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SURVEY AND SUMMARY

Addressing cancer signal transduction pathways with antisense and siRNA oligonucleotides

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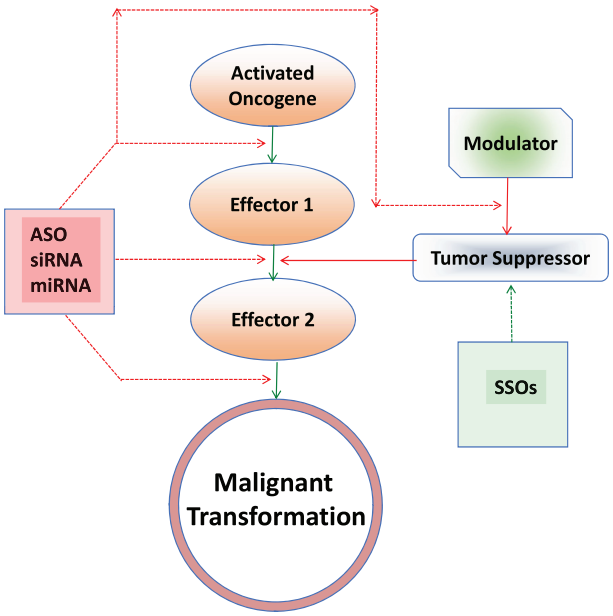
ABSTRACT

Signal transduction pathways play key roles in the initiation, progression and dissemination of cancer. Thus, signaling molecules are attractive targets for cancer therapeutics and enormous efforts have gone into the development of small molecule inhibitors of these pathways. However, regrettably, there has been only moderate progress to date, primarily in connection with the RAS signaling pathway. Oligonucleotide-based drugs potentially offer several advantages for addressing signaling pathways, including their exquisite selectivity and their ability to exploit both enzymatic and nonenzymatic targets. Nonetheless, there are problems inherent in the oligonucleotide approach, not the least being the challenge of effectively delivering these complex molecules to intracellular sites within tumors. This survey article will provide a selective review of recent studies where oligonucleotides were used to address cancer signaling and will discuss both positive aspects and limitations of those studies. This will be set in the context of an overview of various cancer signaling pathways and small molecule approaches to regulate those pathways. The survey will also evaluate the challenges and opportunities implicit in the oligonucleotide-based approach to cancer signaling and will point out several possibilities for future research.

GRAPHICAL ABSTRACT



INTRODUCTION

Aberrations in signaling pathways frequently underlie the initiation and progression of cancer. Activation or overexpression of oncogenes or loss or inhibition of tumor suppressor genes connected to signal transduction can lead to the multiple manifestations of cancer (1). This includes both changes to the tumor cells themselves and alterations of the tumor microenvironment. Hallmark features of cancer cells include enhanced cell proliferation, resistance to programmed cell death, altered metabolism and changes in cell fate and differentiation such as the epithelial–mesenchymal transition often seen in carcinomas (2,3). Tumors can also influence their local and distant microenvironments through signaling processes that affect angiogenesis, inflammation and modulation of potential metastatic sites

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(4,5). In considering therapeutic intervention in cancer signal transduction, it is important to recall that these pathways are highly convoluted, with multiple interconnections between pathways as well as numerous feedback and feed-forward control mechanisms (6). Thus, perturbation of one pathway can have unintended consequences for other pathways; this reality has often impeded the development of therapies directed toward signaling. A powerful concept for cancer therapeutics has been the idea of 'oncogene addiction' whereby tumor cells become highly dependent on the activated state of a particular oncogene (7). However, there are limitations on this approach, including the emergence of resistance to oncogene-directed therapy (8). Another complexity relates to the existence in many tumors of cancer stem cells whose properties are markedly different from the bulk cell population. These differences often include modifications of signaling pathways and altered responses to therapy (9).

Cancer signaling pathways offer many potential opportunities for oligonucleotide-based therapeutics. Indeed, there has been interest in this possibility since the earliest days of research on antisense oligonucleotides (ASOs) (10). However, progress has been slow due to many factors, not the least being the complexity of cancer-related signaling. This survey will focus on recent developments in the application of siRNA oligonucleotides, ASOs and splice-switching oligonucleotides (SSOs) to the regulation of signal transduction in cancer. It will deal solely with efforts directed at core cytosolic signaling pathways and will not discuss upstream ligands and receptors nor downstream mechanisms of cell cycle control, cell death or cell differentiation. An initial overview of individual pathways and small molecule inhibitors of those pathways will precede discussion of oligonucleotide-based therapeutic approaches.

CANCER SIGNALING PATHWAYS

The following sections provide simplified descriptions of some of the key signaling pathways involved in cancer and explore how they have been addressed with small molecule drugs. This will provide context for the subsequent discussion of oligonucleotide-based approaches.

RAS-related signaling

RAS GTPases are molecular switches that play a critical role in many cancers (11). In normal cells, RAS is activated by receptor tyrosine kinases whose autophosphorylation recruits guanine nucleotide exchange factors such as SOS (Son of Sevenless) to the plasma membrane where they can interact with membrane-bound RAS converting it to its active GTP-bound state. Conversely, GTPase-activating proteins such as neurofibromin 1 return RAS to its inactive GDP-bound state. Activated RAS interacts with multiple downstream effectors setting up signaling cascades that regulate many cellular activities, including proliferation, survival, metabolism and cytoskeletal organization. Initial RAS effectors contain weakly homologous RAS-binding domains (RBDs) that interact with RAS and trigger conformational changes that lead to activation of the effector. The two RAS signaling pathways most prominently associated with cancer are the MAP kinase pathway regulating cell proliferation and the phosphoinositide 3-kinase (PI3K) pathway that regulates cell metabolism and survival (Figure 1).

In the MAP kinase pathway (12), activated RAS binds to the RBD of a member of the RAF family of serine–threonine kinases, thus relieving an auto-inhibition and activating the kinase. RAF then activates MEK, a dual-specificity kinase, which in turn activates the ERK MAP kinase. ERK can enter the nucleus and phosphorylate several transcription factors, including ELK-1 and MYC leading to transcriptional activation of genes that positively regulate the cell cycle.

PI3Ks catalyze the phosphorylation of PIP2 to PIP3 that serves as a second messenger for numerous downstream processes (2,13). There are multiple PI3Ks in mammals, but several of them are activated via the interaction of activated RAS with an RBD in the p110 kinase subunit. The activity of PI3Ks is countered by inositol lipid phosphatases, particularly PTEN, which has an important role as a tumor suppressor. A key downstream effector of PI3K is the AKT serine/threonine kinase that influences both cell survival and cell metabolism. Thus, AKT-mediated phosphorylation of FOXO transcription factors (14) prevents their activation of pro-apoptotic genes and thus enhances cell survival. AKT also modulates the mTORC1 complex (15) that senses nutrient levels and coordinates metabolism and protein synthesis. Thus, AKT phosphorylates and inhibits the tumor suppressor proteins TSC1 and TSC2 that in turn inhibit Rheb, a GTPase that is an important regulatory component of the mTOR complex.

Activating mutations in RAS and its downstream effectors are present in many types of cancer. Mutations in RAS, particularly the KRAS and NRAS isoforms, are prevalent in pancreatic, colorectal and lung cancers (11,12), whereas mutations in B-RAF are common in melanoma and colorectal cancer (12,16). Activating mutations have also been identified in PI3K, particularly in the p110α subunit, and are associated with cancers of the breast, colon, stomach, cervix, prostate and lung (13,17). However, a major aspect of the role of PI3K in cancer involves the loss or inactivation of the PTEN tumor suppressor that leads to dysregulation of the PI3K pathway (18).

Small molecule modulation of Ras and its downstream effectors

RAS inhibitors. Enormous efforts have gone into the search for drugs that control cancer by affecting signaling pathways. Obviously, RAS itself has been a major target in this effort, but thus far only modest success has been attained (19,20). RAS normally associates with the plasma membrane through a farnesyl lipid linked to a cysteine residue in its COOH terminus. A number of farnesyl transferase inhibitors were developed, but these led to disappointing results in clinical trials. One reason for this is the existence of an alternative lipidation pathway involving geranylgeranyl transferases. A more recent approach has been to directly address activating mutations in RAS. Thus, compounds were developed that cause irreversible allosteric inhibition of KRAS G12C, thereby trapping RAS in its inac-

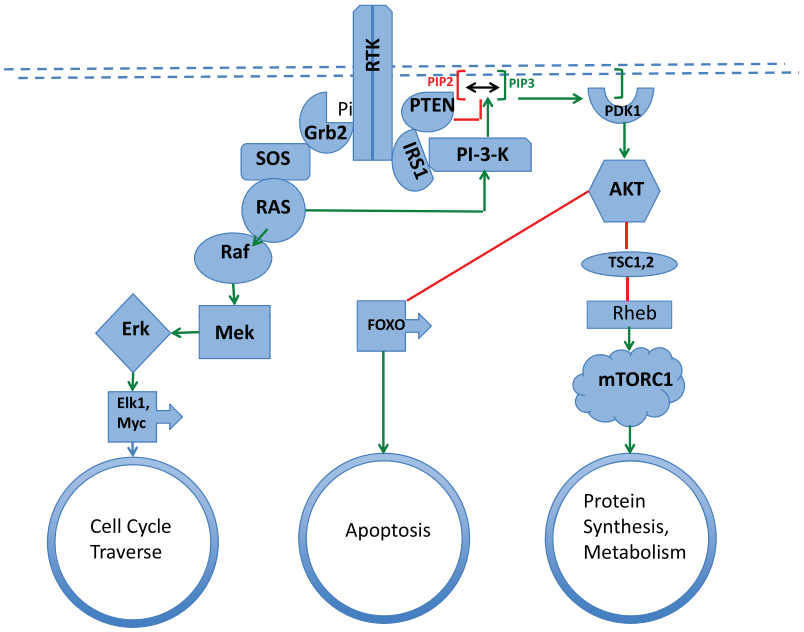

Figure 1. The RAS signaling pathway. This figure and Figures 2–4 present simplified versions of complex pathways. There are multiple additional connections within each pathway that are not depicted as well as interconnections between pathways. RAS signaling in cancer has two major aspects: the MAP kinase pathway and the PI3K pathway. These regulate cell cycle control, apoptosis, cell metabolism and protein synthesis. Green arrows indicate activation, while red lines indicate inhibition. *Abbreviations*: RTK, receptor tyrosine kinase; Grb2, growth factor receptor-bound protein, an adaptor protein; SOS, Son of Sevenless, a guanine nucleotide exchange factor for RAS; RAS, a small GTPase; Raf, a serine–threonine kinase; Mek, Map kinase/Erk kinase, a dual-specificity kinase; Erk, extracellular signal-regulated kinase, a serine–threonine kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10, a lipid phosphatase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent protein kinase 1, a serine–threonine kinase activated by PIP3; AKT, a serine–threonine kinase; TSC1,2, tuberous sclerosis proteins, GTPase-activating proteins for Rheb; Rheb, RAS homolog enriched in brain, a small GTPase; mTORC1, mechanistic target of rapamycin, a multiprotein complex regulating metabolism; FOXO, forkhead box O, transcription factors involved in control of apoptosis.

tive GDP-bound state. Two phase I clinical trials of this type of compound are underway. However, it is unclear whether this progress can be replicated for other activating mutations, such as the more common G12D mutation, where a reactive cysteine is not available.

RAF and MEK inhibitors. In contrast to the situation with RAS itself, there has been substantial progress in the development of inhibitors in the MAP kinase pathway. A key finding was discovery of the V600E BRAF mutation in a majority of melanomas (16). In contrast to wild-type (WT) RAFs that require RAS interaction and dimerization for activation, BRAF V600E is constitutively active as a monomer (21). A number of ATP-competitive selective inhibitors of BRAF have been developed and two (vemurafenib and dabrafenib) have been approved by the FDA. These molecules dramatically changed the therapeutic landscape in melanoma (22,23). Vemurafenib and dabrafenib as single agents both produce objective responses in ∼50% of melanoma patients with BRAF V600E mutations and also provide improved survival. However, these drugs are much less effective in colon cancers and non-small cell lung cancers that also have the V600E mutation. Additionally, patients treated with these agents as monotherapy rapidly develop resistance that is likely due to reactivation of downstream elements in the MAP kinase pathway. Another issue is the paradoxical activation of WT BRAF by these drugs leading to activation of the MAP kinase cascade and potentially to tumor formation in tissues that lack V600E BRAF.

Inhibitors of the MEK kinase have also been developed. While these molecules have been used as monotherapy, their most important role is in combination with RAF inhibitors (22,24). Two compounds (trametinib and cobimetinib) have been approved by the FDA for use in melanoma, while several other Mek inhibitors are at various stages of clinical evaluation. The use of BRAF and MEK inhibitors in combination has at least partially overcome some of the limitations of monotherapy with V600E targeted drugs, particularly the reactivation of the MAP kinase pathway. Studies are also underway on direct inhibitors of ERK itself, but these are still at an early stage of clinical development. There has also been substantial work on the use of RAF and MEK inhibitor combinations in cancers other than melanoma. Despite these advances, melanoma remains a dire disease. A recent retrospective analysis of the dual use of dabrafenib plus trametinib in melanoma found an overall 5-year survival of 34% (25) demonstrating that new therapies are badly needed in this disease as well as in other cancers.

In that vein, two recent papers suggest an exciting new approach to cancer therapy via the RAS pathway (26,27). RAS-driven pancreatic cancers are essentially refractory to RAF and MEK inhibitors. However, combined use of a MEK inhibitor and an inhibitor of autophagy led to a synergistic cytotoxic effect both in cell culture and in animal tumor models. This approach of dual inhibition of two distinct but interconnected pathways mirrors the traditional approach of cytotoxic chemotherapy but with greater preci-

sion. It will be very interesting to see whether this approach can be extended to other tumors and whether it can advance to clinical trials.

PI3K inhibitors. As mentioned earlier, activating mutations of PI3K play a role in multiple cancers and thus there has been a great deal of work on PI3K inhibitors (17,28). Drugs under development include isoform-specific inhibitors, pan-PI3K inhibitors and dual PI3K/mTOR inhibitors. Thus far, two drugs have been approved by the FDA for use in lymphomas and leukemias. Idelalisib is an isoform-selective inhibitor, while duvelisib is a pan-PI3K inhibitor. Overall, PI3K inhibitors have had only modest effects as single agents and their use has been compromised by substantial toxicities, including autoimmune reactions (28).

Thus, over the last decade there has been substantial progress in the use of inhibitors of the RAS pathway in treatment of cancer, particularly in melanoma but in other cancers as well. Nonetheless, many challenges remain including heterogeneous efficacy, rapid emergence of resistance and unacceptable toxicities. Thus, innovative approaches to therapy for cancers that involve the RAS pathway are very much needed.

WNT pathway signaling

The importance of the WNT pathway in cancer has been appreciated ever since the discovery that mutations in adenomatous polyposis coli (APC), a key component of the pathway, are found in 80–90% of colon cancers (29). WNT signaling involves a multimolecular destruction complex that regulates the intracellular levels of the dual function protein β-catenin (30,31) (Figure 2). The formation of cadherin adherens junction in epithelial cells requires the presence of cytosolic β-catenin, while in the nucleus this protein interacts with TCF/LEF transcription factor to activate genes associated with cell cycle traverse. Appropriate levels of β-catenin are controlled by a protein complex that includes the structural proteins Axin 1 and APC, casein kinase 1, glycogen synthase kinase 3 (GSK3) and β-catenin itself. The phosphorylation of β-catenin marks it for recognition by the ubiquitin ligase β-TrCP that then ubiquitinates β-catenin and triggers its destruction by the proteasome.

Binding of WNT to its cell surface receptors FZD and LRP5/6 triggers disassembly of the cytosolic β-catenin destruction complex. The WNT-bound receptors recruit the cytosolic protein DVL allowing membrane docking of AXIN 1 and its associated kinases. This leads to reduced degradation of β-catenin and its accumulation in the nucleus. Recently, a second important Wnt-related regulatory pathway has been identified that involves enzymes termed tankyrases (TNKS) (32). The TNKS bind to AXIN 1 and catalyze the addition of poly(ADP-ribose) moieties. The modified AXIN 1 is then ubiquitinated by the RNF146 E3 ligase leading to its proteosomal degradation and thus disruption of the β-catenin destruction complex.

Small molecules affecting the WNT pathway

Despite the importance of the WNT pathway in cancer, there has been only modest progress in the development of inhibitors (31,33). A number of TNKS inhibitors have been developed, but these displayed concerning gastrointestinal toxicities (34). Inhibitors of binding between FZD and DVL have been described, as well as GSK3 inhibitors, but are at an early stage of development. Thus, there seem to be opportunities for development of novel approaches to therapeutically modulate the WNT pathway.

Notch and Hedgehog signaling

Cell-to-cell signaling through the NOTCH pathway is fundamental to cell fate decisions in developmental processes and also plays an important role in cancers (35,36). The interactions of NOTCH ligands on one cell with NOTCH on an adjacent cell lead to proteolytic release of the NOTCH intracellular domain (NICD) that then migrates to the nucleus to interact with CSL transcription factors (Figure 3). NOTCH ligands are transmembrane proteins that in mammals comprise three delta-type ligands (Dll1–3) and two jagged ligands (Jag1 and Jag2). The four mammalian NOTCH receptors are also transmembrane glycoproteins. Ligand–receptor interaction causes conformational changes that allow sequential cleavage of NOTCH first by an ADAM protease and then by γ-secretase leading to release of the NICD. The signaling outputs of this relatively simple system are remarkably complex and context dependent and may reflect different outputs from different ligand–receptor pairs as well as epigenetic distinctions among cells. While NOTCH may play a direct oncogenic role in some cancers such as T-cell lymphomas, more commonly its impact is on the tumor microenvironment (35,37). Thus, NOTCH ligand–receptor interactions can take place between tumor cells and adjacent stromal cells or between different lineages within the tumor cell population. For example, NOTCH signaling, particularly that involving the Jag1 ligand, can play a role in providing a favorable niche for cancer stem cells.

Hedgehog signaling (Figure 4) also plays a role in multiple developmental processes, while abnormalities in this pathway have been linked to several types of cancer, including basal cell carcinoma, medulloblastoma, and breast, lung, prostate and pancreatic cancers (38,39). Hedgehog signaling in mammals is associated with primary cilia that are microtubule-based structures at the cell surface (40,41). Signaling is activated by three hedgehog ligands, the best known being Sonic hedgehog (SHH). In the absence of SHH binding to its transmembrane receptor PTCH, the GPCR-like protein SMO is inhibited by PTCH and signaling is quiescent. SUFU proteins act with cytosolic kinases to keep transcription factors Gli1–3 in their repressor form. In the presence of SHH, inhibition is relieved and Smo migrates to the primary cilium and initiates the downstream signaling cascade. This results in the activation and nuclear translocation of Gli1–3.

Small molecules that affect Notch and Hedgehog signaling

Small molecule development in the NOTCH pathway has primarily focused on γ-secretase inhibitors (37,42). These molecules have shown promising results in early phase clinical trials. However, since γ-secretase has over 90 substrates

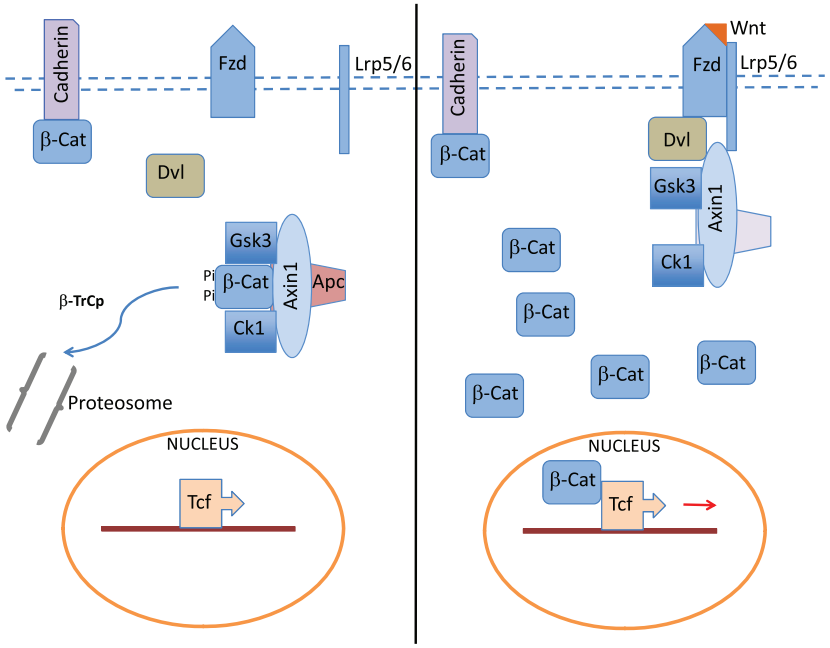

Figure 2. The Wnt signaling pathway. The key element of the Wnt pathway, β-catenin, has dual functions. It is both a transcriptional activator in the nucleus that regulates cell growth and also a key component of cell adhesion junctions. A multiprotein complex in the cytosol regulates the intracellular levels of β-catenin. *Abbreviations*: cadherins; a family of cell–cell adhesion proteins; Wnt, a family of polypeptide mediators; Fzd, Frizzled, a receptor for Wnt; Lrp5/6, lipoprotein receptor-related protein 5/6, a co-receptor for Wnt; Axin 1, a structural protein; APC, adenomatous polyposis coli, a structural protein; Ck1; casein kinase 1, a serine–threonine kinase; Gsk3, glycogen synthase kinase 3, a serine–threonine kinase; β-TrCP, β-transducin repeat-containing protein, a ubiquitin ligase; Tcf, TCF/LEF, T-cell factor/lymphoid enhancer factor, transcription factors.

including many important transmembrane proteins other than Notch, the potential for off-target effects is high. In patients, the dose-limiting toxicity is diarrhea due to intestinal goblet cell metaplasia. There has been a good deal of drug development activity for the Hedgehog pathway focusing on antagonists of SMO (37–39) but also including inhibitors of Gli. Currently, two SMO antagonists (vismodegib and sonidegib) are FDA approved for use in advanced basal cell cancer. Hedgehog pathway inhibitors are also being clinically evaluated in a variety of other cancers as single agents or in conjunction with standard chemotherapy. While there are many variations, the overview is that these molecules are not very effective in unselected patient cohorts but may be more successful in patients whose disease clearly has a Hedgehog-driven component.

OLIGONUCLEOTIDE APPROACHES TO CANCER SIGNALING

Basic aspects of oligonucleotide therapeutics

Over the last decade, oligonucleotide-based therapeutics has evolved from basic research to clinical reality with FDA approval of seven drugs, including two ASOs, two siRNA oligonucleotides and three SSOs (43,44). Progress has largely been based on advances in oligonucleotide chemistry that have improved stability, increased efficacy and reduced off-target and immunostimulatory effects (45–48). Some notable examples have been the development of methoxyethoxy (49), linked nucleic acid (50) and constrained ethyl (c-Et) (47) modifications that have markedly increased binding affinity, as well as backbone modifications such as the uncharged morpholino structure that has proven useful for SSOs (51).

Despite successes, a major remaining challenge for oligonucleotide-based therapeutics concerns the effective delivery of these molecules to their intracellular sites of action (52). This problem has two aspects. The first is to obtain sufficient accumulation of oligonucleotide in the tissue of interest. The second is to overcome nonproductive trapping of oligonucleotide within endosomal compartments. Although systemically administered oligonucleotides distribute broadly to tissues other than the central nervous system, there is great variability in tissue uptake with liver and kidney being predominant (49). Enormous efforts have been devoted to improving the delivery of oligonucleotides, particularly siRNA, primarily involving the use of various cationic lipid or polymer nanoparticles (53,54). However, most nanoparticles can exit from the bloodstream only at sites where the vascular endothelium has gaps of 100 nm or more, thus limiting nanoparticle delivery to the liver, spleen and some rapidly growing tumors, but not to many other tumors (55,56). There is also much interest in ligand–oligonucleotide conjugates that can interact with specific receptors and thus potentially allow tissue-selective targeting (57). Thus, carbohydrate conjugates of siRNAs and ASOs have shown dramatically increased uptake into the liver via the hepatic asialoglycoprotein receptor (58). The extent to which this approach will work with other receptors in other tissues, particularly tumors, is not entirely clear at this point but is a key area to explore.

The second major delivery issue concerns the intracellular trafficking of oligonucleotides (59–61). Whether ad-

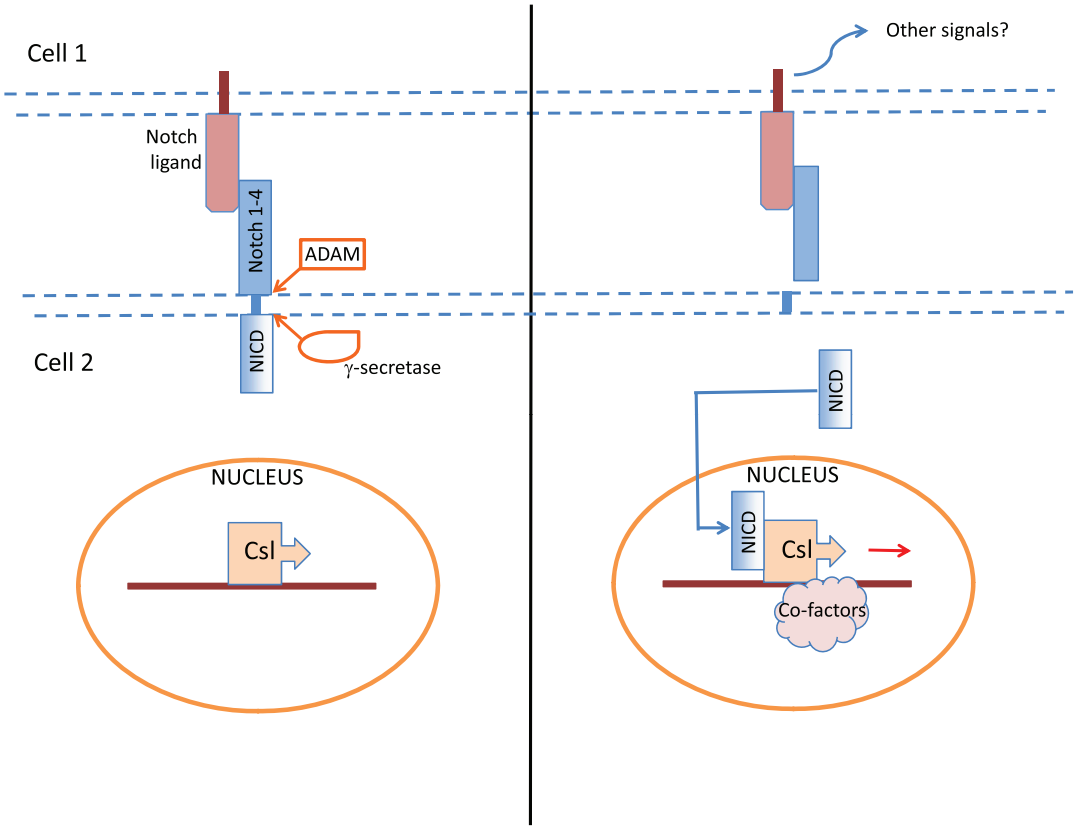

Figure 3. The Notch pathway. Notch signaling involves cell-to-cell communication. Notch ligands on an adjacent cell interact with Notch causing it to be cleaved by two proteases. This releases the Notch intracellular domain that then migrates to the nucleus to interact with transcription factors. *Abbreviations*: Notch ligands, transmembrane proteins of the Delta or Jagged type; ADAM, a disintegrin and metalloproteinase, an extracellular protease; γ-secretase, a membrane-associated protease complex; NICD, Notch intracellular domain; Csl, CBF1, Suppressor of Hairless, Lag-1, a transcription factor.

ministered as 'free' molecules or associated with nanoparticles, oligonucleotides are taken up via endocytosis and then traffic to intracellular membrane-bound compartments including early and late endosomes and lysosomes. Within these compartments, oligonucleotides are pharmacologically inert since they cannot access their molecular targets in the cytosol or nucleus. Certain types of nanoparticle carriers, particularly cationic lipoplexes (53), can facilitate oligonucleotide escape from endosomes. Additionally, another approach has evolved recently that utilizes endosome-destabilizing small molecules to promote oligonucleotide release and thus increased effectiveness (62,63).

In summary, while great progress has been made, there still remain important challenges to therapeutic use of oligonucleotides. These include off-target effects at the nucleic acid level, toxicities due to interactions with proteins (64) and, most importantly, the efficient intracellular delivery of these molecules to their intracellular sites of action.

Oligonucleotides in cancer

There is a very large literature describing use of various types of oligonucleotides in cancer and a number of clinical trials are currently underway. However, there has been more limited work done regarding oligonucleotide modulation of cancer signaling pathways. Several recent reviews address various aspects of the broad potential role of oligonucleotides in cancer therapy. Thus, an article by Chen *et al.* provides an overview of siRNA-based approaches and lists a number of recent clinical trials of siRNA in cancer (65). A review by Yamakawa *et al.* focuses on pancreatic cancer but describes several types of potential oligonucleotide therapies (66). Lee *et al.* discuss both potential siRNA targets in cancer and delivery strategies (67). A review by Martinez-Montiel *et al.* explores the role of alternative RNA splicing in cancer, discusses potential use of SSOs and provides information on current clinical trials (68). In the context of these broader views of oligonucleotides in cancer, this survey will focus on oligonucleotides that address cancer signaling pathways.

Oligonucleotide modulation of the RAS pathway

As discussed earlier, there has been limited progress in developing small molecule inhibitors of RAS and its effectors. In a sense, this presents an opportunity for deploying oligonucleotide approaches to this challenging problem. While there has been more success with small molecules for the enzymes of the MAP kinase and PI3K pathways, there still remain difficulties. Table 1 summarizes a selection of recent investigations of the use of ASOs or siRNA to address the overall RAS pathway. A few of these will be discussed in more detail below.

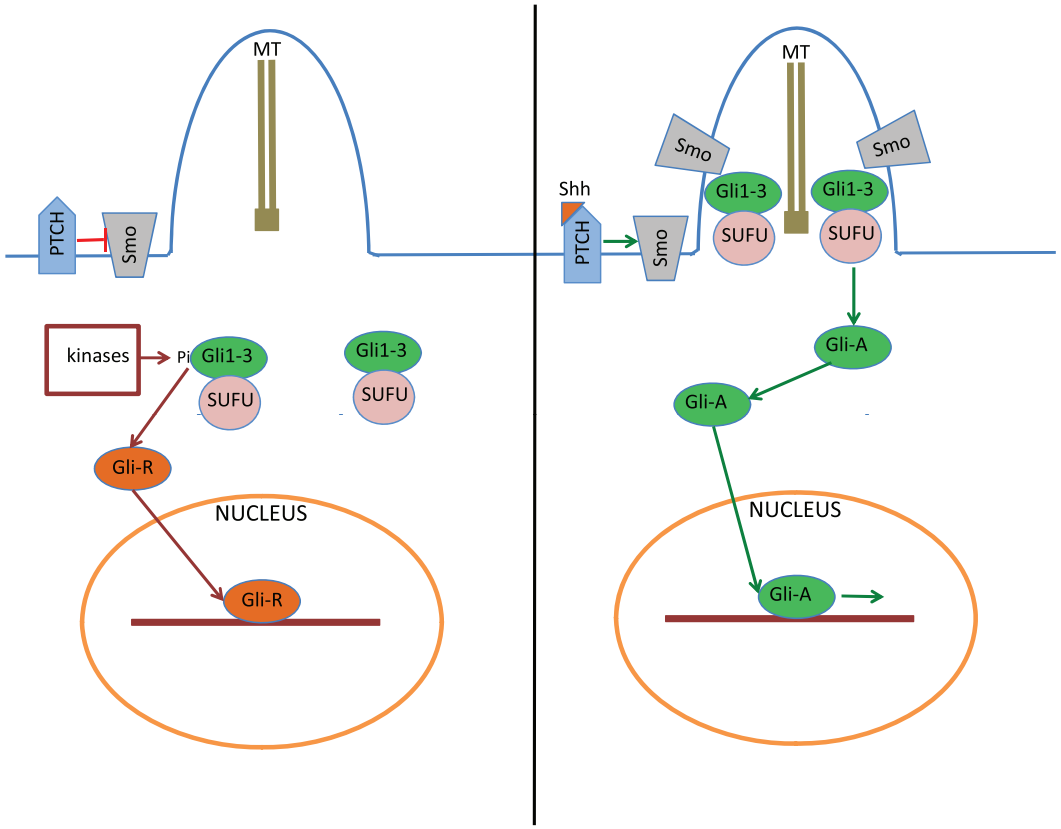

Figure 4. The Hedgehog pathway. The interaction of hedgehog ligands with their membrane receptor Patched controls the activation state of Gli family transcription factors. *Abbreviations*: Shh, Sonic hedgehog; PTCH, Patched, the receptor for hedgehog ligands; Smo, Smoothened, an intermediate protein in the pathway; Gli1–3, glioma-associated oncogene homolog, transcriptional regulators; SUFU, Suppressor of fused, a negative regulator of the pathway; MT, microtubules.

As indicated in Table 1, during the last few years there have been many publications concerning effects of oligonucleotides, especially siRNA, on RAS-driven cancers. However, progress has been limited as only a few of these studies have matured to clinical investigations. Many studies have relied on use of commercially available unmodified siRNAs that are known to be quite unstable as opposed to the highly modified siRNAs that have successfully progressed through clinical trials (48). Another concern is that while several studies have specifically targeted mutant forms of RAS, in most cases there was not definitive proof that the siRNA could discriminate the mRNAs of mutant versus WT RAS. Designing siRNAs to discriminate a single base change is very challenging. While this has been accomplished in highly controlled *in vitro* studies (83,84), whether it has been attained in a complex *in vivo* situation is less clear. In these contexts, it is interesting to delve into a few studies selected from Table 1.

Investigators from Ionis Pharmaceuticals have described AZD4785, a cEt-modified ASO that targets the 3′ untranslated region of human KRAS (69). Thus, the ASO equally affects mutant and WT forms. When tested in cell lines with mutant or WT Ras, the ASO inhibited the MAP kinase pathway and cell growth in KRAS mutant cells but not WT cells. Apparently, the cell lines chosen displayed 'oncogene addiction' in that they were highly dependent on the mutant form of KRAS. Further, while use of a Mek inhibitor triggered positive feedback phosphorylation of Mek on activating residues, this was not observed for the ASO. AZD4785 was administered systemically, without any delivery agents, in KRAS mutant lung cancer xenograft models. This resulted in substantial reduction of intratumoral KRAS and an inhibition of tumor growth. Similar observations were made in a patient-derived xenograft model. However, both of these studies used quite high doses of ASO (50 mg/kg) as well as extended dosing intervals (5× weekly, 4 weeks). In this study, no observations were made concerning the possibility of additional positive feedback loops induced by RAS depletion, for example the well-known activation of EGFR leading to PI3K stimulation via relief of a negative feedback loop from ERK (85). Another issue is whether this ASO would be effective in tumors that have an activated RAS but where oncogene addiction is not strongly manifested. A phase I study of AZD4785 has been completed (NCT03101839), but it is not clear whether further development is contemplated.

A group from MD Anderson Cancer Center published a rather remarkable study involving the use of exosomes to deliver KRAS G12D siRNA to pancreatic tumors (74). Exosomes were prepared from fibroblasts and loaded with the siRNA via electroporation. The exosomes contained CD47, a membrane protein that discourages phagocytosis by monocytes and macrophages, thus leading to long circulation times and potentially allowing increased tumor up-

Table 1. Oligonucleotides targeting Ras and its downstream effectors: selected examples illustrating the molecular target, cancer type, type of oligonucleotide used and approach to *in vivo* delivery

Molecular target	Cancer type	Oligo type	*In vivo* delivery modality	Cell studies	Animal model	Other	Reference
KRAS	NSCLC	c-Et ASO	None	WT and KRAS mutant cell lines	KRAS mutant xenografts	Clinical trial NCT03101839	(69)
KRAS G12D	Pancreatic	Plasmid for bi-shRNA	Lipid nanoparticle	WT and KRAS mutant reporters	KRAS mutant xenografts		(70)
KRAS	Ovarian	Poly-siRNA	Chitosan nanoparticle	Ovarian cancer cells	Allograft tumor model	Co-use of PI3K inhibitor	(71)
KRAS	Pancreatic	siRNA	Peptide nanoparticle	Pancreatic and colon cancer cells	Murine pancreatic tumor tumor		(72)
KRAS G12S	NSCLC	siRNA	IgG and Poloxamer nanoparticle	A549 cells	A549 xenograft	Co-use of erlotinib	(73)
KRAS G12D	Pancreatic	siRNA	Exosomes	PANC-1 cells	PANC-1 xenografts, genetically engineered mouse tumor	Clinical trial NCT03608631	(74)
KRAS	Lung	siRNA	Novel nanoparticles	Lung adenocarcinoma cells	Genetically engineered mouse tumor		(75)
KRAS	Lung, colon	siRNA	Neutral liposomes	A549 cells	Lung, colon xenografts		(76)
KRAS	Colon	siRNA	Anti-EGFR antibody/protamine	Various	Colon xenografts		(77)
KRAS G12D	Pancreatic	siRNA	Sustained release capsule		Xenografts and syngeneic pancreatic tumors	Clinical trial NCT01188785	(78)
BRAF	Thyroid	siRNA	Nanoparticle with near-infrared (NIR) fluorescence	BRaf mutated cell lines	BRaf mutated thyroid tumor xenografts		(79)
BRAF	Melanoma	siRNA	Liposomes topical	BRaf mutated melanoma cells			(80)
BRAF	Melanoma	siRNA	Gold nanorods	BRaf mutated melanoma cells	BRaf mutated xenografts		(81)
PI3K	Colon	siRNA		PI3K and Ras mutated colon cancer cells			(82)

take. The siRNA exosomes reduced KRAS G12D mRNA and downstream signaling in mutant PANC-1 cells but not in tumor cells with WT RAS. In PANC-1 xenografts, daily treatment with siRNA exosomes almost entirely reduced tumor growth and greatly extended survival; this was paralleled by a reduction in KRAS G12D mRNA and in ERK activation. Similar, if slightly less impressive, observations were made in a genetically engineered pancreatic tumor model. Although observations were made regarding differential effects on mutant and WT KRAS RNA during cell studies, these were not confirmed at the protein level. The siRNA used in these studies was apparently unmodified raising questions about stability. A concern with all studies involving the potential therapeutic use of exosomes relates to the difficulty of reproducibly scaling up production of these complex entities (86,87), although this group has made a strong effort in that direction (88). A phase I clinical trial of this approach has been initiated (NCT03608631). It will be interesting to see whether these impressive studies in model systems hold up in the clinic, noting that other initial reports of exciting results with exosome delivery have apparently not progressed very far (89).

Investigators at Harvard have explored therapy of aggressive anaplastic thyroid cancer using nanoparticle delivery of siRNA (79). They developed a hybrid nanoparticle that includes BRAF siRNA, a cationic lipid, an NIR emitting polymer and polyethylene glycol. The NIR characteristics provided the ability to noninvasively image uptake of the nanoparticles into tissues. Nanoparticles with BRAF siRNA were used to treat BRAF V600E thyroid tumor cells and were found to reduce cell growth and inhibit ERK activation. Treatment of thyroid cancer xenografts and orthotopic grafts showed reduced tumor growth and the reduction of intratumoral BRAF. In this study, the effects on BRAF reduction and tumor growth inhibition were somewhat modest. Additionally, there was no attempt to explore the feedback mechanisms often seen with other inhibitors of RAF kinases.

Thus, while there have been some very encouraging reports on the use of oligonucleotides to inhibit the Ras pathway in cancer, only a few of those have matured to the level of clinical trials. Currently, there are 71 clinical trials involving siRNA and 186 involving ASOs listed on the ClinicalTrials.gov website, but very few involve RAS pathway signaling. Additionally, perusal of the websites of several leading antisense and siRNA companies did not identify clinical trials involving the RAS pathway. Thus, despite numerous academic publications on this topic, there has been only modest progress in translating these studies to the clinical environment. Nonetheless, it should be noted that most of the studies to date have not fully benefited from recent advances in the chemistry of siRNAs nor from newer approaches to selective delivery involving conjugation with targeting ligands.

Oligonucleotide modulation of the Wnt, Notch and Hedgehog pathways

Focus on these pathways for cancer therapy has come later than for the RAS pathway. Thus, the literature on oligonucleotide effects on the Wnt, Notch and Hedgehog pathways is more limited. Some of the recent literature is summarized in Table 2.

Many of the studies using oligonucleotides in the WNT, NOTCH and Hedgehog pathways, while interesting, were at a very early stage of development. Perhaps the most complete report is from investigators at Dicerna Pharmaceuticals who targeted β-catenin (90). Using siRNAs that are Dicer substrates and a cationic lipid nanoparticle, they examined delivery of the siRNA to tumors, reduction of β-catenin mRNA levels and inhibition of tumor growth. Several colon and liver cancer xenograft and metastatic models were used. This report also included extensive analysis of the design of the nanoparticles used. Interestingly, significant tumor inhibition was observed in WNT-dependent colon and hepatic tumors but not those that were WNT independent. A second report using this technology explored the interaction between siRNA-mediated β-catenin inhibition and the effectiveness of checkpoint inhibitors in syngeneic mouse tumors (91). One weakness of these reports was a limited amount of information regarding possible toxicities and off-target effects. Currently, no studies using this approach are listed in ClinicalTrials.gov.

Despite the limited progress thus far, it would seem that the WNT, NOTCH and Hedgehog pathways offer many possibilities for the development of therapeutic oligonucleotides. Within these pathways are several key targets that are nonenzymatic proteins and thus difficult to address with small molecules but easily addressed with antisense or siRNA. Especially in the NOTCH and Hedgehog pathways there are distinct isoforms of signaling proteins whose mRNAs can be selectively modulated by oligonucleotides, whereas selective modulation at the protein level would be challenging. Thus, hopefully the near future will see additional progress in this area.

SUMMARY AND ANALYSIS

Perturbations of intracellular signaling pathways are intimately involved in the initiation and progression of many types of cancer. Accordingly, the ability to modulate these pathways is an obvious goal for cancer therapeutics. Unfortunately, there has been only limited progress toward this goal. The development of small molecule inhibitors has often been frustrated by the complexity of cancer signaling pathways. Inhibition at one locus can sometimes trigger compensating feedback processes leading to resistance, such as that seen with BRAF inhibitors. Blocking key multifunctional proteins such as RAS or β-catenin can lead to undesired effects on normal cells and tissues. Small molecule drugs can often be designed to inhibit enzymes, but more rarely to affect nonenzymatic signaling pathway proteins (although there are exceptions such as the inhibitors of SMO in the Hedgehog pathway).

Conceptually, oligonucleotide-based drugs, because of their exquisite specificity, should be able to overcome some of the limitations of small molecule therapeutics in cancer. For example, ASOs and siRNAs targeting KRAS rather than NRAS or HRAS have been described in several publications and it may even be possible, although difficult, to discriminate mutant from WT KRAS (Table 2). Likewise, it is possible to target individual NOTCH isoforms in

Table 2. Oligonucleotides targeting the Wnt, Notch and Hedgehog pathways: selected examples illustrating the molecular target, cancer type, type of oligonucleotide used and approach to *in vivo* delivery

Molecular target	Cancer type	Oligo type	*In vivo* delivery modality	Cell studies	Animal model	Other	Reference
β-Catenin	Several	Dicer substrate siRNA	Lipid nanoparticles	Several cancer cell types	Xenografts of colon, hepatic cancers also metastatic models		(90)
β-Catenin	Melanoma	Dicer substrate siRNA	Lipid nanoparticles		Syngeneic mouse tumors	Used with checkpoint blockade antibodies	(91)
β-Catenin		Morpholino and PNA SSOs		HEK293		Produced dominant negative β-catenin	(92)
HOTAIR lncRNA	Cervical	siRNA		HeLa		SiHOTAIR modulates Wnt signaling	(93)
Frizzled	Esophageal	siRNA		Esophageal carcinoma cells			(94)
NOTCH-1, β-catenin, Stat3	Breast	siRNA		MCF7		Stronger cell growth inhibition with siRNA combinations	(95)
NOTCH-3	Ovarian	siRNA		SKOV3		siNOTCH reduces paclitaxel resistance	(96)
NOTCH-1	Breast	shRNA		MCF7, MDA-MB231			(97)
Notch-2	Bladder	shRNA		Bladder cancer cell lines	Orthotopic xenograft	Stable expression of shNOTCH reduces tumor growth	(98)
Sonic hedgehog	Breast	siRNA		MCF-7, SKBR-3		siSHH and small molecule inhibitor of Gli1 synergize	(99)
Gli1	Lung cancer	siRNA		SBC-5		Combined siGli1 and kinase inhibition block cell growth	(100)

that pathway or individual Gli transcription factors in the Hedgehog pathway. The ability to target either enzymatic or nonenzymatic pathway components is clearly an asset for oligonucleotide-based approaches.

Despite these advantages, oligonucleotide modulation of cancer signaling pathways has progressed only slowly. A major impediment concerns the ability to effectively deliver oligonucleotides to intracellular targets in tumors. Much of the work on siRNA in cancer has involved use of nanoparticles (53,65). This can be an effective strategy in rapidly growing tumors in mice where a highly abnormal intratumoral vasculature allows escape of the nanoparticles to the tumor parenchyma. However, it may be less effective in more slowly growing tumors, including many human tumors, where the vascular abnormalities are not as extreme (56,101). ASOs are often administered as 'free' molecules but here tumor uptake may be low as compared to hepatic or kidney uptake (102). An emerging approach to this problem is the use of ASOs or chemically stabilized siRNAs conjugated to ligands that bind specific cell surface receptors (57,58,103). In some instances, there are well-known cell surface markers for particular tumor types that could be exploited for tumor-selective delivery, although this is not always the case (104,105). Some types of nanoparticles have the ability to overcome endosomal trapping of oligonucleotides by causing endosome membrane destabilization (53), but this is not true of 'free' oligonucleotides or ligand–oligonucleotide conjugates that lack that ability. Thus, new strategies are needed to overcome the limited tumor uptake of oligonucleotides while still dealing with the endosome entrapment issue.

Based on these considerations, there are some hypothetical strategies that may advance the use of oligonucleotides in modulating cancer signaling pathways. First, focus on nonenzymatic pathway components. There is vast medicinal chemistry expertise on the design of, for example, kinase inhibitors. It is not clear that oligonucleotides will offer advantages over small molecules in that context. Second, use conjugates to selectively deliver oligonucleotides to cells where a particular pathway is active. For example, in the Hedgehog pathway ligand-bound Patched is internalized via endocytosis (39). Thus, an oligonucleotide with a ligand for Patched may be taken up more effectively in cells where Hedgehog components are strongly expressed. The oligonucleotide could be designed to downregulate one of the intracellular Hedgehog pathway components. Third, consider simultaneously addressing targets in two interconnected pathways. Recent work has shown that dual inhibition of the MAP kinase and autophagy pathways can lead to synergistic effects in pancreatic cancer cells (26,27). Similar dual inhibition approaches could be used with oligonucleotides, especially for pathways where good small molecule inhibitors are lacking. This is also consistent with traditional strategies in cancer chemotherapy where several drugs with different molecular mechanisms are used to attain therapeutic effects while distributing toxicity to different tissues. An approach that has not been substantially pursued to date is to use oligonucleotides to manipulate levels of tumor suppressor proteins. This might be done via ASO or siRNA inhibition of expression of proteins that negatively modulate tumor suppressors. Alternatively, in some cases it may be possible to use SSOs to increase expression of tumor suppressors as has been done for other types of proteins (106,107). Finally, as suggested by the history of tyrosine kinase inhibitors in cancer (108), it will be important to develop therapeutic oligonucleotides that are appropriate for specific cohorts of cancer patients based on the molecular pathology of their disease, rather than expecting to find oligonucleotide drugs that work for large, unselected populations.

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