the merits of each approach to preparing conjugates. Direct online synthesis offers the most straightforward and versatile option available for preparing oligonucleotide conjugates. The ability to control the synthesis allows for the incorporation of single or multiple conjugate groups at predefined positions anywhere in the sequence. The major limitation to this method is that the conjugate groups (with the appropriate protecting groups) must be compatible with the conditions used for automated oligonucleotide synthesis and deprotection. In cases where the desired conjugate is incompatible with conditions for automated synthesis, solution phase post-synthesis conjugation can be employed. However, solution phase methods may be less efficient and require more intensive purification. Additionally, functional oligonucleotides can be prepared as aptamer chimeras for specific cell receptor mediated targeting (Zhou and Rossi, 2011).

The copper(1)-catalyzed azide-alkyne cycloaddition is the most prominent example of a group of reactions named “click-reactions” and has emerged as a highly efficient and versatile method for preparing conjugates. A recent review provides an up to date account of the use of click chemistry in RNA modification and other novel methods for site specific labeling (Phelps et al., 2012). Alkyne derivatized nucleotide phosphoramidite bases are now available for solid phase synthesis that enable single or multiple conjugates to be introduced by click reaction (Yamada et al., 2011). The recent development of azide functionalized oligonucleotide by using the 2'-azido H-phosphonate monomer in chemical synthesis of RNA (Fauster et al., 2012) and the 5'-triphosphate of 5-(3-azidopropyl) uridine that can be incorporated during enzymatic synthesis (Rao et al., 2012; Winz et al., 2012) has further expanded the utility of click chemistry for preparing conjugates with the versatility of using either azide or alkyne derivatives of prospective labels.

Lipid conjugates

One important thrust has been the development of conjugates with cholesterol or other lipophilic moieties that promote association with plasma lipoproteins or albumin and can thus enhance uptake into the liver and elsewhere via lipoprotein receptors (Krutzfeldt et al., 2005; Wolfrum et al., 2007; Uno et al., 2011). A recent study showed that double tailed, saturated lipid conjugated to an oligonucleotide significantly improved cellular uptake in a variety of tumor cell types (Ugarte-Urbe et al., 2013). Impressively, the conjugate significantly surpassed the cellular uptake achieved by transfection in HeLa cells. Furthermore, the authors demonstrated that the effects of the conjugate could be diminished in CR3 receptor knockout cells or through treatment with soluble fibrinogen thus suggesting another receptor that can be directly targeted through conjugation. However, another recent study demonstrates that while lipophilic moieties, cholesterol or docosanoic acid, improved antisense oligonucleotide efficacy in silencing Bcl-2, they were susceptible to the presence of human serum albumin (Felber et al., 2012).

Targeted conjugates

Recently there has been a surge of interest in additional receptor-targeted ligand–oligonucleotide conjugates. For example, our laboratory has reported on RGD peptide conjugates of SSOs or of siRNA that can be delivered to melanoma cells via the $\alpha v\beta 3$ integrin (Alam et al., 2008, 2010, 2011). We have also examined bombesin conjugates that are targeted to the BB2 receptor, a member of the GPCR superfamily that is overexpressed on prostate cancer cells (Ming et al., 2010). Additionally we have also targeted oligonucleotides to tumor cells using anisamide, a high affinity small molecule ligand for the sigma receptor (Nakagawa et al., 2010). As mentioned above, targeting TLRs is an interesting possibility. Thus in one study an unmethylated CpG oligonucleotide known to bind to TLR9 was chemically conjugated to a siRNA, resulting in enhanced uptake by dendritic cells, macrophages and B-cells, all known to express TLR9, as well as “knockdown” of endogenous and reporter genes (Kortylewski et al., 2009). This approach has recently been extended to targeting malignant hematopoietic cells (Zhang et al., 2013).

Carbohydrate moieties can also be useful ligands for targeting; some of the earlier work in this area has been reviewed previously (Juliano et al., 2012b). More recently, in T-cell lymphocytes, the conjugation of aminoglucosamine to a peptide nucleic acid (PNA) targeting HIV-1 TAR significantly improved the cellular uptake of the PNA (Das et al., 2012). In addition, the amino sugar conjugate reduced luciferase activity greater than naked PNA and scrambled PNA conjugate controls. In another study, the conjugation of galactose–PEG improved the delivery of an aptamer targeting the hepatitis C virus (HCV) to mouse liver tissue while modestly enhancing the ability of the aptamer to reduce HCV replication (Lee et al., 2013).

Nucleic acid aptamers provide another important tool for receptor specific delivery of conjugated oligonucleotides. Thus a pioneering report involved chimeric oligonucleotides comprised of an aptamer that bound with high affinity to the prostate specific membrane antigen (PSMA) receptor found in prostate cancer cells and siRNAs that affected key survival genes such as *Plk1* and *Bcl2* (McNamara et al., 2006). The aptamer–siRNA conjugates were taken up selectively by cells that expressed PSMA receptor, caused “knockdown” of the target messages in cell culture, and displayed antitumor activity when locally administered. A chemically optimized version of a PMSA aptamer–*Plk1*siRNA chimera displayed antitumor activity against PMSA expressing tumors when given systemically (Dassie et al., 2009). In another very interesting study, aptamer–siRNA chimeras inhibited tumor growth *in vivo* using siRNAs directed against genes involved in nonsense mediate mRNA decay and thus in immune regulation of tumors (Pastor et al., 2010). Additional interesting examples of aptamer–siRNA chimeras have also been described (Wheeler et al., 2011).

In summary, a number of studies have validated the concept that monomeric ligand–oligonucleotide conjugates can produce pharmacological effects in both cell culture and in animals, in the absence of any transfection agents. A further overview of receptor targeting of oligonucleotides is found in a recent review (Ming, 2011).

Uptake and Trafficking of Oligonucleotides Incorporated into Nanocarriers

A widely used strategy for promoting delivery of antisense, siRNA or other types of oligonucleotides is to incorporate the

nucleic acid into some form of nanoparticle; potentially this can help to overcome biological barriers, increase cell uptake, and enhance escape from membrane compartments (Akhtar and Benter, 2007; Juliano et al., 2009; Whitehead et al., 2009; Tamura and Nagasaki, 2010). Lipid based nanocarriers have proven to be very efficacious for the delivery of siRNA to the liver (Love et al., 2010). Additionally, a variety of polymeric nanoparticles (Kabanov and Vinogradov, 2009) and other types of nanocarriers (Petros and DeSimone, 2010) have been developed for siRNA delivery. Functional delivery of siRNA to tumors is very challenging; however, targeted lipid based nanoparticles have displayed substantial promise in this setting (Tseng et al., 2009). A residual concern relates to possible toxicities associated with the cationic polymers or lipids typically used to form nanocarriers for oligonucleotide delivery (Lv et al., 2006; Akhtar, 2010). There is a vast literature on use of nanoparticles as oligonucleotide delivery agents; here we will focus on a relatively few reports that have mechanistically addressed issues of cellular uptake and trafficking.

One study has challenged the conventional view that cationic lipid carriers functionally deliver siRNA via endocytosis followed by escape from endosomes (Lu et al., 2009). This investigation found that only a minor component of the cell-associated siRNA that contributed to knockdown function, and that this component probably came from fusion between the siRNA lipoplexes and the plasma membrane. The study is employed both chemical inhibitors and molecular reagents such as dominant negative versions of dynamin and caveolin to probe uptake pathways. Another interesting report examined the uptake and trafficking of siRNA associated with perfluorocarbon nanoparticles (Kaneda et al., 2010) and found that delivery was via formation of cell–nanoparticle hemifusion complexes followed by lipid raft mediated internalization. Our laboratory has compared the uptake and trafficking pathways of SSOs delivered via cationic lipids or via polyethylenimine, a cationic polymer (Ming et al., 2011). The study included use of pharmacological inhibitors, colocalization with known markers of internalization, and use of molecular reagents to modulate uptake and trafficking. Interestingly, in agreement with the study on siRNA delivery discussed above (Lu et al., 2009), functional delivery of antisense associated with lipoplexes was apparently due to fusion at the plasma membrane, while delivery via polyplexes took place through an unconventional form of endocytosis.

Gold nanoparticles have also been used as nanocarriers for oligonucleotide delivery. The so-called “spherical nucleic acids (SNAs)” are polyanionic structures comprised of densely packed and highly oriented oligonucleotides that are attached to the surface of gold nanoparticles via metal–thiol dative bonds. These nanoparticles can effectively enter more than 50 different cell types without the aid of auxiliary transfection agents and are able to produce antisense and RNA interference activity (Zheng et al., 2012). Mechanism studies have been followed to elucidate endocytosis pathways of SNAs (Patel et al., 2010; Choi et al., 2013). The latter study took advantage of the great resolution of gold particles under transmission electron microscopy and of molecular tools that can manipulate endocytotic process. It demonstrated that the SNAs can bind strongly to class A scavenger receptors and undergo rapid cellular uptake via a lipid-raft-dependent, caveolae-mediated pathway. Evidence also showed that the SNAs enter early endosomes. However, it is silent on how

they traffic after early endosomes and more importantly how they exit from the endosomal compartment.

Targeting ligands have been engineered into nanoparticle delivery systems to improve the effectiveness and reduce potential side effects of therapeutic oligonucleotides (Ming, 2011). Unfortunately, in spite of a substantial number of reports demonstrating superior pharmacological outcomes than the nontargeted nanoparticles, few mechanistic studies have been reported to elucidate possible intracellular pathways that lead to the greater effectiveness of the targeted delivery systems. In one recent study, multiple RGD-morpholino conjugates were linked to a single molecule of human serum albumin and resulted in integrin-targeted nanoconjugates (Ming et al., 2013b). The ultra-small (13nm) nanoparticles showed superior delivery of oligonucleotides into tumor cells and deep penetration throughout 3-dimensional tumor spheroids (Ming et al., 2013b). The RGD targeted nanoconjugates undergo dynamin-dependent endocytosis (unpublished data) and then traffic to Rab7-positive late endosomes and Lamp1-positive lysosomes (Ming et al., 2013b). However, the endosomal release mechanism is still unresolved.

Recently two articles appeared in the same issue of *Nature Biotechnology* that provide the most sophisticated analysis to date of the cellular uptake and intracellular trafficking of siRNA associated with nanocarriers (Gilleron et al., 2013; Sahay et al., 2013). In both cases, the siRNA was incorporated into (two different) lipid nanoparticles that had shown excellent efficacy for siRNA delivery *in vivo*. Both studies used advanced imaging techniques and chemical and molecular tools to manipulate uptake and trafficking pathways. One study suggests an initial phase of uptake via clathrin mediated endocytosis, which then triggers a more robust uptake via macropinocytosis (Gilleron et al., 2013). This study found a low level of siRNA escape (~2%) from endosomes via gradual release rather than a bursting process, and that the most likely site of escape is from an early endosomal compartment. The other study (Sahay et al., 2013) found evidence for substantial recycling of siRNA to the external medium via exocytosis from a late endosomal/lysosomal compartment. The transmembrane glycoprotein NPC1, which is present on multivesicular late endosomes, is known to be involved in trafficking of lipids. This study implicated NPC1 in recycling of the lipid/siRNA complex via a Rab27a-dependent pathway; for example, they found a substantial improvement in siRNA potency in NPC1^{-/-} cells or due to knockdown of Rab27a. The two studies differ in many details but they both reveal the importance of intracellular processing in the functional delivery of siRNA. Interestingly these studies seem to counter some of the earlier studies, discussed above, that direct fusion with the plasma membrane accounts for some of the effects of siRNA associated with lipid carriers.

To summarize, both in the case of molecular scale oligonucleotide conjugates and for oligonucleotides associated with nanocarriers there is mounting evidence that the pathways of internalization, trafficking and recycling play key roles in determining the biological effects of the oligonucleotide. This suggests that it will be very productive to continue to investigate uptake and trafficking issues for antisense, siRNA and SSOs and that the knowledge gleaned will be very helpful in therapeutic development of these types of molecules.

Enhancing the Pharmacological Effects of Oligonucleotides Using Small Molecules

Oligonucleotides enter and traffic through cells via dynamic processes that are regulated by a multiplicity of proteins. Their biological actions involve complex RNA-protein interactions involving multiprotein entities such as the RNA-induced silencing complex or the spliceosome. Thus, it stands to reason that the effects of oligonucleotides can be modulated using small molecules that affect either trafficking or the ultimate locus of action. This strategy for improving the pharmacological effectiveness of oligonucleotides has been rather neglected until recently. However, a couple of interesting reports on this subject have now emerged.

SSOs that cause exon skipping in the dystrophin gene have shown promise in treatment of Duchenne muscular dystrophy (Cirak et al., 2011). A recent report showed that the small molecule dantrolene could produce about a 2.5-fold enhancement of SSO-mediated exon skipping (Kendall et al., 2012). Dantrolene acts on the muscle cell ryanodine receptor, a calcium transporter, and is currently used clinically in treatment of malignant hyperthermia. The mechanism by which dantrolene enhances splice correction is unclear but presumably may be a calcium-mediated action on the splicing process.

Our laboratory has pursued the concept of modulating the intracellular trafficking of oligonucleotides to attain improved effects. While it is possible to alter intracellular trafficking using molecular tools such as activated or dominant negative Rab proteins, we felt that a better approach would be to seek small molecules that affect trafficking pathways. In perusing the literature, we were surprised to learn that very few such molecules are known (von Kleist and Hauke, 2012). One report that we found very interesting described a set of small

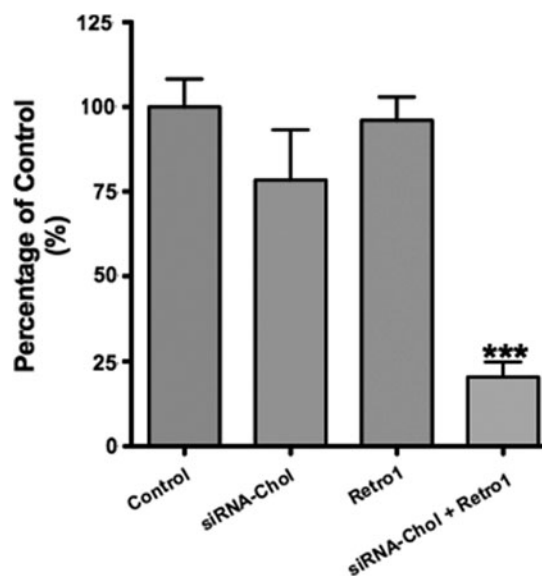


FIG. 3. Retro-1 enhances small interfering RNA effect. Cells that stably express firefly luciferase were treated with a chemically stabilized cholesterol-conjugated siLuc with or without additional treatment with 100 μ M Retro-1. Results are expressed as percentage of luminescence in untreated control cells. *** $p=0.05$. RTKs, receptor tyrosine kinases.

organic molecules termed “retro compounds” that had emerged from a high throughput screen for drugs that would block the action of bacterial toxins (Stechmann et al., 2010). In a collaborative effort with the group that originated these compounds, we tested several of them for the ability to enhance the actions of oligonucleotides. We were pleased to find that the agent Retro-1 produced substantial improvements of the pharmacological effects of both SSOs and classic antisense oligonucleotides (Ming et al., 2013a). In these studies, the oligonucleotides were used in “free” form, without the inclusion of transfection agents. Through use of confocal microscopy and markers for specific membrane compartments, we showed that Retro-1 could trigger rapid release of oligonucleotides from late endosomes, with subsequent accumulation of SSOs or AS in the nucleus. Retro-1 also provided a modest enhancement of the action of a SSO in a xenograft tumor model. In our published studies, we reported that Retro-1 did not affect the actions of siRNA; however, those studies used conventional siRNA that was too unstable to provide a robust effect. Since then we have found that Retro-1 provides a strong enhancement of the actions of siRNA when the oligonucleotide is stabilized by chemical modification (see Fig. 3). Despite these promising developments, Retro-1 is not an ideal agent for oligonucleotide pharmacology. It is active only at high micromolar concentrations and it is very lipophilic thus making its *in vivo* use difficult. For these reasons, it would seem valuable to undertake additional screening, as well as using medicinal chemistry approaches, to seek molecules that enhance oligonucleotide actions, but with better “drug-like” characteristics.

Summary

A variety of strategies are being pursued to influence the cellular uptake and subcellular trafficking of oligonucleotide with the goal of improving pharmacological effects. Most of the work is still at an early stage and much more information is needed concerning the intracellular fates of oligonucleotides linked to various ligands or nanocarriers. Additionally, with a few notable exceptions, most of the work thus far on mechanisms of uptake and trafficking of oligonucleotides has been done in simple tissue culture settings, with very limited *in vivo* data. Performing mechanistic studies in animals is challenging but new technologies such as intravital two-photon microscopy will be very helpful. Additionally, use of more complex three-dimensional tissue culture models (e.g., tumor spheroids) may provide insights into cellular processes that are absent or altered in simple two-dimensional culture.

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Author Disclosure Statement

No competing financial interests exist.

References

AKHTAR, S. (2010). Cationic nanosystems for the delivery of small interfering ribonucleic acid therapeutics: a focus on toxicogenomics. *Expert Opin. Drug Metab. Toxicol.* **6**, 1347–62.

AKHTAR, S., and BENTER, I.F. (2007). Nonviral delivery of synthetic siRNAs *in vivo*. *J Clin Invest* **117**, 3623–32.

ALAM, M.R., DIXIT, V., KAN, H., LI, Z.B., CHEN, X., TREJO, J., FISHER, M., and JULIANO, R.L. (2008). Intracellular delivery of an anionic antisense oligonucleotide via receptor-mediated endocytosis. *Nucleic Acids Res.* **36**, 2764–2776.

ALAM, M.R., MING, X., DIXIT, V., FISHER, M., CHEN, X., and JULIANO, R.L. (2010). The biological effect of an antisense oligonucleotide depends on its route of endocytosis and trafficking. *Oligonucleotides* **20**, 103–109.

ALAM, M.R., MING, X., FISHER, M., LACKEY, J.G., RAJEEV, K.G., MANOHARAN, M., and JULIANO, R.L. (2011). Multi-valent cyclic RGD conjugates for targeted delivery of small interfering RNA. *Bioconjug. Chem.* **22**, 1673–81.

ALAM, M.R., MING, X., NAKAGAWA, O., JIN, J., and JULIANO, R.L. (2013). Covalent conjugation of oligonucleotides with cell-targeting ligands. *Bioorg. Med. Chem.* **21**, 6217–6223.

APLIN, A.E., STEWART, S.A., ASSOIAN, R.K., and JULIANO, R.L. (2001). Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J Cell Biol.* **153**, 273–282.

ARMBRUSTER, B.N., and ROTH, B.L. (2005). Mining the receptorome. *J. Biol. Chem.* **280**, 5129–5132.

BENIMETSKAYA, L., LOIKE, J.D., KHALED, Z., LOIKE, G., SILVERSTEIN, S.C., CAO, L., EL KHOURY, J., CAI, T.Q., and STEIN, C.A. (1997). Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nat. Med.* **3**, 414–420.

BENNETT, C.F., and SWAYZE, E.E. (2010). RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* **50**, 259–293.

BOBRIE, A., COLOMBO, M., RAPOSO, G., and THERY, C. (2011). Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic* **12**, 1659–1668.

BRETSCHER M.S. (1992). Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. *EMBO J* **11**, 405–410.

BRODEUR, G.M., MINTURN, J.E., HO, R., SIMPSON, A.M., IYER, R., VARELA, C.R., LIGHT, J.E., KOLLA, V., and EVANS, A.E. (2009). Trk receptor expression and inhibition in neuroblastomas. *Clin. Cancer Res.* **15**, 3244–3250.

BURNETT, J.C., and ROSSI, J.J. (2012). RNA-based therapeutics: current progress and future prospects. *Chem. Biol.* **19**, 60–71.

BUTLER, M., CROOKE, R.M., GRAHAM, M.J., LEMONIDIS, K.M., LOUGHEED, M., MURRAY, S.F., WITCHELL, D., STEINBRECHER, U., and BENNETT, C.F. (2000). Phosphorothioate oligodeoxynucleotides distribute similarly in class A scavenger receptor knockout and wild-type mice. *J. Pharmacol. Exp. Ther.* **292**, 489–496.

BUTLER, M., STECKER, K., and BENNETT, C.F. (1997). Cellular distribution of phosphorothioate oligodeoxynucleotides in normal rodent tissues. *Lab. Invest.* **77**, 379–388.

CABRERA-VERA, T.M., VANHAUWE, J., THOMAS, T.O., MEDKOVA, M., PREININGER, A., MAZZONI, M.R., and HAMM, H.E. (2003). Insights into G protein structure, function, and regulation. *Endocr. Rev.* **24**, 765–781.

CAI, H., REINISCH, K., and FERRO-NOVICK, S. (2007). Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. *Dev. Cell* **12**, 671–682.

CASWELL, P.T., CHAN, M., LINDSAY, A.J., MCCAFFREY, M.W., BOETTIGER, D., and NORMAN, J.C. (2008). Rab-coupling protein coordinates recycling of alpha5beta1 integrin and EGFR1 to promote cell migration in 3D microenvironments. *J. Cell Biol.* **183**, 143–155.

- CASWELL, P.T., VADREVU, S., and NORMAN, J.C. (2009). Integrins: masters and slaves of endocytic transport. *Nat. Rev. Mol. Cell Biol.* **10**, 843–853.
- CHOI, C.H., HAO, L., NARAYAN, S.P., AUYEUNG, E., and MIRKIN, C.A. (2013). Mechanism for the endocytosis of spherical nucleic acid nanoparticle conjugates. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 7625–7630.
- CIRAK, S., ARECHAVALA-GOMEZA, V., GUGLIERI, M., FENG, L., TORELLI, S., ANTHONY, K., ABBS, S., GARRALDA, M.E., BOURKE, J., WELLS, D.J. et al. (2011). Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet* **378**, 595–605.
- CLAGUE, M.J., LIU, H., and URBE, S. (2012). Governance of endocytic trafficking and signaling by reversible ubiquitylation. *Dev. Cell* **23**, 457–467.
- CORNELIO, D.B., ROESLER, R., and SCHWARTSMANN, G. (2007). Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. *Ann. Oncol.* **18**, 1457–1466.
- DAS, I., DESIRE, J., MANVAR, D., BAUSSANNE, I., PANDEY, V.N., and DECOU, J.L. (2012). A peptide nucleic acid-aminosugar conjugate targeting transactivation response element of HIV-1 RNA genome shows a high bioavailability in human cells and strongly inhibits tat-mediated transactivation of HIV-1 transcription. *J. Med. Chem.* **55**, 6021–6032.
- DASSIE, J.P., LIU, X.Y., THOMAS, G.S., WHITAKER, R.M., THIEL, K.W., STOCKDALE, K.R., MEYERHOLZ, D.K., MCCAFFREY, A.P., MCNAMARA, J.O., 2nd, and GIANGRANDE, P.H. (2009). Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. *Nat. Biotechnol.* **27**, 839–849.
- DE GIER, J. (1993). Osmotic behaviour and permeability properties of liposomes. *Chem. Phys. Lipids* **64**, 187–196.
- DEAMER, D.W., and BRAMHALL, J. (1986). Permeability of lipid bilayers to water and ionic solutes. *Chem. Phys. Lipids* **40**, 167–188.
- DELEAVEY, G.F., WATTS, J.K., and DAMHA, M.J. (2009). Chemical modification of siRNA. *Curr. Protoc. Nucleic Acid Chem.* **Chapter 16**, Unit 16.3.
- DESGROSELLIER, J.S., and CHERESH, D.A. (2010). Integrins in cancer: biological implications and therapeutic opportunities. *Nat. Rev. Cancer* **10**, 9–22.
- DOHERTY, G.J., and MCMAHON, H.T. (2009). Mechanisms of endocytosis. *Annu. Rev. Biochem.* **78**, 857–902.
- DRAKE, M.T., SHENOY, S.K., and LEFKOWITZ, R.J. (2006). Trafficking of G protein-coupled receptors. *Circ. Res.* **99**, 570–582.
- DUXBURY, M.S., ASHLEY, S.W., and WHANG, E.E. (2005). RNA interference: a mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. *Biochem. Biophys. Res. Commun.* **331**, 459–463.
- EDIN, M.L., and JULIANO, R.L. (2005). Raf-1 serine 338 phosphorylation plays a key role in adhesion-dependent activation of extracellular signal-regulated kinase by epidermal growth factor. *Mol. Cell Biol.* **25**, 4466–4475.
- FAUSTER, K., HARTL, M., SANTNER, T., AIGNER, M., KREUTZ, C., BISTER, K., ENNIFAR, E., and MICURA, R. (2012). 2'-Azido RNA, a versatile tool for chemical biology: synthesis, X-ray structure, siRNA applications, click labeling. *ACS Chem. Biol.* **7**, 581–589.
- FELBER, A.E., BAYO-PUXAN, N., DELEAVEY, G.F., CASTAGNER, B., DAMHA, M.J., and LEROUX, J.C. (2012). The interactions of amphiphilic antisense oligonucleotides with serum proteins and their effects on *in vitro* silencing activity. *Biomaterials* **33**, 5955–5965.
- FILMORE, D. (2004). Its a GPCR world. *Mod. Drug Discovery* **7**, 24–28.
- GARRISON, J.C., ROLD, T.L., SIECKMAN, G.L., FIGUEROA, S.D., VOLKERT, W.A., JURISSON, S.S., and HOFFMAN, T.J. (2007). *In vivo* evaluation and small-animal PET/CT of a prostate cancer mouse model using ⁶⁴Cu bombesin analogs: side-by-side comparison of the CB-TE2A and DOTA chelation systems. *J. Nucl. Med.* **48**, 1327–1337.
- GEARY, R.S. (2009). Antisense oligonucleotide pharmacokinetics and metabolism. *Expert Opin. Drug Metab. Toxicol.* **5**, 381–391.
- GILLERON, J., QUERBES, W., ZEIGERER, A., BORODOVSKY, A., MARSICO, G., SCHUBERT, U., MANYGOATS, K., SEIFERT, S., ANDREE, C., STOTER, M., et al. (2013). Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat. Biotechnol.* **31**, 638–646.
- GILMAN, A.G. (1995). Nobel lecture. G proteins and regulation of adenylyl cyclase. *Biosci. Rep.* **15**, 65–97.
- GOH, L.K., HUANG, F., KIM, W., GYGI, S., and SORKIN, A. (2010). Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. *J. Cell Biol.* **189**, 871–883.
- GURTOVENKO, A.A., ANWAR, J., and VATTULAINEN, I. (2010). Defect-mediated trafficking across cell membranes: insights from in silico modeling. *Chem. Rev.* **110**, 6077–6103.
- HANSS, B., LEAL-PINTO, E., TEIXEIRA, A., CHRISTIAN, R.E., SHABANOWITZ, J., HUNT, D.F., and KLOTMAN, P.E. (2002). Cytosolic malate dehydrogenase confers selectivity of the nucleic acid-conducting channel. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 1707–1712.
- HANYALOGLU, A.C., and VON ZASTROW, M. (2008). Regulation of GPCRs by endocytic membrane trafficking and its potential implications. *Annu. Rev. Pharmacol. Toxicol.* **48**, 537–568.
- HENNE, W.M., BUCHKOVICH, N.J., and EMR, S.D. (2011). The ESCRT pathway. *Dev. Cell* **21**, 77–91.
- HOPKINS, A.L., and GROOM, C.R. (2002). The druggable genome. *Nat. Rev. Drug Discov.* **1**, 727–730.
- HOUSTON, D., OHNO, M., NICHOLAS, R.A., JACOBSON, K.A., and HARDEN, T.K. (2006). [³²P]2-iodo-N⁶-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate ([³²P]MRS2500), a novel radioligand for quantification of native P2Y₁ receptors. *Br. J. Pharmacol.* **147**, 459–467.
- HOWES, M.T., MAYOR, S., and PARTON, R.G. (2010). Molecules, mechanisms, and cellular roles of clathrin-independent endocytosis. *Curr. Opin. Cell Biol.* **22**, 519–527.
- HUGHSON, F.M., and REINISCH, K.M. (2010). Structure and mechanism in membrane trafficking. *Curr. Opin. Cell Biol.* **22**, 454–460.
- HUOTARI, J., and HELENIUS, A. (2011). Endosome maturation. *EMBO J.* **30**, 3481–3500.
- HUTAGALUNG, A.H., and NOVICK, P.J. (2011). Role of Rab GTPases in membrane traffic and cell physiology. *Physiol. Rev.* **91**, 119–149.
- HWANG, J.Y., PARK, J., KANG, B.J., LUBOW, D.J., CHU, D., FARKAS, D.L., SHUNG, K.K., and MEDINA-KAUWE, L.K. (2012). Multimodality imaging *in vivo* for preclinical assessment of tumor-targeted doxorubicin nanoparticles. *PLoS One* **7**, e34463.
- HYNES, N.E., and LANE, H.A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer* **5**, 341–354.

- HYNES, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673–687.
- IMAI, Y., LEUNG, C.K., FRIESEN, H.G., and SHIU, R.P. (1982). Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture. *Cancer Res.* **42**, 4394–4398.
- JOHANNES, L., and WUNDER, C. (2011). Retrograde transport: two (or more) roads diverged in an endosomal tree? *Traffic* **12**, 956–962.
- JULIANO, R., BAUMAN, J., KANG, H., and MING, X. (2009). Biological barriers to therapy with antisense and siRNA oligonucleotides. *Mol. Pharm.* **6**, 686–695.
- JULIANO, R.L., ALAHARI, S., YOO, H., KOLE, R., and CHO, M. (1999). Antisense pharmacodynamics: critical issues in the transport and delivery of antisense oligonucleotides. *Pharm. Res.* **16**, 494–502.
- JULIANO, R.L., MING, X., and NAKAGAWA, O. (2012a). Cellular uptake and intracellular trafficking of antisense and siRNA oligonucleotides. *Bioconjug. Chem.* **23**, 147–157.
- JULIANO, R.L., MING, X., and NAKAGAWA, O. (2012b). The chemistry and biology of oligonucleotide conjugates. *Acc. Chem. Res.* **45**, 1067–1076.
- JULIANO, R.L., REDDIG, P., ALAHARI, S., EDIN, M., HOWE, A., and APLIN, A. (2004). Integrin regulation of cell signalling and motility. *Biochem. Soc. Trans.* **32**, 443–446.
- KABANOV, A.V., and VINOGRADOV, S.V. (2009). Nanogels as pharmaceutical carriers: finite networks of infinite capabilities. *Angew Chem. Int. Ed. Engl.* **48**, 5418–5429.
- KANEDA, M.M., SASAKI, Y., LANZA, G.M., MILBRANDT, J., and WICKLINE, S.A. (2010). Mechanisms of nucleotide trafficking during siRNA delivery to endothelial cells using perfluorocarbon nanoemulsions. *Biomaterials* **31**, 3079–3086.
- KARJALAINEN, M., KAKKONEN, E., UPLA, P., PALORANTA, H., KANKAANPAA, P., LIBERALI, P., RENKEMA, G.H., HYYPIA, T., HEINO, J., and MARJOMAKI, V. (2008). A Raft-derived, Pak1-regulated entry participates in alpha2beta1 integrin-dependent sorting to caveosomes. *Mol. Biol. Cell* **19**, 2857–2869.
- KAWAI, T., and AKIRA, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637–650.
- KENDALL, G.C., MOKHONOVA, E.I., MORAN, M., SEJBUK, N.E., WANG, D.W., SILVA, O., WANG, R.T., MARTINEZ, L., LU, Q.L., DAMOISEAUX, R., et al. (2012). Dantrolene enhances antisense-mediated exon skipping in human and mouse models of Duchenne muscular dystrophy. *Sci. Transl. Med.* **4**, 164ra160.
- KERR, M.C., and TEASDALE, R.D. (2009). Defining macropinocytosis. *Traffic* **10**, 364–371.
- KIM, C., YE, F., and GINSBERG, M.H. (2011). Regulation of integrin activation. *Annu. Rev. Cell Dev. Biol.* **27**, 321–345.
- KIMPLE, A.J., BOSCH, D.E., GIGUERE, P.M., and SIDEROVSKI, D.P. (2011). Regulators of G-protein signaling and their Galpha substrates: promises and challenges in their use as drug discovery targets. *Pharmacol. Rev.* **63**, 728–749.
- KIRPOTIN, D.B., DRUMMOND, D.C., SHAO, Y., SHALABY, M.R., HONG, K., NIELSEN, U.B., MARKS, J.D., BENZ, C.C., and PARK, J.W. (2006). Antibody targeting of long-circulating lipid nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res.* **66**, 6732–6740.
- KOCH, S., and CLAESSEON-WELSH, L. (2012). Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb. Perspect. Med.* **2**, a006502.
- KOLE, R., KRAINER, A.R., ALTMAN, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nat. Rev. Drug Discov.* **11**, 125–1240.
- KOLLER, E., VINCENT, T.M., CHAPPELL, A., DE, S., MANOHARAN, M., and BENNETT, C.F. (2011). Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. *Nucleic Acids Res.* **39**, 4795–4807.
- KORTYLEWSKI, M., SWIDERSKI, P., HERRMANN, A., WANG, L., KOWOLIK, C., KUJAWSKI, M., LEE, H., SCUTO, A., LIU, Y., YANG, C., et al. (2009). *In vivo* delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. *Nat. Biotechnol.* **27**, 925–932.
- KRAUSE, D.S., and VAN ETTEN, R.A. (2005). Tyrosine kinases as targets for cancer therapy. *N. Engl. J. Med.* **353**, 172–187.
- KRUTZFELDT, J., RAJEWSKY, N., BRAICH, R., RAJEEV, K.G., TUSCHL, T., MANOHARAN, M., and STOFFEL, M. (2005). Silencing of microRNAs *in vivo* with ‘antagomirs’. *Nature* **438**, 685–689.
- LAJOIE, P., and NABI, I.R. (2010). Lipid rafts, caveolae, and their endocytosis. *Int. Rev. Cell Mol. Biol.* **282**, 135–163.
- LEE, C.H., LEE, Y.J., KIM, J.H., LIM, J.H., KIM, J.H., HAN, W., LEE, S.H., NOH, G.J., and LEE, S.W. (2013). Inhibition of hepatitis C virus (HCV) replication by specific RNA aptamers against HCV NS5B RNA replicase. *J. Virol.* **87**, 7064–7074.
- LEMMON, M.A., and SCHLESSINGER, J. (2010). Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117–1134.
- LEVITZKI, A., ATLAS, D., and STEER, M.L. (1974). The binding characteristics and number of beta-adrenergic receptors on the turkey erythrocyte. *Proc. Natl. Acad. Sci. U. S. A.* **71**, 2773–2776.
- LI, J., WANG, Y., ZHU, Y., and OUPICKY, D. (2013). Recent advances in delivery of drug-nucleic acid combinations for cancer treatment. *J. Control. Release* **172**, 589–600.
- LI, S., HUANG, S., and PENG, S.B. (2005). Overexpression of G protein-coupled receptors in cancer cells: involvement in tumor progression. *Int. J. Oncol.* **27**, 1329–1339.
- LIU, X., WANG, Y., and HNATOWICH, D.J. (2011). A nanoparticle for tumor targeted delivery of oligomers. *Methods Mol. Biol.* **764**, 91–105.
- LONNBERG, H. (2009). Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjug. Chem.* **20**, 1065–1094.
- LOVE, K.T., MAHON, K.P., LEVINS, C.G., WHITEHEAD, K.A., QUERBES, W., DORKIN, J.R., QIN, J., CANTLEY, W., QIN, L.L., RACIE, T., et al. (2010). Lipid-like materials for low-dose, *in vivo* gene silencing. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 1864–1869.
- LU, J.J., LANGER, R., and CHEN, J. (2009). A novel mechanism is involved in cationic lipid-mediated functional siRNA delivery. *Mol. Pharm.* **6**, 763–771.
- LV, H., ZHANG, S., WANG, B., CUI, S., and YAN, J. (2006). Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release* **114**, 100–109.
- MARGADANT, C., MONSUUR, H.N., NORMAN, J.C., and SONNENBER, G.A. (2011). Mechanisms of integrin activation and trafficking. *Curr. Opin. Cell Biol.* **23**, 607–614.
- MARLIN, F., SIMON, P., SAISON-BEHMOARAS, T., and GIOVANNANGELI, C. (2010). Delivery of oligonucleotides and analogues: the oligonucleotide conjugate-based approach. *ChemBiochem* **11**, 1493–500.
- MCNAMARA, J.O., 2nd, ANDRECHEK, E.R., WANG, Y., VILES, K.D., REMPEL, R.E., GILBOA, E., SULLENGER, B.A., and GIANGRANDE, P.H. (2006). Cell type-specific delivery of

- siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol.* **24**, 1005–1015.
- METTLEN, M., PUCADYIL, T., RAMACHANDRAN, R., and SCHMID, S.L. (2009). Dissecting dynamin's role in clathrin-mediated endocytosis. *Biochem. Soc. Trans.* **37**, 1022–1026.
- MIACZYNSKA, M., and BAR-SAGI, D. (2010). Signaling endosomes: seeing is believing. *Curr. Opin. Cell Biol.* **22**, 535–540.
- MILLARD, M., ODDE, S., and NEAMATI, N. (2011). Integrin targeted therapeutics. *Theranostics* **1**, 154–188.
- MING, X. (2011). Cellular delivery of siRNA and antisense oligonucleotides via receptor-mediated endocytosis. *Expert Opin. Drug Deliv.* **8**, 435–449.
- MING, X., ALAM, M.R., FISHER, M., YAN, Y., CHEN, X., and JULIANO, R.L. (2010). Intracellular delivery of an antisense oligonucleotide via endocytosis of a G protein-coupled receptor. *Nucleic Acids Res.* **38**, 6567–6576.
- MING, X., CARVER, K., FISHER, M., NOEL, R., CINTRAT, J.C., GILLET, D., BARBIER, J., CAO, C., BAUMAN, J., and JULIANO, R.L. (2013a). The small molecule Retro-1 enhances the pharmacological actions of antisense and splice switching oligonucleotides. *Nucleic Acids Res.* **41**, 3673–3687.
- MING, X., CARVER, K., and WU, L. (2013b). Albumin-based nanoconjugates for targeted delivery of therapeutic oligonucleotides. *Biomaterials* **34**, 7939–7949.
- MING, X., SATO, K., and JULIANO, R.L. (2011). Unconventional internalization mechanisms underlying functional delivery of antisense oligonucleotides via cationic lipoplexes and polyplexes. *J. Control. Release* **153**, 83–92.
- MOLITORIS, B.A., DAGHER, P.C., SANDOVAL, R.M., CAMPOS, S.B., ASHUSH, H., FRIDMAN, E., BRAFMAN, A., FAERMAN, A., ATKINSON, S.J., THOMPSON, J.D., et al. (2009). siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury. *J. Am. Soc. Nephrol.* **20**, 1754–1764.
- MOSSALAM, M., DIXON, A.S., and LIM, C.S. (2010). Controlling subcellular delivery to optimize therapeutic effect. *Ther. Deliv.* **1**, 169–193.
- MULGREW, K., KINNEER, K., YAO, X.T., WARD, B.K., DAMSCHRODER, M.M., WALSH, B., MAO, S.Y., GAO, C., KIENER, P.A., COATS, S., et al. (2006). Direct targeting of alphavbeta3 integrin on tumor cells with a monoclonal antibody, Abegrin. *Mol. Cancer Ther.* **5**, 3122–3129.
- NAKAGAWA, O., MING, X., HUANG, L., and JULIANO, R.L. (2010). Targeted intracellular delivery of antisense oligonucleotides via conjugation with small-molecule ligands. *J. Am. Chem. Soc.* **132**, 8848–8849.
- NAPIONE, L., PAVAN, S., VEGLIO, A., PICCO, A., BOFFETTA, G., CELANI, A., SEANO, G., PRIMO, L., GAMBA, A., and BUSSOLINO, F. (2012). Unraveling the influence of endothelial cell density on VEGF-A signaling. *Blood* **119**, 5599–5607.
- NGUYEN, J., and SZOKA, F.C. (2012). Nucleic acid delivery: the missing pieces of the puzzle? *Acc. Chem. Res.* **45**, 1153–1162.
- NIU, G., and CHEN, X. (2011). Why integrin as a primary target for imaging and therapy. *Theranostics* **1**, 30–47.
- OVERHOFF, M., and SCZAKIEL, G. (2005). Phosphorothioate-stimulated uptake of short interfering RNA by human cells. *EMBO Rep.* **6**, 1176–1181.
- PARACHONIAK, C.A., and PARK, M. (2012). Dynamics of receptor trafficking in tumorigenicity. *Trends Cell Biol.* **22**, 231–240.
- PARSONS, J.T. (2003). Focal adhesion kinase: the first ten years. *J. Cell Sci.* **116**, 1409–1416.
- PASTOR, F., KOLONIAS, D., GIANGRANDE, P.H., and GILBOA, E. (2010). Induction of tumour immunity by targeted inhibition of nonsense-mediated mRNA decay. *Nature* **465**, 227–230.
- PATEL, P.C., GILJOHANN, D.A., DANIEL, W.L., ZHENG, D., PRIGODICH, A.E., and MIRKIN, C.A. (2010). Scavenger receptors mediate cellular uptake of polyvalent oligonucleotide-functionalized gold nanoparticles. *Bioconjug. Chem.* **21**, 2250–2256.
- PETROS, R.A., and DESIMONE, J.M. (2010). Strategies in the design of nanoparticles for therapeutic applications. *Nat. Rev. Drug Discov.* **9**, 615–227.
- PFEFFER, S.R. (2007). Unsolved mysteries in membrane traffic. *Annu. Rev. Biochem.* **76**, 629–645.
- PFEFFER, S.R. (2013). Rab GTPase regulation of membrane identity. *Curr. Opin. Cell Biol.* **25**, 414–419.
- PHELPS, K., MORRIS, A., and BEAL, P.A. (2012). Novel modifications in RNA. *ACS Chem. Biol.* **7**, 100–109.
- PIERSCHBACHER, M.D., and RUOSLAHTI, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30–33.
- POST, S.R., HILAL-DANDAN, R., URASAWA, K., BRUNTON, L.L., and INSEL, P.A. (1995). Quantification of signalling components and amplification in the beta-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes. *Biochem. J.* **311**, 75–80.
- RAO, H., SAWANT, A.A., TANPURE, A.A., and SRIVATSAN, S.G. (2012). Posttranscriptional chemical functionalization of azide-modified oligoribonucleotides by bioorthogonal click and Staudinger reactions. *Chem. Commun. (Camb.)* **48**, 498–500.
- ROBBINS, M., JUDGE, A., and MACLACHLAN, I. (2009). siRNA and innate immunity. *Oligonucleotides* **19**, 89–102.
- SAHAY, G., QUERBES, W., ALABI, C., ELTOUKHY, A., SARKAR, S., ZURENKO, C., KARAGIANNIS, E., LOVE, K., CHEN, D., ZONCU, R., et al. (2013). Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. *Nat. Biotechnol.* **31**, 653–658.
- SCHWARTZ, M.A. (2010). Integrins and extracellular matrix in mechanotransduction. *Cold Spring Harb. Perspect. Biol.* **2**, a005066.
- SCZEKAN, M.M., and JULIANO, R.L. (1990). Internalization of the fibronectin receptor is a constitutive process. *J Cell Physiol* **142**, 574–80.
- SHEPARD, H.M., JIN, P., SLAMON, D.J., PIROT, Z., and MANEVAL, D.C. (2008). SHEPARD, H.M., JIN, P., SLAMON, D.J., PIROT, Z., and MANEVAL, D.C. (2008). Herceptin. *Handb. Exp. Pharmacol.* **181**, 183–219.
- SHIMAOKA, M., and SPRINGER, T.A. (2003). Therapeutic antagonists and conformational regulation of integrin function. *Nat. Rev. Drug Discov.* **2**, 703–716.
- SHUKLA A.K., XIAO K., LEFKOWITZ R.J. (2011). Emerging paradigms of beta-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem. Sci.* **36**, 457–469.
- SINGH, Y., MURAT, P., and DEFRANCO, E. (2010). Recent developments in oligonucleotide conjugation. *Chem. Soc. Rev.* **39**, 2054–2070.
- SORKIN, A., and VON ZASTROW, M. (2009). Endocytosis and signalling: intertwining molecular networks. *Nat. Rev. Mol. Cell Biol.* **10**, 609–622.
- SPANG, A. (2009). On vesicle formation and tethering in the ER-Golgi shuttle. *Curr. Opin. Cell Biol.* **21**, 531–536.
- STECHMANN, B., BAI, S.K., GOBBO, E., LOPEZ, R., MERER, G., PINCHARD, S., PANIGAI, L., TENZA, D., RAPOSO, G., BEAUMELLE, B., et al. (2010). Inhibition of retrograde transport protects mice from lethal ricin challenge. *Cell* **141**, 231–242.

- STEIN, C.A., HANSEN, J.B., LAI, J., WU, S., VOSKRESENSKIY, A., HOG, A., WORM, J., HEDTJARN, M., SOULEIMANIAN, N., MILLER, P., et al. (2010). Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. *Nucleic Acids Res.* **38**, e3.
- STENMARK, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell. Biol.* **10**, 513–525.
- STREULI, C.H., and AKHTAR, N. (2009). Signal co-operation between integrins and other receptor systems. *Biochem J* **418**, 491–506.
- SZABO, A.M., HOWELL, N.R., PELLEGRINI, P., GREGURIC, I., and KATSIFIS, A. (2012). Development and validation of competition binding assays for affinity to the extracellular matrix receptors, alpha(v)beta(3) and alpha(IIb)beta(3) integrin. *Anal. Biochem.* **423**, 70–77.
- TAMURA, A., and NAGASAKI, Y. (2010). Smart siRNA delivery systems based on polymeric nanoassemblies and nanoparticles. *Nanomedicine (Lond.)* **5**, 1089–1102.
- TOHIDKIA, M.R., BARAR, J., ASADI, F., and OMIDI, Y. (2012). Molecular considerations for development of phage antibody libraries. *J. Drug Target* **20**, 195–208.
- TSANG, S.Y., MOORE, J.C., HUIZEN, R.V., CHAN, C.W., and LI, R.A. (2007). Ectopic expression of systemic RNA interference defective protein in embryonic stem cells. *Biochem. Biophys. Res. Commun.* **357**, 480–486.
- TSENG, Y.C., MOZUMDAR, S., and HUANG, L. (2009). Lipid-based systemic delivery of siRNA. *Adv. Drug Deliv. Rev.* **61**, 721–731.
- UGARTE-URIBE, B., GRIJALVO, S., BUSTO, J.V., MARTIN, C., ERITJA, R., GONI, F.M., and ALKORTA, I. (2013). Double-tailed lipid modification as a promising candidate for oligonucleotide delivery in mammalian cells. *Biochim. Biophys. Acta* **1830**, 4872–4884.
- UNO, Y., PIAO, W., MIYATA, K., NISHINA, K., MIZUSAWA, H., and YOKOTA, T. (2011). High-density lipoprotein facilitates *in vivo* delivery of alpha-tocopherol-conjugated short-interfering RNA to the brain. *Hum. Gene Ther.* **22**, 711–719.
- VARKOUHI, A.K., SCHOLTE, M., STORM, G., and HAISMA, H.J. (2011). Endosomal escape pathways for delivery of biologicals. *J. Control. Release* **151**, 220–228.
- VON KLEIST, L., and HAUCKE, V. (2012). At the crossroads of chemistry and cell biology: inhibiting membrane traffic by small molecules. *Traffic* **13**, 495–504.
- WATTS, J.K., DELEAVEY, G.F., and DAMHA, M.J. (2008). Chemically modified siRNA: tools and applications. *Drug Discov. Today* **13**, 842–855.
- WHEELER, L.A., TRIFONOVA, R., VRBANAC, V., BASAR, E., MCKERNAN, S., XU, Z., SEUNG, E., DERUAZ, M., DUDEK, T., EINARSSON, J.I., et al. (2011). Inhibition of HIV transmission in human cervicovaginal explants and humanized mice using CD4 aptamer-siRNA chimeras. *J. Clin. Invest.* **121**, 2401–2412.
- WHITEHEAD, K.A., LANGER, R., and ANDERSON, D.G. (2009). Knocking down barriers: advances in siRNA delivery. *Nat. Rev. Drug Discov.* **8**, 129–138.
- WICKSTROM, S.A., and FASSLER, R. (2011). Regulation of membrane traffic by integrin signaling. *Trends Cell Biol.* **21**, 266–273.
- WINSTON, W.M., SUTHERLIN, M., WRIGHT, A.J., FEINBERG, E.H., and HUNTER, C.P. (2007). *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 10565–10570.
- WINZ, M.L., SAMANTA, A., BENZINGER, D., and JASCHKE, A. (2012). Site-specific terminal and internal labeling of RNA by poly(A) polymerase tailing and copper-catalyzed or copper-free strain-promoted click chemistry. *Nucleic Acids Res.* **40**, e78.
- WOLFRUM, C., SHI, S., JAYAPRAKASH, K.N., JAYARAMAN, M., WANG, G., PANDEY, R.K., RAJEEV, K.G., NAKAYAMA, T., CHARRISE, K., NDUNGO, E.M., et al. (2007). Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. *Nat. Biotechnol.* **25**, 1149–1157.
- XU, S., OLENYUK, B.Z., OKAMOTO, C.T., and HAMM-ALVAREZ, S.F. (2013). Targeting receptor-mediated endocytotic pathways with nanoparticles: rationale and advances. *Adv. Drug Deliv. Rev.* **65**, 121–138.
- YAMADA, T., PENG, C.G., MATSUDA, S., ADDEPALLI, H., JAYAPRAKASH, K.N., ALAM, M.R., MILLS, K., MAIER, M.A., CHARISSE, K., SEKINE, M., et al. (2011). Versatile site-specific conjugation of small molecules to siRNA using click chemistry. *J. Org. Chem.* **76**, 1198–1211.
- ZHANG, Q., HOSSAIN, D.M., NECHAEV, S., KOZLOWSKA, A., ZHANG, W., LIU, Y., KOWOLIK, C.M., SWIDERSKI, P., ROSSI, J.J., FORMAN, S., et al. (2013). TLR9-mediated siRNA delivery for targeting of normal and malignant human hematopoietic cells *in vivo*. *Blood* **121**, 1304–1315.
- ZHANG, Y., QU, Z., KIM, S., SHI, V., LIAO, B., KRAFT, P., BANDARU, R., WU, Y., GREENBERGER, L.M., and HORAK, I.D. (2011). Down-modulation of cancer targets using locked nucleic acid (LNA)-based antisense oligonucleotides without transfection. *Gene Ther.* **18**, 326–333.
- ZHENG, D., GILJOHANN, D.A., CHEN, D.L., MASSICH, M.D., WANG, X.Q., IORDANOV, H., MIRKIN, C.A., and PALLER, A.S. (2012). Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 11975–11980.
- ZHOU, J., and ROSSI, J.J. (2011). Cell-specific aptamer-mediated targeted drug delivery. *Oligonucleotides* **21**, 1–10.

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