GROWTH HORMONE SPLICING AND TREATMENT OF DISEASE USING RNA INTERFERENCE

By

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To my parents, Thamineh and Hashem,

thank you for your continual support,

encouragement and love.

To Sean, having you by my side

made this possible, thank you

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Δ3	*GH-1* transcripts lacking exon 3
20R	Rescue mice co-expressing Δ3 and shRNA-17.5 (sh-20 line)
25R	Rescue mice co-expressing Δ3 and shRNA-17.5 (sh-25 line)
AAV	Adeno-associated virus
ACTH	Adrenocorticotropic hormone
AtT-20	Mouse adrenocorticotropic hormone secreting cell line
BBB	Blood brain barrier
bp	Base pairs
BPS	Branch point sequence
cDNA	Complementary DNA
DCSV	Dense core secretory vesicles
dsRNA	Double stranded RNA
DSX	Drosophila *doublesex* gene
EM	Electron micrograph
ER	Endoplasmic reticulum
ESE	Exonic splicing enhancer
GH	Growth hormone
GH-1	Human growth hormone gene
GH3	Rat somatotroph cell line
GHD	Growth hormone deficiency
GHR	Growth hormone receptor

GHRH	Growth hormone releasing hormone
hGH	Human growth hormone
hnRNP	Heterogeneous nuclear ribonucleoproteins
IGF-1	Insulin-like growth factor 1
IGHD	Isolated growth hormone deficiency
IGHD I	Isolated growth hormone deficiency type I
IGHD II	Isolated growth hormone deficiency type II
ISE	Intronic splicing enhancer
kb	Kilobase
kDa	Kilodalton
LH	Luteinizing hormone
LCR	Locus control region
miRNA	microRNA
NMD	Nonsense-mediated mRNA decay
nt	Nucleotide
OTE	Off target effect
PEG	Polyethyleneglycol
PEI	Polyethylenimine
PCR	Polymerase chain reaction
Pit-1	Pituitary-specific transcription factor
PRL	Prolactin
PTC	Premature termination codon
Py	Polypyrimindine tract

RISC	RNA-induced silencing complex
RNAi	RNA interference
RNP	Ribonucleoprotein
RRM	RNA recognition motif
RT	Reverse transcription
RT-PCR	Reverse transcriptase PCR
shRNA	Short hairpin RNA
shRNA-17.5	Short hairpin RNA targeting the *GH-1* transcript encoding 17.5-kDa isoform
sh-20	Transgenic shRNA-17.5 mouse line #20
sh-25	Transgenic shRNA-17.5 mouse line #25
siRNA	Small interfering RNA
siRNA-17.5	Small interfering RNA targeting the *GH-1* transcript encoding 17.5-kDa isoform
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoprotein
SR protein	Serine-arginine protein
ssRNA	Single stranded RNA
TSH	Thyroid stimulating hormone
U2AF	U2 snRNP auxiliary factor
UTR	Untranslated region
wt	Wild type

CHAPTER I

INTRODUCTION

Splicing is the regulated removal of introns and the concurrent ligation of exons to produce mature mRNA transcripts. Variability in this tightly regulated process is responsible for an extraordinarily diverse proteome from a relatively small mammalian genome. Alternative splicing can lead to differential exon inclusion or exclusion, as can aberrant splicing, and such transcripts therefore differ from constitutively spliced transcripts. Where mistakes in splicing cause disease, the resulting mutant transcripts appear to be ideal targets for RNA interference (RNAi). In the case of inappropriate exon inclusion, small interfering RNAs (siRNAs) can be targeted to specific exons. When exon skipping prevails, siRNAs can be designed complementary to the specific exon-exon junctions that are not present in normal transcripts. The human growth hormone gene, *GH-1*, nicely illustrates these points. Constitutive splicing of all 5 exons produces the normal hormone but aberrant skipping of exon 3 can lead to the production of a dominant negative isoform and associated Isolated Growth Hormone Deficiency type II (IGHD II). This thesis describes research into understanding the causes of exon 3 skipping and shows that siRNAs targeting the unique exon 2-exon 4 sequence in mutant transcripts can prevent onset of IGHD II in a mouse model.

Splicing

Splice Site Selection

The efficient excision of introns and accurate splicing of exons is required for mature transcripts to correctly encode protein. The importance of correct splicing cannot be understated: mistakes that make a wrong protein can rob the cell of the function of that protein by altering its structure/function or prevent the mRNA from even being translated. Any one of these outcomes could have severe negative consequences. A conserved series of sequence elements are recognized by the spliceosome, a macromolecular complex that catalyzes the splicing reaction. These cis-acting signals include the 5' splice site, 3' splice site, branch point sequence, and polypyrimidine tract (Figure 1). The consensus sequences for these elements are summarized in Table 1. Importantly, these elements are highly conserved in lower eukaryotes, such as the yeast *Saccharomyces cerevisiae,* but not in higher eukaryotes. It is thought that this level of sequence conservation in *S. cerevisiae* may explain the relative simplicity and lack of alternative splicing in yeast and it most likely reflects the small number of intron-containing genes (Hodges et al., 1997). As such, alternative splicing is more abundant in higher organisms. Another difference between higher and lower eukaryotes involves the relative size of exons. In yeast, the average length of exons is long, generally greater than 1 kb, though in vertebrates they are much shorter (~140 nucleotides, accounting for 1.1% of the genome). Conversely, vertebrate introns are typically large, averaging greater

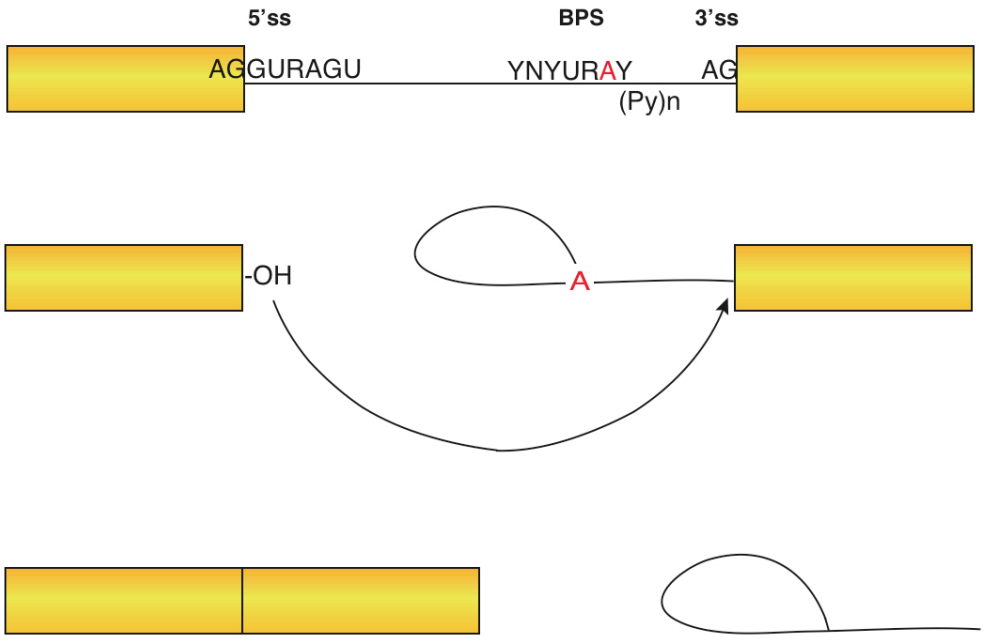


Figure 1. Splicing reaction. Conserved splicing elements include the 5' and 3' splice sites and a branch point sequence followed by the polypyrimidine tract. Splicing occurs in two sequential *trans*-esterification reactions, culminating in ligation of two exons and removal of an intron lariat, which is debranched and degraded.

Table 1. Splice site consensus sequences. Consensus splice sites in vertebrates and the yeast, *S. cerevisiae*. The intron-exon boundary is denoted by │ and the branch point adenosine is shown in bold. R, purine; Y, pyrimidine; N, purine or pyrimidine.

	Vertebrates	Yeast
5' splice site	AG│GURAGU	GUAUGU
Branch point sequence	YNYUR**A**Y	UACUA**A**C
3' splice site	YAG│G	YAG

than 3 kb in length and representing 25% of the genome whereas their yeast counterparts are on average only 250 nucleotides in length (Consortium, 2001; Hawkins, 1988; Venter et al., 2001). This discrepancy in size poses yet another conundrum for the splicing machinery in higher eukaryotes: how to define an exon within the "sea" of introns (Black, 1995)?

Spliceosome Assembly

Canonical splices sites are recognized by multiple trans-acting factors, most notably five small nuclear ribonucleoprotein (snRNP) complexes. The core components of these complexes are short RNA molecules, snRNAs, which associate with common and specific proteins to form snRNPs (Lührmann, 1988; Will and Luhrmann, 1997; Will and Lührmann, 2001). Spliceosome assembly results from the sequential and dynamic organization of the snRNPs that recognize the cis-acting splicing signals and assemble in a step-wise manner, forming discrete complexes culminating in spliceosome formation and catalysis of splicing (Figure 2).

The first, or early complex, E, is initiated by U1 snRNP recognizing the 5' splice site via base-pairing between the 5' end of the U1 snRNA and the 5' splice site sequence (Mount et al., 1983; Reed and Palandjian, 1997; Ruby and Abelson, 1988; Seraphin and Rosbash, 1989). Simultaneously, U2 auxiliary factor (U2AF) interacts with the polypyrimidine tract and the 3' splice site through its 65-kDa and 35-kDa subunits and splicing factor 1 (SF1) binds to the branch point sequence (Krämer and Utans, 1991; Zamore and Green, 1989; Zamore

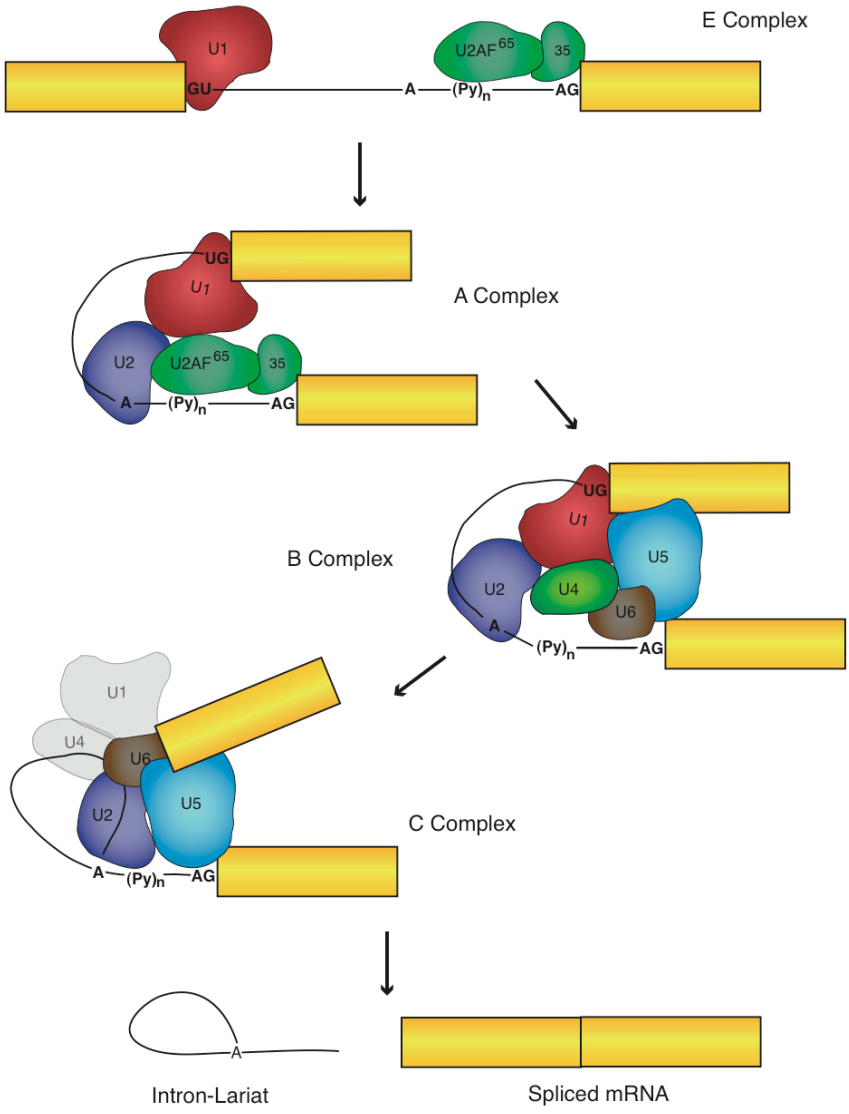


Figure 2. Spliceosome assembly. Spliceosome assembly occurs in a step-wise manner with the formation of four discrete complexes, E, A, B and C. It is in the C complex where the two sequential *trans*-esterification reactions occur.

and Green, 1991). The second, or A complex is ATP dependent. Here, U2AF recruits U2 snRNP to base-pair with the branch point sequence (Chabot and Steitz, 1987; Das et al., 2000; Query et al., 1994; Ruskin et al., 1988; Valcárcel et al., 1996). U2 snRNA replaces SF1, displacing a protein-RNA interaction in favor of an RNA-RNA interaction (Liu et al., 2001). The next complex, B, forms when the U4/U6-U5 tri snRNP is recruited, where U5 snRNA interacts with sequences at both the 5' and 3' splice sites (Behrens and Lührmann, 1991; Newman et al., 1995). It is thought that the interaction of U5 maintains the proximity of these sites within the catalytic core of the spliceosome (Sontheimer and Steitz, 1993; Wyatt et al., 1992). The final, catalytic complex, C, involves a series of rearrangements of assembled proteins, RNA-protein interactions, and RNA-RNA interactions. These include destabilization of U4/U6 base-pairing, allowing U6 to base-pair with the 5' splice site by displacing U1 and also to interact with U2 snRNA, forming a U2/U6 helix (Staley and Guthrie, 1999; Wassarman and Steitz, 1993; Wassarman and Steitz, 1992). Following these rearrangements, U1 and U4 are displaced and the two steps of splicing occur.

Splicing Reaction

Splicing occurs in two sequential *trans*-esterification reactions (Figure 1) within the catalytic core of the spliceosome (Reed and Palandjian, 1997; Staley and Guthrie, 1998). In the first step, there is a nucleophilic attack on the phosphodiester bond at the 5' splice site by the 2'-hydroxl of the conserved branch point adenosine residue to generate a lariat intermediate. The free 3'-

hydroxyl of the first exon then attacks the phosphodiester bond at the 3' splice site to link the two exons. The intron lariat, with a 2'-5' phosphodiester linkage, is released and is subsequently debranched, degraded, and recycled. The mature mRNA is then exported to the cytoplasm and spliceosomal components are recycled for new rounds of splicing.

Alternative Splicing

A surprising result from the human genome sequencing project was that the number of estimated genes (30-35,000) turned out to be far fewer than originally predicted based on expressed sequence tags (ESTs; 100,000-150,000 genes) (Consortium, 2001; Venter et al., 2001). Recent analysis puts the number of protein-coding genes at approximately ~20,500 (Clamp et al., 2007). Alternative splicing, the regulated process of differential inclusion or exclusion of regions of the mRNA, accounts in large part for this discrepancy. This molecular phenomenon is arguably the most important source of proteomic diversity in higher eukaryotes with the ability to generate many different protein isoforms from a single RNA transcript. The majority (60-75%) of human and mouse genes are subject to alternative splicing (Consortium, 2001; Johnson et al., 2003; Kan et al., 2001; Modrek et al., 2001; Zavolan et al., 2003). Alternative splicing analysis of human chromosome 22 has led to estimates that 60% of genes produce at least two transcripts (Hide et al., 2001; Modrek and Lee, 2002). These estimates are likely conservative; with increased molecular capabilities to isolate and sequence cDNAs, it is expected that alternative splicing events will be

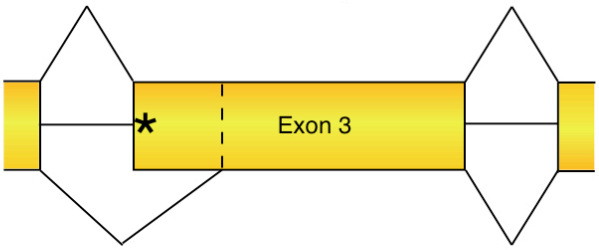
detected at an even higher frequency. Interestingly, approximately one-third of alternative splicing events introduce premature termination codons (PTCs), which cause targeted degradation of transcripts by nonsense-mediated mRNA decay (NMD) (Lewis et al., 2003). In effect, this is an on-off splicing switch, regulated by NMD.

Alternative splicing represents an efficient expansion of the genome by producing several functional proteins from a single transcript. This is accomplished via several mechanisms including the use of alternative 5' or 3' splice sites (also called cryptic splice sites), intron retention, exon skipping or inclusion, and selection between mutually exclusive exons (Figure 3). These mechanisms have a variety of consequences, including disease (Figure 3). Additionally, alternative splicing events can also occur in a development and/or tissue-specific manner (Black, 2003; Lopez, 1998).

The spliceosome faces a tremendous task in efficiently and accurately identifying splice sites. This challenge is exacerbated in higher eukaryotes as exons are much smaller than introns and the splice site signals that define exon-intron interfaces are poorly conserved (Cartegni et al., 2002). Additionally, in a given transcript, there can be several sequences that represent potential splice sites that match consensus sequences better than the bona fide sites. Such sequences are common in introns and define "pseudo-exons," which are not normally included in the mature mRNA (Sun and Chasin, 2000). The spliceosome has to differentiate between these and the correct splicing signals to maintain splicing fidelity. Such degeneracy is a double-edged sword in that

Figure 3. Types of alternative splicing with disease-related examples. (A) IGHD II is caused by exon 3 skipping in the *GH-1* gene. There is also a cryptic splice site in exon 3 that produces mRNAs lacking the first 45 nucleotides of exon 3. Mutations at the 5' end of exon 3 can result in loss of this portion of exon 3 to produe a 20-kDa GH isoform. (B) Exon 9 of the Wilms' tumor suppressor (*WT1*) gene has two alternative 5' splice sites. These two sites are nine nucleotides apart and code for the amino acid sequence KTS. Normally, the proximal 5′ splice site is favored resulting in production of the +KTS isoform. In Fraiser syndrome, mutations inactivate the proximal 5' splice site and result in –KTS isoform production (Klamt et al., 1998). (C) Intron 8 of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene contains variable length poly U,G and poly U tracts downstream of the branch point. The lengths of these tracts affect exon 9 inclusion. Exclusion causes atypical cystic fibrosis (Noone and Knowles, 2001). (D) The fibroblast growth factor receptor 2 (*FGFR2*) gene has mutually exclusive exons (IIIb and IIIc) which are included in epithelia and mesenchyme, respectively. In prostate cancer, loss of the exon IIIb containing isoform in epithelia promotes an epithelial to mesenchymal transition, which corresponds to a transition from a well-differentiated tumor to an aggressive tumor (Carstens et al., 1997). (E) Fish-eye disease is caused by a mutation two base pairs upstream of the branch point in intron 4 of the lecithin:cholesterol acetyltransferase (*LCAT*) gene, which results in intron 4 retention (Kuivenhoven et al., 1996). For all examples, the wild-type splicing pattern is shown above and the aberrant splicing pattern is shown below.

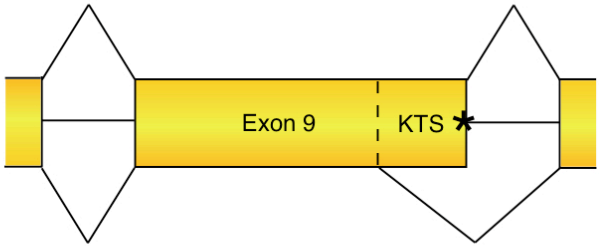
Alternative 3' ss: IGHD II; GH1 gene



WT; 22-kDa

20-kDa

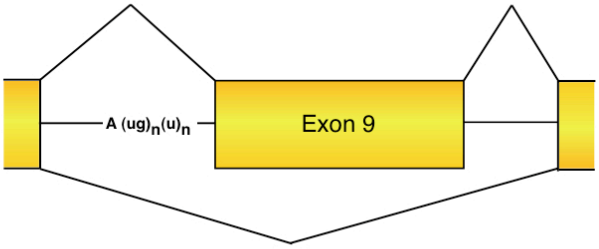
Alternative 5' ss: Frasier Syndrome; WT1 gene



+ KTS

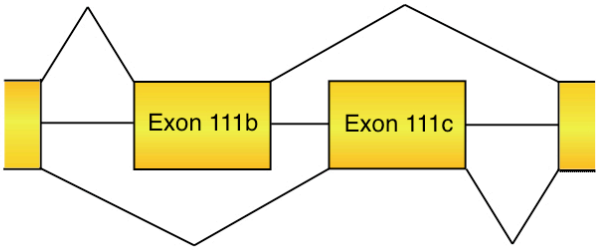
- KTS

Exon Skip vs. Exon Inclusion: Atypical Cystic Fibrosis; CFTR gene



Poly $(U)_n$: n=5, E9 skipped
n=7, 9, E9 included
Poly $(UG)_n$: n large (~13), E9 skipped
n small (~9), E9 included

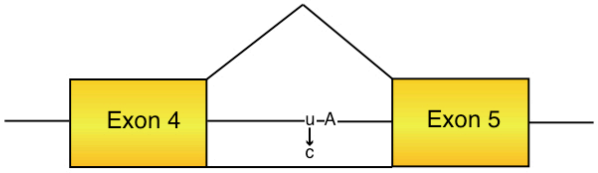
Mutually Exclusive Exons: Prostate Cancer; FGFR2 gene



Exon 111b: Epithelial

Exon 111c: Mesenchymal

Intron retention: Fish-eye Disease; LCAT gene



these ‘weaker’ splice sites (those conforming less to the consensus sequence) allow alternative splicing to occur. However, as a result, they are harder to recognize by the splicing machinery. The canonical splice site sequences are not sufficient for exon definition in a majority of higher organisms (Lim and Burge, 2001). To maintain splicing fidelity, additional *cis*-acting elements are required. These include splicing enhancers and silencers that influence splice site choice by recruitment of *trans*-acting factors to the pre-mRNA. Enhancers promote splicing by recruiting a one or more members of a family of serine-arginine-rich proteins (SR proteins) whereas silencers typically inhibit splicing via interactions with heterogeneous nuclear ribonucleoproteins (hnRNPs) (Burd and Dreyfuss, 1994; Cartegni et al., 2002; Liu et al., 1998; Smith and Valcarcel, 2000). The two pathways often compete as binding of hnRNPs to silencers can inhibit splicing by antagonizing SR proteins and/or snRNPs (Eperon et al., 2000; Mayeda and Krainer, 1992; Wang et al., 2006).

Splicing Enhancers

Splicing enhancers are short RNA sequences that are most commonly recognized by SR proteins to promote spliceosome assembly (Blencowe, 2000; Graveley, 2000; Liu et al., 1998). They promote both constitutive and regulated splicing and can reside within exons or introns (exonic splicing enhancers (ESEs) or intronic splicing enhancers (ISEs)). The majority of enhancers comprise short, 6-10 nucleotide, purine-rich motifs though other types of enhancers have been identified (A/C-rich enhancers) (Blencowe, 2000; Cartegni et al., 2002; Cooper

and Mattox, 1997; Coulter et al., 1997; Schaal and Maniatis, 1999). Enhancers have been identified through mutations that block splicing, via computational analyses, and through identification of SR protein binding sequences. Although often purine-rich, short, and degenerate, it appears that the specific sequence is more important than overall purine content (Tanaka et al., 1994).

SR proteins play a crucial role for both constitutive and alternative splicing. There are ten core SR proteins and each contains one or two N-terminal RNA recognition motifs (RRMs) plus a C-terminal RS domain (Birney et al., 1993; Fu, 1995). Binding to certain RNA sequences via RRMs determines substrate specificity. The RS domain consists of several arginine-serine dipeptides that facilitate protein-protein interactions with core spliceosome components or other *trans*-acting factors (Tacke and Manley, 1999). Exonic enhancers direct U2AF and U2 binding to the 3' splice site and U1 binding to the 5' splice site via interactions with SR proteins, forming a 'bridge' of splicing factors across the exon. This model of exon definition predominates in higher eukaryotes due to moderate exon size (50-250 nucleotides) and large intron size (Berget, 1995). An intron-definition model exists in organisms with large exons and small introns (Kennedy et al., 1998; Sterner et al., 1996). SR proteins can also stimulate exon definition by antagonizing nearby silencers (Kan and Green, 1999).

Splicing in Disease

Mistakes in splicing can cause disease directly, modify the severity of the disease phenotype, or be linked to disease susceptibility (Wang and Cooper, 2007). It is also becoming apparent that genetic variation affecting splicing efficiency contributes significantly to disease. It is thought that 50-60% of mutations that cause disease do so by affecting splicing (Cartegni et al., 2002; López-Bigas et al., 2005; Maquat, 2001; Pagenstecher et al., 2006). Exonic mutations are generally thought to be pathogenic due to introduction of missense or nonsense mutations or by altering the reading frame and hence the final protein product. However, it is likely that a fraction of these mutations affect splicing (Pagani et al., 2005). Likewise, intronic mutations can also affect splicing efficiency though these are rarely analyzed for disease susceptibility since introns are non-coding.

In addition to mutations within splicing motifs, mutations within essential splicing factors can also cause disease. Two examples are spinal muscular dystrophy (SMA) and retinitis pigmentosum (Briese et al., 2005; Mordes et al., 2006). Both diseases are caused by mutations in proteins required for snRNP assembly and function. The former results from loss of the SMN-1 protein (survival of motor neuron) that is involved in cytoplasmic snRNP assembly. As a result, loss of SMN-1 impairs snRNP production (Winkler et al., 2005). Complete loss of SMN-1 causes SMA. Retinitis pigmentosum is a common form of blindness caused by mutations in one of more than 30 genes including three

dominant genes required for U4/U6-U5 tri-snRNP assembly and function (Chakarova et al., 2002; McKie et al., 2001; Vithana et al., 2001).

Phenotypic variability due to splicing mutations can also influence disease severity. In the *CFTR* gene, a single nucleotide polymorphism (SNP) in intron 19 creates a variably spliced exon whose inclusion increases cystic fibrosis severity (Chiba-Falek et al., 1998). In the *OAS1* (2',5' oligoadenylate synthetase) gene, a G→A mutation in intron 6 shifts the 3' splice site, exacerbating a Type I Diabetes phenotype (Field et al., 2005). Two polymorphisms in the 3'UTR of *CTLA-4* (cytotoxic T lymphocyte antigen 4) cause skipping of exon 3 to produce a soluble protein that lacks a critical ligand-binding domain. Both mutations have been implicated in a host of autoimmune diseases such as Graves' Disease and autoimmune hypothyroidism (Ueda et al., 2003). These examples highlight the importance of splicing fidelity to prevent disease and underscore the importance of efficient and precise splicing.

RNA Interference

The molecular phenomenon of RNA interference (RNAi) was first characterized a decade ago in *Caenorhabditis elegans* (Fire et al., 1998) and subsequently in mammalian cells (Elbashir et al., 2001). RNAi uses small RNAs to trigger RNA silencing via sequence specific base-pairing with an mRNA to induce mRNA degradation or translational repression (Figure 4). Broadly, there are two classes of small RNAs involved in RNAi and they differ based on their

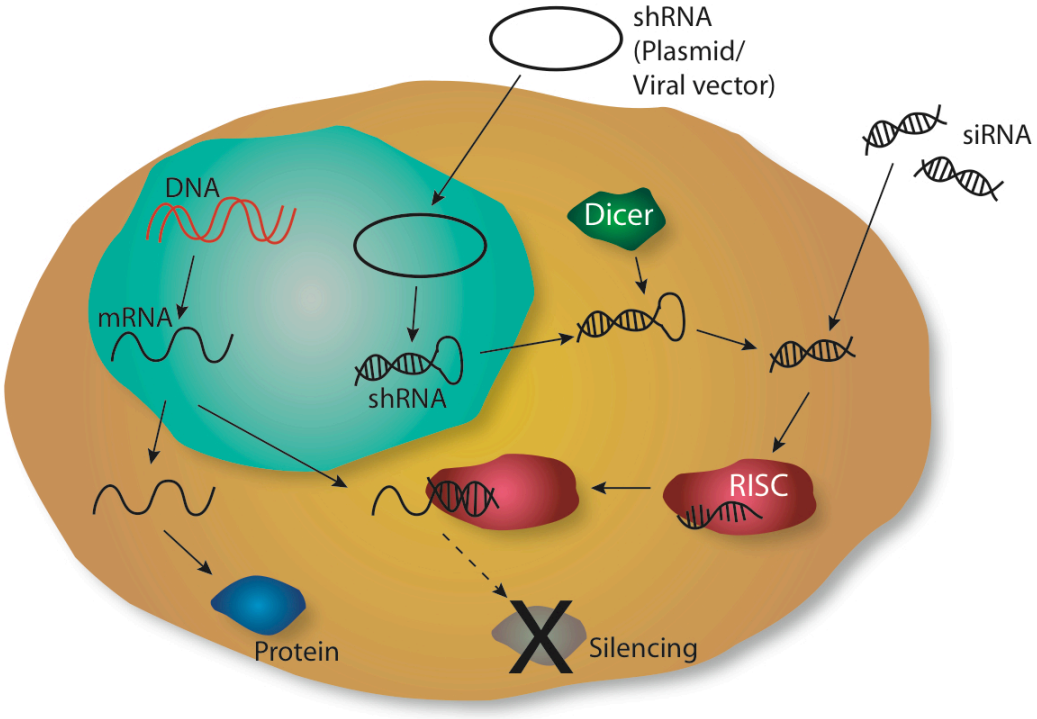


Figure 4. RNAi pathway and expression of exogenous RNAi effector molecules. Short hairpin RNAs (shRNAs) expressed from a vector are transcribed in the nucleus to produce 60-70 nt hairpin precursors, akin to miRNA precursors. These are exported to the cytoplasm where they are cleaved by Dicer and are loaded into the RNA-induced silencing complex (RISC) and can locate and silence their target mRNA. Alternatively, ~21 nt siRNAs can be introduced directly into the cytoplasm where they can be incorporated into RISC.

origin: microRNAs (miRNAs) and siRNAs. The former are excised from endogenous genomic transcripts and the latter are generated from exogenous double-stranded RNAs (dsRNAs). Since its discovery, RNAi has proven to be a potent molecular tool for both reverse genetics and for use as a potential therapeutic strategy to treat disease.

Small Interfering RNAs

It is thought that the RNAi machinery evolved as an innate immune response to viral attack. Long viral dsRNAs are cleaved by an RNase III enzyme, Dicer, into small 21-25 nt duplexes called siRNAs (Bernstein et al., 2001; Zamore et al., 2000). Cleavage by Dicer leaves a characteristic 2 nt 3' overhang on each strand. In addition, each strand has a 5' phosphate and a 3' hydroxyl. One strand of the siRNA duplex, the one exhibiting the lower free energy at its 5' end, is loaded into an RNA-induced silencing complex (RISC) (Khvorova et al., 2003; Schwarz et al., 2003). This guide strand directs silencing of complementary mRNAs in a sequence-specific manner by cleavage of the mRNA. Cleavage of the mRNA occurs between bases 10 and 11 relative to the 5' end of the siRNA, allowing subsequent degradation of the cleaved mRNA (Elbashir et al., 2001; Orban and Izaurralde, 2005).

microRNAs

miRNAs are an endogenous class of eukaryotic genes whose mature product resembles siRNAs. They function to fine-tune gene expression during

development and differentiation (Bartel and Chen, 2004). Primary miRNAs (pri-miRNAs) are generally transcribed as long RNA polymerase II transcripts (Lee et al., 2004) that are cleaved in the nucleus by a microprocessor that includes an RNase III enzyme, Drosha, and a dsRNA-binding protein, DCGR8 (Gregory et al., 2004; Lee et al., 2003). The resulting ~70 nt stem-loop product, the precursor miRNA (pre-miRNA), is exported to the cytoplasm via the nuclear karyopherin, Exportin-5 (Lund et al., 2004; Yi et al., 2003). The pre-miRNA is then cleaved by Dicer, akin to long dsRNAs, to produce small ~22 nt dsRNA duplexes (Bernstein et al., 2001). Importantly, cleavage by both Drosha and Dicer at either end of the duplex defines the termini of the mature miRNA and leaves 2 nt 3' overhangs. In concert with Dicer, TRBP (HIV-1 TAR RNA-binding protein) and PACT (protein activator of PKR), the mature miRNA is loaded into RISC (Chendrimada et al., 2005; Lee et al., 2006). RISC is the effector complex for RNAi, the catalytic components of which are the Argonaute (AGO) family of proteins. AGO2 is the endonucleolytic component of human RISC and is the only catalytically active member of the Argonaute family in mammals (Liu et al., 2004). In the case of siRNAs, AGO2 is also responsible for cleaving the passenger strand of the siRNA duplex prior to the guide strand loading into RISC (Matranga et al., 2005; Rand et al., 2005). For miRNAs, the passenger strand is released.

MicroRNAs generally target the 3' UTRs of mRNAs. Typically, and unlike siRNAs, miRNAs do not exhibit perfect base-pairing with their target mRNA and as such it seems that miRNAs function by repressing translation rather than by

mRNA cleavage. These mRNAs accumulate in specific cytoplasmic entities known as processing bodies (P-bodies) (Liu et al., 2005). Transcripts that are associated with P-bodies can either be degraded or can return to translation (Parker and Sheth, 2007). On occasion where there is perfect complementarity between the miRNA and its target, cleavage of the mRNA ensues (Yekta et al., 2004). Nucleotides 2-8 at the 5' end of the miRNA are referred to as the seed region and usually show perfect complementarity with the target mRNA. It is this region of the mature miRNA that appears to be most important for the interaction between the two RNA species (Lewis et al., 2005). Computer algorithms usually emphasize seed pairing in identifying potential targets.

Therapeutic RNAi

The beauty of the sequence specificity of RNAi is that it lends itself well to therapeutic applications since many disorders are caused by inappropriate gene expression. Much research has progressed in this direction as RNAi has the potential to treat a multitude of genetic disorders.

Therapeutic RNAi effector molecules

Exogenous siRNAs can be introduced into cells via two mechanisms, each exploiting the endogenous RNAi pathway. First, siRNAs can be directly administered as synthetic 21 nt dsRNA duplexes with 2 nt 3' overhangs that mimic Dicer substrates and are directly incorporated into RISC (Figure 5a). Second, vectors expressing short hairpin RNAs (shRNAs) can be delivered to

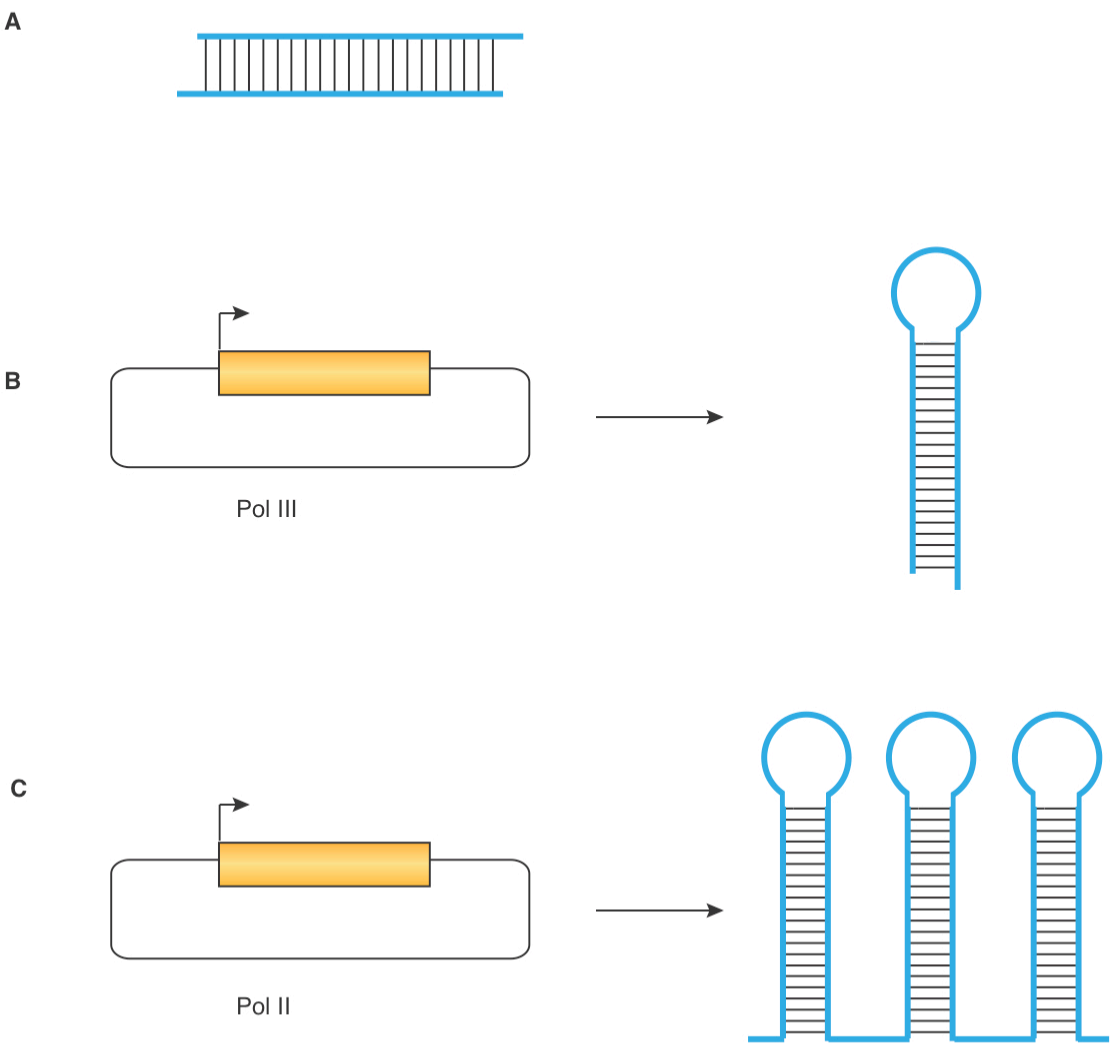


Figure 5. RNAi effector molecules. (A) siRNAs can be delivered exogenously as 21-nt duplexes. (B) shRNAs can be expressed from a Pol III promoter such that the 3' 2-nt overhang is produced by a transcription termination sequence. (C) shRNAs can be expressed as long Pol II transcripts expressing one or more hairpins. These hairpins are processed as miRNAs with initial cleavage by Drosha.

cells (Brummelkamp et al., 2002). shRNA precursors imitate miRNA precursors by forming stem-loop structures recognized by Dicer. shRNAs can be expressed from either Pol II or Pol III promoters (Figure 5b,c). The former allows tissue specific expression. Additionally, several shRNAs can be expressed from a single polycistronic transcript, thus potentially increasing potency by enabling knock-down of a single mRNA by different shRNAs (Sarnow et al., 2006). Expression from a constitutive Pol III promoter such as H1 or U6 yields a transcript that mimics a pre-miRNA and can be directly exported to the cytoplasm via Exportin 5 (Kim and Rossi, 2007). RNAi induced by siRNAs tends to be transient, effective only as long as the siRNA is present. Conversely, RNAi triggered by shRNAs is effective for as long as the shRNA continues to be transcribed (Brummelkamp et al., 2002; Paddison et al., 2002).

siRNA Design

The first step in designing therapeutic siRNAs is to identify potential sequences that allow knock-down of specific target mRNAs, usually via a bioinformatic approach. This is followed by *in vitro* testing to determine silencing efficacy at the lowest concentration and to eliminate potential off-target effects (OTEs; discussed below). The siRNA strand with lower free energy at its 5' end is incorporated into RISC to activate the complex. In siRNA drug design, strand selection can be manipulated by making a single nucleotide substitution at the end of the duplex to favor incorporation into RISC (Schwarz et al., 2003). More potent siRNAs generally have a moderate-to-low GC content (30-52%); if the GC

content is too high, there may be difficulty in unwinding and if it is too low it may not interact well with its target mRNA (Boese et al., 2005; Reynolds et al., 2004). A lack of secondary structure and low internal stability are also characteristics of optimal siRNAs (Boese et al., 2005; Reynolds et al., 2004). It is also desirable to design siRNAs against conservesd mRNA sequences (de Fougerolles et al., 2007). As an example, the Human Immunodeficiency Virus (HIV) can mutate rapidly to evade host immune responses and also RNAi (Scherer et al. 2007). Therefore, an siRNA that targets a conserved region of the mRNA can reduce this (Naito et al. 2007). For all siRNAs, regardless of design, innate immune responses must be evaded as well as avoiding potential OTEs and saturation of endogenous host RNAi components (discussed in depth below).

In higher vertebrates, the introduction of long dsRNAs (>30 bp) activates Protein Kinase R (PKR) and induces an interferon (IFN) response, directing cessation of global gene expression (Manche et al., 1992). There are a limited number of reports that small siRNA duplexes can also trigger this innate immune response, as Toll-like receptors (TLRs) expressed in endosomes can recognize both single- and double-stranded RNAs to elicit an IFN response (Seth et al., 2006). In particular TLR3, TLR7 and TLR8 have been shown to activate such a response to exogenous siRNAs *in vivo* in mice and *in vitro* in human blood (Hornung et al., 2005; Kim and Rossi, 2007; Marques and Williams, 2005). GU-rich immunostimulatory sequence motifs recognized by TLRs should be avoided in siRNA design – 5'-GUCCUUCAA-3' and 5'-UGUGU-3' (Hornung et al., 2005; Judge et al., 2005). Immune stimulation by synthetic siRNAs can be completely

abrogated by incorporation of 2'-*O*-methyl uridine or guanosine nucleotides into one strand of the siRNA duplex (Judge et al., 2006).

Avoiding complementarity between the siRNA and untargeted mRNAs is the key to avoiding off-target silencing. miRNA seed sequences usually have increased complementarity with the 3'UTR of target mRNAs, copmpared to the 3' end of the miRNA. As such, particular attention should be addressed in avoiding base-pairing between the 5' region of an siRNA and any off-target mRNAs (Birmingham et al., 2006; Jackson et al., 2003; Jackson et al., 2006b). A recent report showed that a simple 2'*O*-methyl ribosyl substitution at position 2 of the guide strand reduced silencing of all partially complementary mRNAs but did not affect silencing of the perfectly matched target transcript (Jackson et al., 2006a).

Grimm and colleagues recently showed that abundant shRNA expression using adeno-associated virus (AAV) vectors caused toxicity in mice due to saturation of Exportin-5, which prevented nuclear export of endogenous liver miRNAs (Grimm et al., 2006). Lower hepatic expression of shRNAs via AAV-vectors does not appear to be toxic (Narvaiza et al., 2006). Expression of shRNAs from lentiviral vectors in primary lymphocytes with different Pol III promoters demonstrated that expression via U6 caused gradual cytotoxicity whereas that by H1 showed reduced expression but no cell death and was still able to silence the CCR5 (chemokine receptor-5) target mRNA (An et al., 2006). Considering this, siRNAs appear more desirable than shRNAs as they are introduced into the RNAi pathway at a later point and therefore are not as likely

to be detrimental by causing toxicity due to saturation of endogenous RNAi components.

Stability of siRNAs

Although double stranded, siRNAs are still rapidly degraded by serum RNAses with a half-life of minutes in human plasma (Choung et al., 2006; Layzer et al., 2004). Additionally the molecular mass of siRNAs is smaller than the threshold for glomerular filtration leading to premature removal by renal clearance (Dykxhoorn and Lieberman, 2006). For use as a viable therapeutic, various chemical modifications have recently been developed to increase stability and improve efficacy without adversely affecting biological activity. Importantly, Chiu and Rana showed that the 2'-hydroxyl (2'-OH) that differentiates RNA from DNA is not required for RNAi (Chiu and Rana, 2003). Modifications of the 2'-OH group have successfully increased persistence of siRNAs compared to non-modified counterparts. These include addition of 2'-*O*-methyl-(2'-*O*-me)-purines, 2'-fluoro-(2'-*F*)-pyrimidines or locked nucleic acid (LNA) nucleotides (Allerson et al., 2005; Braasch et al., 2003; Chiu and Rana, 2003; Choung et al., 2006; Czauderna et al., 2003; ElmÈn et al., 2005) (Figure 6a,b). Phosphorothioate linkages on the siRNA also increase stability by preventing nuclease activity while retaining wild-type activity (Amarzguioui et al., 2003; Choung et al., 2006). Extensive modifications of siRNAs against Hepatitis B virus (2'-*F* on all pyrimidines and 2'-*O*-me on all purines plus a 3' terminal phosphorothioate linkage) increased the half-life from 5 minutes (unmodified) to 3 days in 90%

human serum yet still inhibited viral replication (Morrissey et al., 2005a). Other additions include terminal peptide sequences on the siRNAs, which can increase both stability and uptake (Chiu et al., 2004). In most cases, modifications that confer exonuclease resistance appear generally well tolerated and silencing activity is retained. Additional stability against nucleases can also occur depending on the mode of siRNA delivery and packaging, such as liposomes or nanoparticles.

RNAi Delivery

Efficient and effective delivery of RNAi effector molecules is the most challenging obstacle in the development of therapeutic RNAi. Various methods have been developed to deliver both shRNAs and siRNAs.

Viral Delivery of shRNAs

shRNAs can be produced from viral expression vectors and although this gene therapy approach has its limitations, especially regarding safety issues (ESGT, 2006; Cavazzana-Calvo and Fischer, 2007), it may be the best option for long-term delivery and treatment of chronic diseases (Kim and Rossi, 2007). There are three types of viral vectors, each with advantages and disadvantages: lentivirus vectors, adenovirus, and adeno-associated virus (AAV). Lentiviral vectors transduce both dividing and non-dividing cells and exert stable shRNA expression with broad host tropism (Sumimoto and Kawakami, 2007). However, they require integration of the shRNA transgene into the genome, with potential

insertional mutagenic consequences. shRNA delivery via adenovirus vectors is advantageous due to robust shRNA expression and also the potential ability to treat cancers by generating tumor-specific variants that conditionally replicate in and lyse transformed cells (Yoo et al.). However, these vectors can be strongly immunogenic (Grimm and Kay, 2007). Adenoviral and AAV vectors express transgenes episomally with no or very low (AAV) levels of genomic integration (Grimm and Kay, 2007). AAV vectors efficiently infect a wide variety of dividing or quiescent cells and have already been clinically studied in multiple tissues (Manno et al., 2006). The ability to pseudotype shRNA genomes with a huge array of AAV capsids (both natural and synthetic) can confer tissue specificity (Grimm and Kay, 2003). Disadvantages to use of adenoviral and AAV vectors include somewhat moderate shRNA expression and the necessity for repeat administrations that can be strongly immunogenic (Kim and Rossi, 2007).

Non-Selective delivery of siRNAs

Early RNAi therapeutic studies delivered synthetic siRNAs (or vectors encoding shRNAs), using high-pressure, large volume, tail vein injections in mice to target these RNAs to the liver, kidney, spleen, pancreas and lung (Lewis et al., 2002; Lewis and Wolff, 2005; McCaffrey et al., 2002). The elevated venous pressures from this method transiently disrupt the plasma membranes of highly vascularized tissues, allowing siRNA uptake (Dykxhoorn and Lieberman, 2006). This hydrodynamic approach is not applicable for humans as it has been shown to cause right-sided heart failure (Dykxhoorn et al., 2006; McCaffrey et al., 2002).

Like all nucleic acids, siRNA duplexes are negatively charged polymers and cannot easily penetrate hydrophobic cell membranes (Kim and Rossi, 2007; Li et al., 2006). Systemic delivery of modified siRNAs or siRNAs conjugated to cholesterol or packaged within a liposomal particle has been achieved by intravenous injection, though this is only clinically relevant if targeting the liver or jejunum (Figure 6c,d). Cholesterol is transported in the circulation via lipoprotein particles, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) (Brown and Goldstein, 1985). Conjugation of cholesterol to the 5' end of the passenger strand increased stability of siRNAs targeted to apoplipoprotein B (ApoB) mRNA after intravenous injection in mice and led to mRNA reduction in both the liver and jejunum (55% and 70%, respectively) (Soutschek et al., 2004). Recent work has shown that mice injected with HDL-cholesterol-siRNA conjugates had an 8-15 fold greater reduction in ApoB protein in the liver, gut and blood than with just cholesterol-siRNA conjugates (Wolfrum et al., 2007). HDL receptors facilitate uptake by the liver, gut, kidney, adrenal glands and ovaries, whereas LDL primarily targets the liver. Null mice lacking the HDL scavenger receptor class B type I (SR-BI) exhibit a substantial reduction in cholesterol-siRNA uptake (Wolfrum et al., 2007). HDL-cholesterol-siRNA complexes are large enough to avoid renal clearance, increasing the overall stability and efficacy.

Liposomes are vesicles with an internal aqueous lumen enclosed by a phospholipid bilayer. When liposomes complex with siRNAs or other nucleic acids, they are referred to as lipoplexes, which in turn can be further adapted to include multiple lipids (de Fougerolles et al., 2007). An important advance in

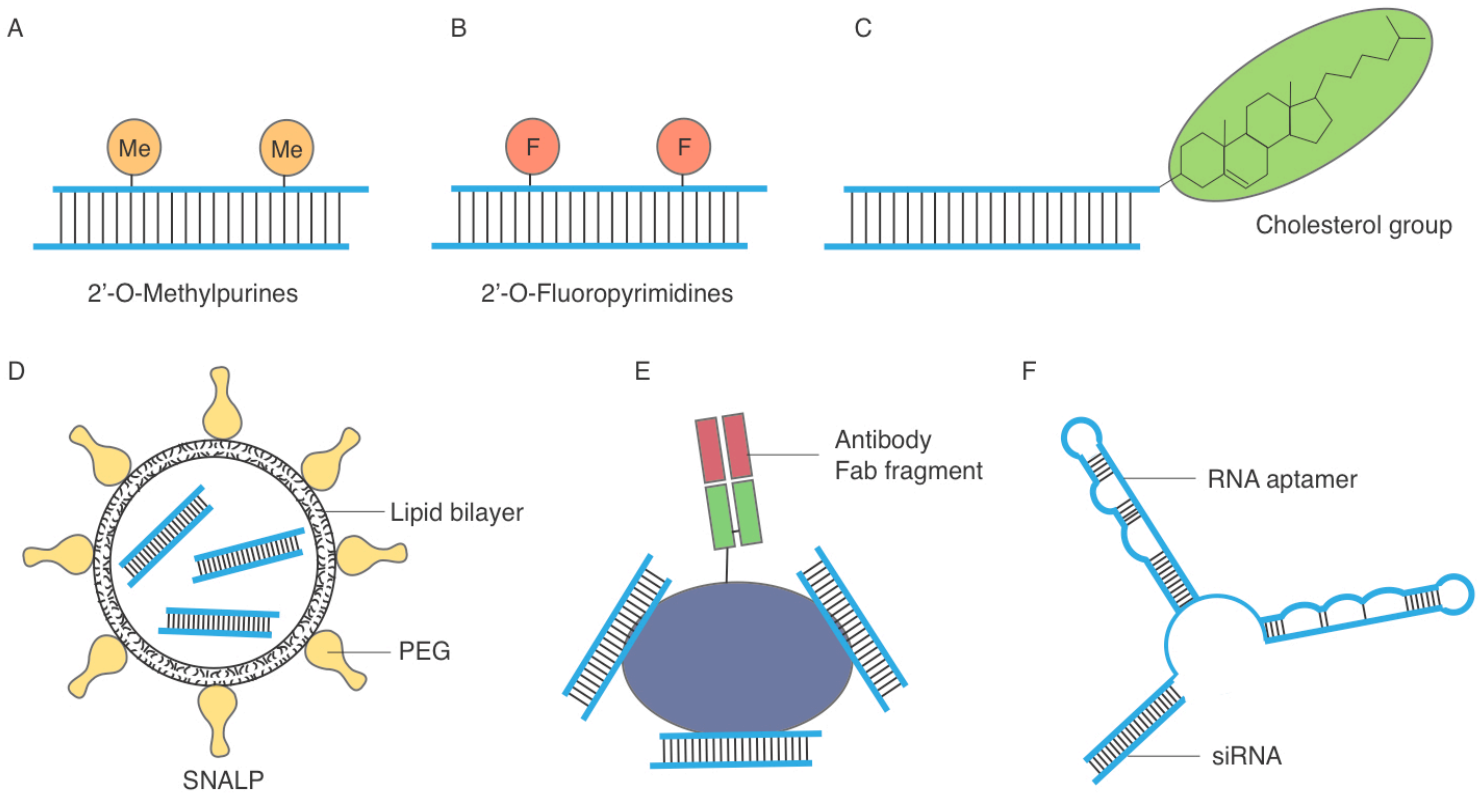


Figure 6. Increasing stability and efficacy of siRNAs. (A), (B) Chemical modifications such as 2'-*O*-methylpurines (A) or 2'-*O*-fluoropyrimidines can be added to increase siRNA stability. (C) Cholesterol can be conjugated to siRNAs for systemic delivery. (D) siRNAs can be encapsulated within liposomes to form stable-nucleic-acid-lipid particles (SNALPs). (E) An antibody-protamine fusion protein can noncovalently bind siRNAs to deliver siRNAs to specific cell-surface receptors. (F) siRNAs linked to an RNA aptamer can target cells that express cell surface receptors that are recognized by the aptamers.

RNAi therapeutics was the first example of siRNA delivery in non-human primates. siRNAs were encapsulated in stable nucleic acid-lipid particles (SNALPs) containing fusogenic and cationic lipids and polyethylene glycol (PEG; Figure 6d), which, upon injection reduced ApoB mRNA levels in the liver for 11 days after a single dose (Zimmermann et al., 2006). Importantly, silencing was achieved using a dose 20x less than that mentioned above with cholesterol-siRNA conjugates (2.5 $mgkg^{-1}$ in cynomologous monkeys versus 50 $mgkg^{-1}$ in mice) (Soutschek et al., 2004; Zimmermann et al., 2006). That SNALPs can be used to target siRNAs to organs other than the liver has not yet been published.

Local injection of lipoplexes has been successfully used to deliver siRNAs to several organs including the eye, CNS, vagina, lung, intestine and also to tumor tissue (Bitko et al., 2005; Bumcrot et al., 2006; Kumar et al., 2006; Luo et al., 2005; Masiero et al., 2007; Palliser et al., 2006; Reich SJ, 2003; Tompkins et al., 2004; Zhang et al., 2006). Most of these deliveries involve direct local injection of the siRNA with the exception of the lung and vagina. Delivery to the lung to treat respiratory syncytial virus (RSV) or influenza was achieved by intranasal instillation (Bitko et al., 2005; Tompkins et al., 2004). For vaginal epithelium delivery, siRNAs were delivered by tissue transfection, mixing the siRNAs with a lipid transfection reagent (Palliser et al., 2006).

Polyethylenimine (PEI) polymers can also be used to deliver siRNAs. These polymers are highly cationic and when endocytosed into cells they disrupt the endosomal pH, leading to osmotic release of the siRNA-polyplex into the cytoplasm (Boussif et al., 1995; de Fougerolles et al., 2007). PEI-siRNA

complexes have been used successfully to treat influenza in mice (Ge et al., 2004) and Ebola in guinea pigs (Geisbert et al., 2006). However, at higher doses, PEI appears to be extremely toxic (de Fougerolles et al., 2007).

Selective delivery of siRNAs

Selective siRNA administration is desirable as it could enable a lower drug dose, thus reducing potential OTEs in non-target tissues (Kim and Rossi, 2007). This has been achieved by packaging siRNAs within nanoparticles coated with receptor-targeting ligands or by coupling siRNAs to antibody fragments and aptamers (Chu et al., 2006; McNamara et al., 2006; Peer et al., 2007; Song et al., 2005; Zhang et al., 2004). siRNAs are delivered in a cell-specific fashion mediated by endocytosis.

RNA aptamers are RNA ligands that bind to cell surface receptors and can be linked to siRNAs either covalently forming an 'all-RNA' molecule (McNamara et al., 2006) or via modular streptavidin bridges that conjugate biotinylated siRNAs (Chu et al., 2006). The advantage of the former is that the ability of specific cell targeting and RNAi are both available from a single RNA molecule (Figure 6e). Although using RNA aptamers may be more simple and flexible, the small size of the aptamers (25-35 bases) means it is a viable approach for local delivery *in vivo* but would not be stable unless further conjugated due to renal clearance and short half-life (de Fougerolles et al., 2007).

Another powerful mode of selective systemic delivery involves the use of nanoparticles. Here, siRNAs are complexed with cationic peptides and polymers

via interactions with the negative phosphate backbone of the RNA (Juliano, 2005). Cell-specific targeting is achieved by coating the nanoparticle with cell-type-specific ligands. In one example, transferrin was covalently linked to short polycations containing cyclodextrin polymers that provide low toxicity and the complex was delivered via low-pressure, low-volume tail vein injections to a mouse model of metastatic Ewing's sarcoma (Hu-Lieskovan et al., 2005). This approach dramatically inhibited tumor growth as compared to control siRNAs in transferrin receptor-expressing tumor cells and, importantly, relapse rates were low compared to chemotherapy (Hu-Lieskovan et al., 2005).

In two other cell-specific delivery approaches, either a plasmid encoding an shRNA within a PEG-PEI nanoparticle was linked to folate, or siRNAs packaged within nanoparticles comprising bacteriophage phi29 pRNA conjugated to folate, were used to target nasopharyngeal carcinoma cells which overexpress the folate receptor (Guo et al., 2006; Hwa Kim et al., 2005). In the latter, phi29 utilizes the 120-bp pRNA to accomplish dsDNA packaging into a preformed procapsid (Garver and Guo, 1997). PEG-PEI nanoparticles containing siRNAs have also been conjugated to Arg-Gly-Asp (RGD) peptide ligands to form 'nanoplexes' that target siRNAs to integrin-expressing tumor cells both in cultured cells and *in vivo* (Schiffelers et al., 2004).

Immunoliposomes, where antibodies are conjugated to liposomes, have also been used to confer specificity. Liposomal complexes encapsulating shRNA expression vectors were conjugated to two receptor-specific monoclonal antibodies that recognize the human insulin receptor and the mouse transferrin

receptor (Zhang et al., 2004). The target was human epidermal growth factor, expressed in human gliomas that were implanted within the brain of mice. Receptor-mediated trancytosis across the blood-brain barrier was achieved via binding of the mouse transferrin antibody to its receptor and receptor-mediated endocytosis via binding of the human insulin antibody and its cognate receptor. Weekly injections of these immunoliposomes resulted in a significant increase in mouse survival time (Zhang et al., 2004).

Protamine is a positively charged, arginine-rich protein that binds nucleic acids with high affinity and is involved in DNA nucleation in sperm. In an elegant study, heavy chain antibody fragments specific for the HIV gp120 protein were conjugated to a protamine fragment bound to siRNAs to selectively deliver siRNAs to HIV-infected cells *in vivo* (Song et al., 2005) (Figure 6f). In a similar approach, an antibody fragment specific to the open conformation of the integrin lymphocyte function-associated antigen-1 (LFA-1) was used to deliver siRNAs. LFA-1 alters its conformation to an open state only in activated cells and this approach specifically targeted activated rather than unstimulated leukocytes (Peer et al., 2007).

Clinical Trials

Currently there are three different RNAi-based therapies that are undergoing clinical trials (de Fougerolles et al., 2007). Two of these, Sirna-027 (Sirna Pharmaceuticals; Merck) and Cand5 (also known as bevasiranib sodium; Opko Health), target vascular endothelial growth factor (VEGF) via intravitrial

injection (de Fougerolles et al., 2007). VEGF plays a pivotal role in ocular angiogenesis, causing age-related macular degeneration (AMD) (Bressler, 2004; Ferrara, 2002). Ocular injection is attractive for ease of delivery with the additional benefits of fewer nucleases and less protection from the immune system. Cand5 began a phase III trial in July 2007 and siRNA-027 is in phase II trials (Perkel, 2007). The third siRNA trial is ALN-RSV01 (Alnylam), an antiviral siRNA that targets RSV, a respiratory viral infection that affects both children and adults. The siRNA targets a viral nucleocapsid gene via intranasal delivery. ALN-RSV01 is currently in phase II trials (Perkel, 2007). Another promising RNAi-based strategy is AKIi-5 (Quark and Silence Therapeutics), a modified siRNA that prevents acute kidney injury (AKI; also known as acute renal failure). AKI develops rapidly (within hours to days) post-surgery with a mortality rate of 65%. AKIi-5 targets p53, a transcription factor associated with DNA repair and apoptosis. Temporary inhibition of p53 during AKI delays apoptosis, allowing natural repair mechanisms to restore normal DNA and cellular intergrity. AKIi-5 is expected to enter phase I clinical trials later this year and will be the first systemic siRNA tested in humans (Quark Pharmaceuticals, Inc. Press Release - http://www.medicalnewstoday.com/articles/89268.php).

In the incredibly short time since the discovery of RNAi, much has been accomplished in characterizing and utilizing this powerful mechanism. That there are current clinical trials employing siRNAs only six years after RNAi was first observed in mammalian cells is truly astounding and it is possible that RNAi as a

treatment for disease may usher in an era similar to the introduction of antibiotics in the 20th century.

Growth Hormone

Growth hormone (GH) is produced in the anterior pituitary gland and promotes postnatal growth of bone and muscle. It is the main determinant of longitudinal growth and overall body size (Isaksson et al., 1982). GH, also known as somatotropin, is composed of 191 amino acids and is synthesized, stored and secreted from somatotrophs, which constitute the major cell type in the anterior pituitary. Regulated expression of GH is essential for normal stature and also for homeostasis of carbohydrate, protein and fat metabolism (Gibney et al., 2003; Jorgensen et al., 2004; Norrelund et al., 2002; Ohlsson et al., 1998).

Anterior Pituitary Hormones

The pituitary gland is composed of two distinct units, the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis) that differ in morphology and function and also embryologically. The anterior pituitary is derived from an epithelial invagination of the oral mucosa known as Rathke's pouch. This gland is connected to the hypothalamus by the pituitary stalk, allowing communication between the two organs. Five distinct cell types comprise the anterior pituitary and each secretes a specific hormone(s). These are somatotrophs (secrete GH), lactotrophs (prolactin; PRL), corticotrophs (adrenocorticotropin; ACTH), thyrotrophs (thyroid-stimulating hormone; TSH) and

gonadotrophs (follicle-stimulating hormone; FSH and luteinizing hormone; LH). Secretion of these hormones is controlled directly by the hypothalamus and is mediated via hypothalamic releasing hormones, which can promote or inhibit specific hormone secretion.

Growth Hormone Genetics

The human GH gene, *GH-1*, resides within a cluster of highly homologous (92-98% nucleotide identity) genes on chromosome 17 (17q23) (Chen et al., 1989; Procter et al., 1998). This 66.5 kb cluster is thought to have arisen from a series of gene duplications and consists of five genes: *GH-1*, chorionic somatomammotropin genes 1 and 2 (*CSH-1* and *CSH-2*), a chorionic somatomammotropin pseuodgene (*CSHP-1*) and a second GH gene (*GH-2*) (Barsh et al., 1983; Chen et al., 1989; Hirt et al., 1987; Miller and Eberhardt, 1983). These genes lie in the same transcriptional orientation and share a similar structure of five exons separated by four small introns (Fiddes et al., 1979; Jacquemin et al., 1990) (Figure 7). *CSH-1*, *CSH-2* and *GH-2* are all expressed in the placenta and contribute to fetal growth (Cooke and Liebhaber, 1995). *GH-2* encodes GH-V (GH-variant) that differs from the protein encoded by *GH-1* by 13 amino acids. GH-V replaces pituitary GH in the maternal circulation during the second half of pregnancy (Frankenne et al., 1988). With the exception of *CSHP*, all genes in the cluster encode a 217 amino acid pre-hormone that is cleaved to yield a mature hormone with 191 amino acids and a molecular weight of 22-kDa (Mullis, 2005).

Somatotrophs constitute 40-50% of cells in the anterior pituitary and 3% of pituitary transcripts encode GH (Chen et al., 1989). There are two main elements that regulate *GH-1* transcription, a highly polymorphic proximal promoter (containing at least 16 single nucleotide polymorphisms; SNPs) and a locus control region (LCR) (Cooke and Liebhaber, 1995; Giordano et al., 1997; Horan et al., 2003; Jones et al., 1995; Wagner et al., 1997). Several *cis*-acting factors have been implicated in regulation of *GH-1* expression, including regulatory sequences within the proximal *GH-1* promoter. The most important of these are binding sites for the pituitary-specific transcription factor, Pit-1, which stimulates *GH-1* expression (Mangalam et al., 1989). Pit-1 regulates important differentiating steps during embryological development of the anterior pituitary (Theill and Karin, 1993). Pit-1 also regulates the expression of PRL and TSH as well as GH. Differences in the proximal promoter between *GH-1* and other related genes in the GH cluster are thought to account for the difference in expression of these genes (Procter et al., 1998). The LCR is located between 7.5–40-kb upstream of *GH-1* and is required for expression of all five genes in the GH cluster (Ho et al., 2002; Jones et al., 1995; Su et al., 2000). This region also contains binding sites for Pit-1, which are responsible for the high level, somatotroph-specific expression of *GH-1* (Shewchuk et al., 1999; Shewchuk et al., 2002).

Growth Hormone Secretion

Human GH is active as a monomeric globular protein with a molecular mass of 22-kDa. The nascent GH precursor polypeptide is 217 amino acids in length and includes an N-terminal signal peptide for translocation into the endoplasmic reticulum (ER). After cleavage of the leader, the remaining 191 amino acids exist as a single polypeptide with two intramolecular disulfide bridges, which fold into a structure containing four anti-parallel α-helices. The first two helices are parallel to each other and anti-parallel to the last pair (Abdel-Meguid et al., 1987; De Vos et al., 1992). From the ER, GH is transported to the Golgi. There are two current models for transport through the Golgi body; the vesicular model and the cisternal maturation model. In the former, secretory proteins traverse the Golgi via distinct vesicles between cisternae before emerging from the *trans*-Golgi (Dannies, 1999; Dannies, 2001). In the maturation model, newly secreted proteins from the ER align with pre-Golgi intermediates and these structures mature into *cis*-cisternae. These cisternae then 'mature' into the *medial*- and then *trans*-Golgi cisternae. The *trans*-Golgi network disintegrates, forming secretory vesicles (Dannies, 1999; Dannies, 2001; Rambourg et al., 1992).

An important aspect of GH secretion, and indeed the secretion of other neuroendocrine hormones, is the ability to rapidly secrete hormone in response to stimuli. This is achieved by packaging of concentrated hormone aggregates within dense-core secretory vesicles (DCSVs), so called due to their dense appearance in electron microscopy (Dannies, 2002). Aggregation begins in the

Golgi and may serve two functions; first to concentrate the GH and also to sort secretory granule proteins (Dannies, 2002). A model for GH aggregation suggests it is induced by acidity; the pH from the ER to the *trans*-Golgi to the secretory granule is 7.2, 6 and 5.5, respectively (Anderson and Pathak, 1985; Wu et al., 2001). All cells have an acidic trans-Golgi lumen but hGH and human PRL do not form aggregates in cell lines that do not store proteins within secretory granules (Lee et al., 2001; Sankoorikal et al., 2002). The presence of both Zn^{2+} and Cu^{2+} ions appears relatively specific to the neuroendocrine secretory pathway and may be required in addition to low pH (Dannies, 2002; Mullis et al., 2002). hGH contains well-defined Zn^{2+} binding sites that induce dimerization (Cunningham et al., 1991). Current hypotheses suggest that while these aggregates form in the *trans*-Golgi, soluble proteins that also occupy the lumen are removed in vesicles leaving condensed aggregates behind (Dannies, 2002).

When GH releasing hormone (GHRH) interacts with its receptor on the somatotroph cell surface, it triggers a series of cascades that culminate in an influx of intracellular Ca2+, stimulating swelling of DCSV and release of GH (Jena et al., 1997; Lin-Su and Wajnrajch, 2002). After stimulation, DCSVs swell and fuse with the porosome, or fusion pore, a basket-like structure within the plasma membrane that contains pores open to the external environment (Anderson et al., 2004; Jena et al., 2002; Jena et al., 1997; Jeremic et al., 2003). At the porosome base t-SNAREs and calcium channels in the membrane facilitate the docking and fusion of secretory vesicles (Wheatley 2007). An

increase in pressure causes release of GH, propelling it into the circulatory system (Cho et al., 2002a; Jena et al., 2002). Studies in pigs show that stimulated GH cells contain twice the number of both empty and partially filled vesicles than resting cells and that the total number of secretory vesicles does not alter after release (Lee et al., 2004). This supports the notion that during secretion, DCSVs transiently dock at the fusion pore to release vesicular content, refuting the classical theory of total fusion of the secretory granule with the plasma membrane (Breckenridge and Almers, 1987; Cho et al., 2002a; Cho et al., 2002b; Lee et al., 2004; Valentijn et al., 1999).

Regulation of Growth Hormone Secretion

GH is secreted from the anterior pituitary in pulsatile bursts that peak at night. Secretion is under the concerted control of three hypothalamic hormones: GH releasing hormone (GHRH), somatostatin and grehlin. GHRH and grehlin are positive regulators, stimulating GH release, while somatostatin is a negative regulator (Anderson et al., 2004). GHRH is mainly expressed in the arcuate nucleus of the hypothalamus and is released from neurosecretory terminals in the median eminence (Frohman and Kineman, 1999). Ghrelin is a natural ligand for the GH-secretagogue receptor, a G protein-coupled receptor, and stimulates GH release directly and also by upregulating GHRH (Caminos et al., 2005; Kojima et al., 1999). It is produced by the stomach, small intestine, and central nervous system (Lee et al., 2002). Unlike GHRH, ghrelin does not stimulate transcription of GH-1 (Barinaga et al., 1983). Somatostatin is a cyclic 14- or 28-

amino acid residue containing peptide that suppresses GH release, but does not seem to affect GH synthesis in the somatotroph (Muller et al., 1999). Somatostatin itself is regulated by GH and GHRH, forming a feedback loop that regulates GH secretion.

Growth Hormone Signaling Pathway

Once secreted into the circulatory system, GH acts on various target cells expressing the GH receptor (GHR), and GH responsiveness is largely dependent on GHR expression (Leung et al., 1987). GHRs are most highly expressed in hepatocytes and to a lesser extent in muscle, bone, kidney, mammary gland, adipose and embryonic stem cells, among other cell types (Kelly et al., 1991). GHRs belongs to the family of hematopoietic cytokine receptors with single-transmembrane domains with a heavily glycosylated extracellular domain (Cooke and Liebhaber, 1995; Cosman, 1993). The receptor is present as a dimer on the cell surface and binds one GH molecule at two distinct sites, surprising since GH itself has no apparent symmetry (Brown et al., 2005; De Vos et al., 1992; Harding et al., 1996; Ross et al., 2001). GH binding induces a conformational change in the GHR, which activates two Janus Kinase 2 (JAK2) molecules. These in turn autophosphorylate multiple tyrosine residues and subsequently phosphorylate GHR, thereby initiating several signaling proteins and pathways (Lanning and Carter-Su, 2006). Activation of JAK2 is thought to be a key step in GH signaling (Argetsinger et al., 1993). The activated GHR-JAK2 complex provides a platform for the Signal Transducers and Activators of Transcription (Stats) family of

proteins. After binding to this complex, Stats 1, 3, 5a and 5b are phosphorylated by JAK2 and subsequently dimerize, translocate to the nucleus, and act as transcription factors for a host of GH-related genes (Herrington et al., 2000; Kurzer, 2003). Binding of JAK2 to GHR also activates signal transduction via the Mitogen Activated Protein Kinase (MAPK) and phosphatidylinositol 3′-kinase (PI3K) pathways (Smit et al., 1999; Zhu et al., 2001). GH-induced signal transduction can also occur independently of the GHR-JAK2 interaction (Zhu et al., 2002).

Prolonged activation of JAK2 can lead to unwanted cell transformation and cancer so it is vital that GH-signaling is precisely regulated. The Suppressor of Cytokines (SOCS) protein family are negative regulators of GH-signaling (Lanning and Carter-Su, 2006). SOCS 1, 2 and 3 achieve this by binding to JAK2, inhibiting its activity and targeting ubiquitination of the GHR-JAK2 complex (Flores-Morales et al., 2006; Hansen et al., 1999; Ram and Waxman, 1999). The GH signaling pathway is also negatively regulated by many protein tyrosine phosphatases (Flores-Morales et al., 2006).

Physiological Effects of Growth Hormone

A major role of growth hormone in stimulating body growth is to stimulate the liver and other tissues to secrete Insulin-like Growth Factor 1 (IGF-1) (D'Ercole and Calikoglu, 2001). IGF-1 is secreted by the liver in response to GH stimulation and low serum IGF-1 levels indicate GH deficiency (GHD) (Clemmons, 2007). GH affects a variety of different tissues. For bone growth, it

promotes chondrocyte proliferation (indirectly, via IGF-1) and differentiation (directly) (Olney, 2003). GH is also involved in the regulation of protein, lipid and carbohydrate metabolism both directly and via IGF-1 (Moller et al., 2007; Norrelund, 2005). IGF-1 is implicated in muscle growth, stimulating both differentiation and proliferation of myoblasts, as well as enhancing amino acid uptake and protein synthesis in muscle (Agnusdei and Gentilella, 2005).

Growth Hormone Deficiency

GHD is thought to occur at a frequency of 1:4,000 to 1:10,000 (Lacey and Parkin, 1974; Lindsay et al., 1993; Rona and Tanner, 1977; Vimpani et al., 1977). Most cases appear to be sporadic, a result of pituitary insult or developmental abnormalities. 5-30% of cases have an affected first-degree relative and are genetic (Cogan et al., 1993; Phillips III, 1995). In addition to *GH-1* mutations, other mutations that cause GHD have been characterized in a series of genes involved in pituitary development and *GH-1* expression. These genes include the transcription factors, *Pit-1, Prop-1* (Prophet of Pit-1), *Hesx-1, Sox-2, Sox-3, Lhx-3* and *Lhx-4* and in the GHR and GHRH-receptor genes (Mullis, 2005; Mullis, 2007).

A GHD phenotype can be either isolated (IGHD) or associated with combined pituitary hormone deficiency, CPHD (Dattani and Robinson, 2000). There are four, well-characterized, distinct familial types of IGHD; IGHD type IA and IB, IGHD II and IGHD III, where clinical phenotypes are restricted to the GH axis (Mullis, 2005; Phillips III, 1995). In addition to short stature, IGHD patients

exhibit other symptoms including delayed skeletal development (though still in proportion to their height), delayed puberty (but normal fertility), truncal obesity, delayed secondary dentition, young facial appearance, and occasional fasting hypoglycemia (Moseley and Phillips III, 2000). IGHD IA and IB are inherited in an autosomal recessive manner. IGHD IA is the most severe form of IGHD I with gross *GH-1* deletions and development of anti-GH antibodies in response to GH treatment (Illig, 1970; Phillips III, 1995; Phillips III et al., 1981). IGHD IB is characterized by low but detectable serum GH levels (<2.5ng/ml) and patients respond well with immunological tolerance to exogenous GH (Mullis, 2005). Mutations causing IGHD IB include missense and splice site mutations in *GH-1* (in intron 4) plus mutations in the GHRH-receptor gene (Moseley and Phillips, 2000; Mullis, 2005). IGHD II is an autosomal dominant form of IGHD, which is characterized by mutations that disrupt *GH-1* splicing and cause skipping of exon 3. Three substitution mutations, R183H, P89L and V110F, are also responsible for an IGHD II phenotype but do not affect *GH-1* splicing (Binder et al., 2001; Deladoey et al., 2001; Duquesnoy, 1998). Rare mutations have also been identified in the *Hesx-1* (homeobox gene expressed in embryonic stem cells) gene that cause IGHD II (Fintini et al., 2006). Five cases of IGHD II have been identified where no mutations have been observed in either *GH-1* or *Hesx-1*, suggesting mutations in other genes could also cause IGHD II (Fintini et al., 2006). IGHD III is a X-linked, recessive disorder that may involve mutations/deletions within the long arm of chromosome X and these might be caused by mutations in the *Btk* (Bruton's tyrosine kinase) gene (Conley et al.,

1991; Duriez et al., 1994). *Btk* mutatons have been associated with IGHD, suggesting that genes critical for GH production exist on the X-chromosome (Stewart et al., 1995).

Growth Hormone Splicing

GH-1 is composed of five exons and four short introns, which can be spliced to produce at least five distinct isoforms (Figure 7) (DeNoto et al., 1981). Constitutive splicing of *GH-1* includes all five exons and these wild-type transcripts encode a 22-kDa protein, which is the major, biologically active form of GH. Of the other isoforms, the 20-kDa is the most abundant (5-10% of normal *GH-1* transcripts) caused by activation of an in-frame cryptic 3' splice site in exon 3 (cryptic transcripts) that results in elimination of the first 45 nt of this exon (amino acids 32-46) (Cooke et al., 1988; Masuda et al., 1988; Procter et al., 1998; Stewart et al., 1992; Tsushima et al., 1999). This isoform is thought to retain much of the biological activity of the wild type, 20-kDa protein (Asada et al., 2000; Ishikawa et al., 2000; Ishikawa et al., 1999; Masuda et al., 1988). Complete skipping of exon 3 (Δ3 transcripts) produces a 17.5-kDa protein, accounting for 1-5% of pituitary *GH-1* transcripts (Lecomte et al., 1987; Procter et al., 1998). This hGH isoform acts in a dominant negative fashion by preventing secretion of the wild type, 22-kDa protein. Transcripts skipping exons 3-4 or exons 2-4, encoding 11.3-kDa or 7.4-kDa proteins, respectively, have also been identified (Palmetshofer et al., 1995).

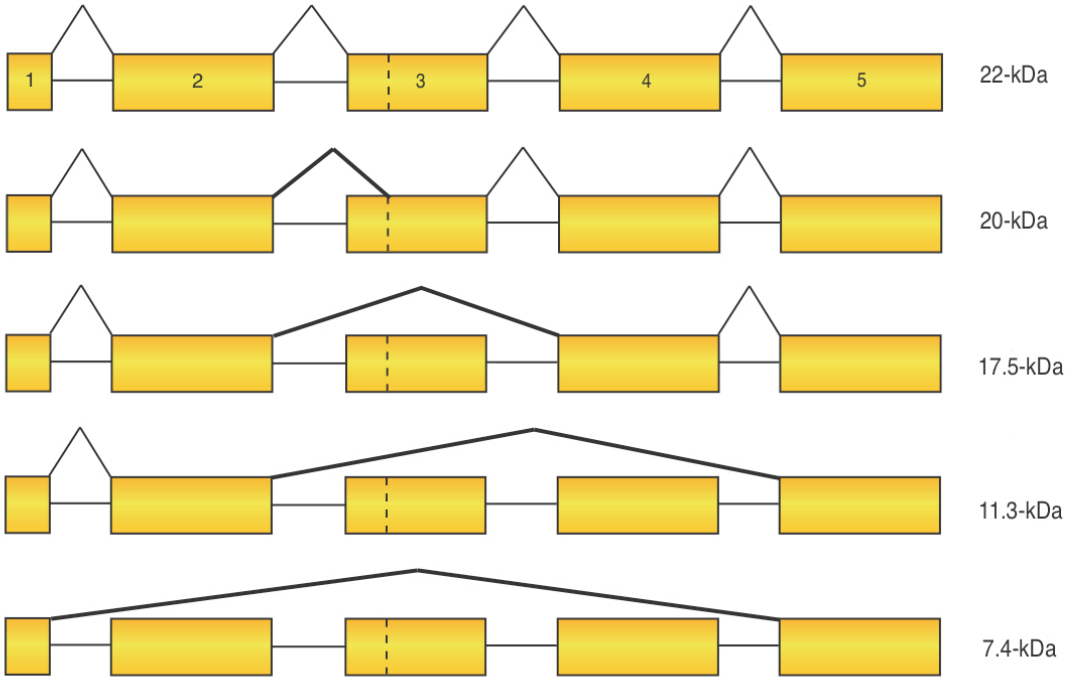


Figure 7. *GH-1* alternative splicing pattern. *GH-1* comprises 5 exons and 4 introns that are constitutively spliced to produce a wild type 22 kDa GH protein. Alternative splicing events give rise to other isoforms, the four most common are depicted. The cryptic splice site is denoted by dashed lines.

The 17.5-kDa GH isoform results from aberrant splicing of the *GH-1* transcript and acts in a dominant negative fashion highlighting the absolute importance of maintaining splicing fidelity. The splice sites within introns 2 and 3 are particularly weak and the cryptic splice site within exon 3 is a stronger splice site than the actual 3' splice site of intron 2. Accurate splicing of *GH-1* transcripts requires the concerted effort of three splicing enhancers (ESE 1, ESE 2 and ISE) to maintain inclusion of the exon (Figure 8). Both ESEs are in exon 3; ESE 1 comprises the first seven nucleotides of the exon and ESE 2 is a 15 nucleotide sequence upstream of the cryptic splice site (E3+19-33) (Moseley et al., 2002; Ryther et al., 2004; Ryther et al., 2003). ESE 1 was first identified as a six nucleotide motif in a family with IGHD II that had an A→G transition at the fifth nucleotide of exon 3 (Moseley et al., 2002). Subsequent deletion analysis extended ESE 1 to include the first seven nucleotides of exon 3, 5'-GAAGAAG-3' (Ryther et al., 2004). ESE 1 promotes definition of exon 3 by activating the 3' splice site of intron 2 and preventing use of the cryptic splice site (Ryther et al., 2004; Ryther et al., 2003). A series of deletions within exon 3 and subsequent splicing analyses identified a second enhancer, ESE 2, that also activates exon 3 inclusion (Ryther et al., 2004). ISE is a nine nucleotide purine-rich sequence within intron 3 (IVS3+26-34), first identified by the presence of two patient mutations, IVS3+28 G→A and IVS3Δ28-45. (Cogan et al., 1997; Cogan et al., 1995; McCarthy and Phillips, 1998). ISE promotes exon 3 definition (Ryther et al., 2003).

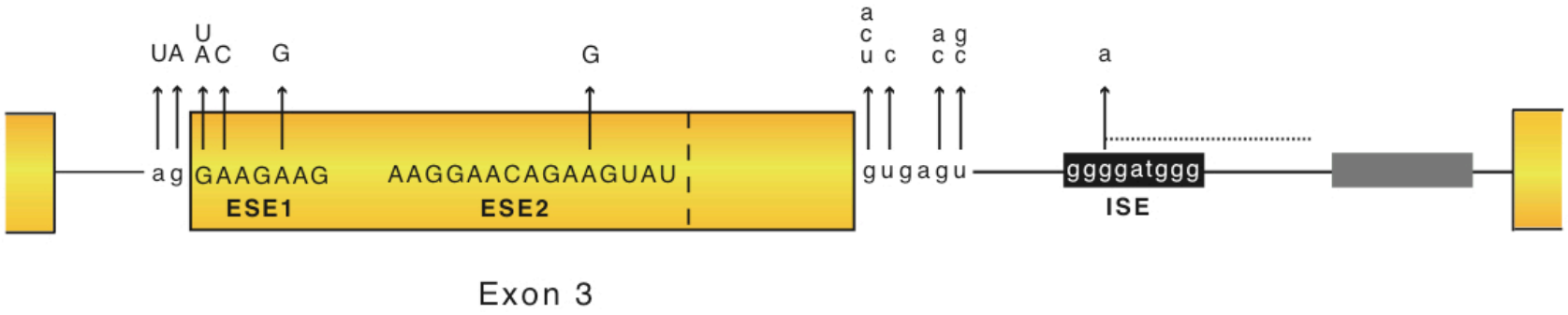


Figure 8. Regulation of *GH-1* exon 3 inclusion. Schematic of exon 3 showing IGHD II-causing point mutations. Mutations that result in exon 3 skipping commonly occur at the exon/intron boundaries flanking exon 3, but they can also occur in enhancer elements within exon 3 (ESE1 and ESE2) and intron 3 (ISE). The cryptic splice site in exon 3 is depicted by a vertical dashed line. A deletion mutation in intron 3 (IVS3 del 28-45) is illustrated by a horizontal dotted line, and another deletion mutation in intron 3 (IVS3 del 56-77) is illustrated by a gray box.

Patients with mutations that disrupt either the splice sites flanking exon 3 or any of the enhancer elements exhibit increased skipping of exon 3 and therefore suffer from IGHD II due to increased production of the 17.5-kDa isoform (Binder and Ranke, 1995; Cogan et al., 1997; Cogan et al., 1995; Fofanova et al., 2003; Hayashi et al., 1999a; Kamijo et al., 1999; McCarthy and Phillips III, 1998; Millar et al., 2003; Missarelli et al., 1997; Moseley et al., 2002; Phillips III and Cogan, 1994; Ryther et al., 2003; Takahashi et al., 2002) reviewed in (Mullis, 2007); Figure 9). These mutations have different effects upon the extent of exon 3 skipping. Those in the splices sites, particularly the 5' splice site of intron 3, usually cause more skipping than mutations that affect enhancers, leading to greater production of the 17.5-kDa protein. Since an increase in the 17.5-kDa isoform correlates with an increase in severity of IGHD II symptoms (see below), it appears that splice site mutations, rather than enhancer, mutations, trigger more severe forms of IGHD II. Patients with splice site mutations have an earlier age of onset and exhibit greater clinical severity. There is also variation in exon 3 skipping and disease severity among mutations at the 5' splice site. Mutations affecting the first two nucleotides (IVS3 +1/+2) always cause exon 3 skipping whereas transcripts with mutations at positions +5/+6 (IVS3 +5/+6) allow minor levels of normal splicing. This observation is explained by the conserved GU dinucleotide at the 5' end of the intron, which pairs with U1 snRNA. Disruption of base-pairing between the mRNA and the snRNA inhibits spliceosome assembly. For mutations at positions at +5/+6, skipping is not as extreme since these nucleotides are not as highly conserved.

Mouse Model of Isolated Growth Hormone Deficiency type II

How the 17.5 kDa isoform acts in a dominant negative fashion is only partially understood. A transgenic mouse model of IGHD II has been created that expresses a human mutant *GH-1* transcript expressed from a cosmid containing the entire *GH-1* LCR, including upstream DNA elements required for somatotroph-specific expression (McGuinness et al., 2003; Ryther et al., 2003). The transgene contains a G to A transition at the 5' splice site of intron 3 (IVS3+1 G→A) that leads to exclusive production of the 17.5-kDa isoform (McGuinness et al., 2003) and exerts a dominant negative effect on wild type mouse GH (mGH). This inhibition is dose-dependent; high copy transgenic mice exhibit far more extreme phenotypes than those with low copy numbers (McGuinness et al., 2003). This correlates with the severity of symptoms observed in IGHD II patients with regard to the amount of exon 3 skipping (Mullis et al., 2005). Even in the presence of two wild type mGH alleles, high copy transgenic mice exhibit severe IGHD II with concomitant reduced weight, severely reduced pituitary GH content, and progressive anterior pituitary hypoplasia (McGuinness et al., 2003; Ryther et al., 2003; Shariat et al., 2007). Overproduction of the 17.5-kDa isoform triggers not only somatotroph death but also destruction of neighboring cells by macrophage invasion, leading to severe hypoplasia and additional anterior pituitary hormone deficiencies (McGuinness et al., 2003). This is evident in electron micrographs where pituitaries from IGHD II mice show a lack of DCSVs and extreme vacuolation (McGuinness et al., 2003; Shariat et al., 2007).

Patients with severe IGHD II often develop additional pituitary hormone deficiencies, particularly ACTH and TSH, which require additional treatment (Mullis et al., 2005). The IGHD II mouse model showed deficiencies in PRL and TSH at weaning, worsening by 8-10 weeks of age compared to non-transgenic littermates. At the later time point, a loss of LH was also evident (McGuinness et al., 2003). Variability in the age of onset and severity of IGHD II among patients is thought to be due to the amount of the 17.5-kDa isoform relative to the wild type, 22-kDa protein and occurs from splicing mutations that generate differential exon 3 skipping (Mullis, 2007; Mullis et al., 2005). This result suggests a threshold and dose-dependence of the amount of the 17.5-kDa isoform above which somototroph death and pituitary defects are triggered (McGuinness et al., 2003).

Exclusion of exon 3 results in loss of amino acids 32-71 in the 17.5-kDa isoform. This deletion corresponds to the entire loop region between helices 1 and 2 (Ultsch et al., 1994). The deletion also disrupts an internal disulfide bridge by elimination of Cys^{53}, leaving an unpaired Cys^{165}. It has been assumed that the 17.5-kDa is strongly misfolded, though mechanisms of how it functions in a dominant negative manner are only just being elucidated. It is not likely that disruption of intra- or intermolecular disulphide bridges is responsible since a compensatory mutation of the unpaired Cys^{165} still results in a mutant GH with a dominant negative effect (Lee et al., 2000) and another mutation, R77C, which disrupts a disulfide bridge does not have a dominant negative effect on wild-type GH (Takahashi et al., 1996). The dominant negative effect upon the wild-type

GH is also seen with missense mutations that do not affect *GH-1* splicing (Binder et al., 2001; Deladoey et al., 2001; Duquesnoy, 1998). One current model proposes that as the 17.5-kDa and wild type GH isoforms progress through the secretory pathway, they form heterodimers that do not efficiently condense in the trans-Golgi, preventing formation of DCSVs (McGuinness et al., 2003) (Figure 9b). It is postulated that these heterodimers accumulate in the Golgi and then back up into the ER, triggering an unfolded protein response (Graves et al., 2001). When proteins misfold, they are often retained in the endoplasmic reticulum (ER) and targeted for degradation (Ellgaard et al., 1999). Cells exhibit a variety of responses to the accumulation of misfolded protein in the ER. This includes the unfolded protein response, which involves transcriptional activation of genes encoding a wide range of proteins necessary for protein folding and secretion (Bernales et al., 2006; Chapman et al., 1998). The model above supports findings in which the 17.5-kDa isoform has been expressed in various cell lines. In transient transfections in neuorendocrine cell lines, the 17.5-kDa isoform decreased intracellular and secreted wild-type forms of GH (Graves et al., 2001; Hayashi et al., 1999b; Lee et al., 2000; McGuinness et al., 2003). The mutant GH isoform had no specific dominant negative effect upon wild-type GH secretion in non-neuroendocrine cell lines, though in COS7 cells, the 17.5-kDa isoform disrupted trafficking of both plasma membrane and secretory proteins (Graves et al., 2001; Lee et al., 2000). Confocal studies showed co-localization of the 17.5-kDa isoform with components of the ER, Golgi and also, albeit minimally compared to wild-type, with secretory granules (Salemi et al., 2006).

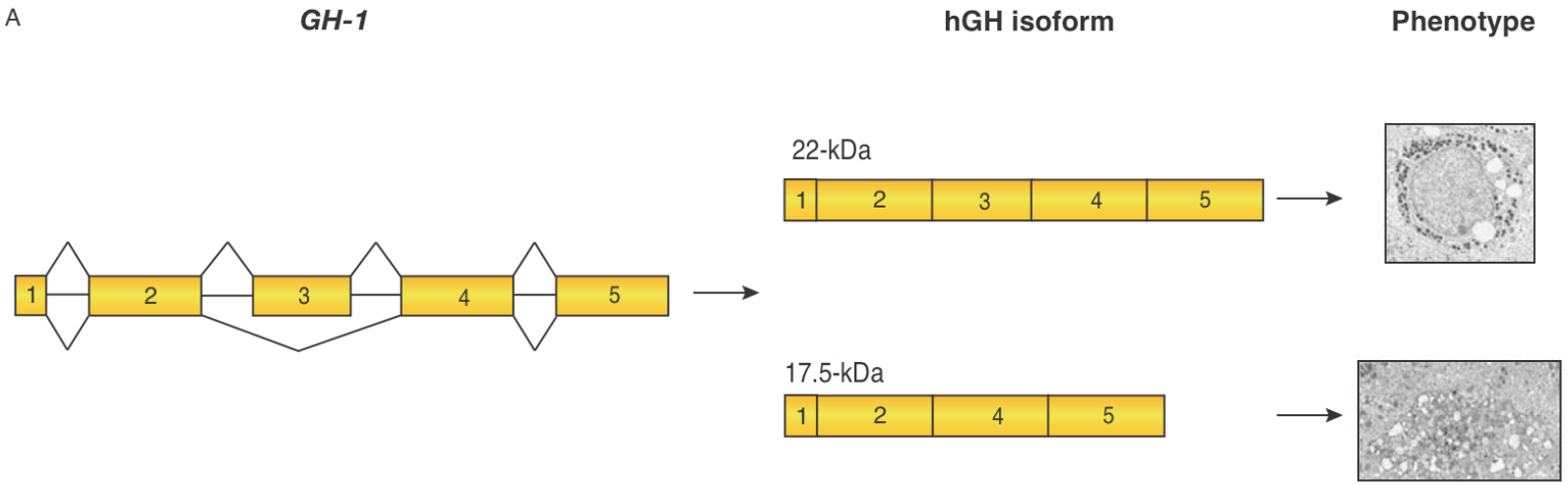


Figure 9. IGHD II pathogenesis (A) Expression of the hGH 17.5-kDa isoform in mice results in an IGHD II phenotype, preventing formation of GH secretory vesicles in somatotroph cells. (B) The 17.5-kDa isoform prevents efficient packaging of wild type GH which eventually overwhelms the degradative capacity of the proteosome, disrupting protein trafficking and leading to cell death and concomitant anterior pituitary hypoplasia.

Recent data in stable somatotroph cell lines expressing both wild type and Δ3 transcripts shows that the 17.5-kDa isoform is irregularly folded and degraded in a proteosome-dependent manner (Kannenberg et al., 2007). Once the degradative capacity of the proteosome is exceeded, toxic GH aggregates form in the cytosol, ER and Golgi (McGuinness et al., 2003). In the mouse model of IGHD II, an increase in GHRH and a decrease in somatostatin were observed, causing increased mutant *GH-1* expression and further accelerating the toxic effects of the 17.5-kDa protein (McGuinness et al., 2003). However, this does not seem to be a problem in patients with IGHD II (Mullis, 2005).

RNA Interference as a Therapy for IGHD II

Transcripts lacking exon 3 possess a unique sequence at the boundary of exon 2 and exon 4 that is not present in any other spliced *GH-1* products. As a result, the Δ3 transcript is an ideal target for knock down by RNAi using siRNAs complementary to this unique sequence (Ryther et al., 2004). As discussed above, while treatment with recombinant GH (rGH) rescues short stature, IGHD II patients often develop additional hormonal deficiencies. Specifically targeting transcripts that encode the 17.5-kDa isoform, a direct cause of IGHD II, is extremely advantageous and highlights a potentially ideal way to treat patients diagnosed with IGHD II caused by mutations that disrupt *GH-1* splicing.

The following work illustrates that RNAi can be used to effectively treat IGHD II in a mouse model of IGHD II. We developed transgenic mice expressing an shRNA complimentary to the Δ3 transcript (shRNA-17.5). These mice were

bred with IGHD II mice, and the resulting progeny were ‘rescued,’ meaning they did not exhibit disease symptoms.

We also identify and characterize a new mutation in *GH-1* in a family suffering with IGHD II. The mutation occurs at the first base of exon 3, corresponding to the 3’ splice site and to ESE 1. Disruption of these splicing elements promotes skipping of exon 3 and an increase in the 17.5-kDa isoform.

CHAPTER II

ISOLATED GROWTH HORMONE DEFICIENCY TYPE II CAUSED BY A DOUBLE ENCRYPTED GH-1 MUTATION THAT HAS UNEXPECTED EFFECTS

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Summary

Context: Genetic mutations that lead to amino acid changes are most commonly believed to be due to altered protein function but such changes can also exert their effects at earlier stages by affecting splicing.

Objective: Exon 3 of the human growth hormone gene (*GH-1*) contains two splicing enhancers that prevent exon skipping. We made use of a novel patient mutation that causes Isolated GH Deficiency type II to determine whether the overall defect was due to a missense mutation, a splice site mutation, or a splicing enhancer mutation.

Design, Setting and Patients: A heterozygous guanine to adenine transition at the first nucleotide of exon 3 (E3 +1 G→A) in *GH-1* was identified in a family presenting with IGHD II. Although this mutation encodes an amino acid substitution, GH-E32K, it also resides within a splicing enhancer and is part of the 3' splice site of exon 3.

Interventions and Results: Interestingly, the E3 +1 G→A causes an increase in skipping of exon 3 transcripts by disrupting an exon splicing enhancer and weakening the 3' splice site. Thus, exon skipping due to production of a dominant negative 17.5-kDa isoform forms the basis of disease for this mutation. Transcripts encoding the 17.5-kDa isoform can be targeted for specific degradation using RNA interference in patient derived lymphoblastoid cell lines.

Conclusion: The predicted GH-E32K amino acid substitution is not responsible for GHD. Instead, the mutation causes aberrant splicing due to disruption of a splicing enhancer within exon 3 coupled to a weakening of the 3' splice site. The

resulting increase in the 17.5-kDa isoform causes IGHD II. This study illustrates that RNA serves as more than just a code for protein production and that it is important to look beyond the protein sequence when assaying genetic mutations.

Introduction

The human GH gene, *GH-1*, comprises five exons that are constitutively spliced to produce the full-length, 22-kDa protein. The majority of circulating GH is translated from mRNAs containing all 5 exons but aberrant splicing of wild type transcripts gives rise to at least 5 other smaller isoforms, the most abundant of which are a 20-kDa isoform and a 17.5-kDa isoform (Procter et al., 1998) (Figure 7). The 20-kDa protein lacks amino acids 32-46 due to activation of an in-frame cryptic splice site within exon 3 but apparently retains full functionality (Stewart et al., 1992). Complete skipping of exon 3 generates the 17.5-kDa isoform that acts in a dominant negative manner (Figure 9). Even in normal individuals, RNAs encoding the 20-kDa and 17.5-kDa isoforms account for approximately 5-10% and 1-5% of *GH-1* transcripts, respectively (Procter et al., 1998).

The 17.5-kDa isoform exerts a dominant negative effect by preventing secretion of wild type GH in both tissue culture cells and in transgenic mice (Hayashi et al., 1999b; Lee et al., 2000; McGuinness et al., 2003; Shariat et al., 2007). Patients with inherited mutations that increase the levels of the 17.5-kDa isoform exhibit Isolated GH Deficiency type II (IGHD II), an autosomal dominant form of GHD. Common characteristics of IGHD II include short stature due to impaired bone elongation, delayed puberty, and, in severe cases, anterior

pituitary hypoplasia with concomitant disruption of the anterior pituitary hormone axis.

Splicing is catalyzed by a macromolecular complex termed the spliceosome whose RNA and protein moieties recognized conserved sequences in the mRNA, namely the 5' splice site, the branch point/polypyrimidine tract, and the 3' splice site (Moore et al., 1993) (Figure 1). In higher eukaryotes these splice sites are poorly conserved and this lack of sequence conservation has at least two consequences. First, exons and introns must be properly recognized to maintain splicing fidelity even when the flanking sites are weak, a daunting task given that exons are typically small and sequences resembling bona fide splice sites can often be found within the sea of RNA that constitutes introns (Black, 1995; Smith and Valcarcel, 2000). Second, regulated splicing is common, involving differential recognition of splice sites, alternative splicing, and the production of an incredibly diverse proteome from a relatively small genome (Modrek and Lee, 2002; Smith and Valcarcel, 2000). For both splicing fidelity and alternative splicing, additional *cis*-acting regulatory elements have been identified that aid identification of the correct splice sites. The two best characterized elements are referred to as splicing enhancers and silencers (Blencowe, 2000; Solis et al., 2008; Weighardt et al., 1996). Splicing enhancers are typically purine-rich and aid exon and intron definition (Blencowe, 2000; Tacke and Manley, 1999).

The splice sites surrounding exon 3 in *GH-1* are relatively weak and require the concerted effort of three splicing enhancers to ensure inclusion

(Ryther et al., 2004). Two of these enhancers reside within exon 3 (Exonic Splicing Enhancers (ESEs) 1 and 2; whereas the third is found in the downstream intron (ISE) (Figure 8). ESE 1 comprises the first seven bases of exon 3 and is required for both proper recognition of the upstream 3' splice site and suppression of the downstream cryptic splice site (Ryther et al., 2003). Suppression of the cryptic splice site is necessary because it is a stronger 3' splice site sequence than the wild type 3' splice site (scores of 91 and 85 respectively, calculated according to Shapiro and Senepathy, 1987; http://genet.sickkids.on.ca/~ali/splicesitescore.html). Molecular analyses of ESE 2 and ISE have shown that both function to ensure proper exon 3 definition to avoid skipping. From an evolutionary perspective, it is interesting that the *GH-1* gene requires the action of multiple enhancer elements to ensure splicing fidelity. Disruption of any of these elements leads to the production of aberrant GH, mostly involving skipping of all or portions of exon 3.

Here, we examine the sequence requirements to maintain accurate splicing of *GH-1* transcripts by characterizing a heterozygous mutation discovered in a family presenting with IGHD II. This mutation is a guanine to adenine transition at the first base of exon 3 (E3+1 G→A) (Figure 10). Our analysis shows that this transition mediates its effects in two ways; by disrupting ESE 1 and by weakening the 3' splice site consensus sequence. While GH is primarily expressed in somatotroph cells in the anterior pituitary, we also detect aberrantly spliced transcripts in patient-derived lymphoblastoid cell lines (LCLs) providing a unique tool to molecularly characterize the effects of specific

mutations in heterozygous settings. Small interfering RNAs (siRNAs) can be introduced into these LCLs to specifically degrade transcripts encoding the 17.5-kDa isoform.

Subjects and Methods

Subjects

We studied DNA from members of a Caucasian family presenting with IGHD II, inherited in an autosomal dominant manner whose pedigree is shown in Figure 10a. Clinical tests and pedigree analysis confirmed autosomal IGHD II. Sequencing of DNA from family members identified a heterozygous E3+1 G→A transition in the *GH-1* gene in affected IGHD II individuals that is predicted to encode an E32K substitution (Figure 10b). Patient I-I was examined and diagnosed at age 2 years 5 months with IGHD II based on short stature, low GH levels, both resting and following stimulation. The clinical data are shown in Table 2. A cranial MRI done prior to treatment showed a hypoplastic anterior pituitary. He was started on subcutaneous GH replacement therapy (Humatrope) at two years and six months and showed a good response to treatment within the first four months. Following therapy, slight scoliosis of the spine and increased truncal adiposity exhibited before treatment have been reduced. Currently, at age five years and three months, the patient is at the 25th percentile for height.

Cell Culture and *In Vivo* Splicing Analyses

Blood samples were obtained from the subject and affected members of his family after obtaining consent. Genomic DNA was isolated from total blood and the *GH-1* gene sequenced (Cogan et al., 2006). The primers used for amplification and subsequent sequencing of *GH-1* were: 5'-CCAGCAATGCTCAGGGAAAG-3', 5'-TGTCCCACCGGTTGGGCATGGCAGGTAGCC-3' and 5'-CTGGGAAATAAGAGGAGGAGAC-3'. LCLs were isolated and transformed as previously described (Oh et al., 2003) and were maintained in RPMI supplemented with 20% FBS. Total RNA was isolated from 2x106 cells using RNeasy (Qiagen) and 3μg was used for cDNA synthesis using SuperScript III (Invitrogen) with oligo d(T) primers. cDNA products were amplified using 32P-labeled *GH-1*-specific primers (Ryther et al., 2003) followed by separation on 6% polyacrylamide gels and exposure to phosphorimager analysis. Amplifying spliced products with the same primers allowed accurate quantitation within the same lane, obviating the need for a loading control. Results are thus shown as a ratio of the three products derived from a single lane.

Mutant constructs were generated from wild type *GH-1* in pXGH5 by reverse PCR (Coolidge and Patton, 1995) using mismatch primers followed by sequence verification. Rat somatotroph GH3 cells were grown in DMEM with 10% FBS and transfected with 1μg of wild type or mutant constructs using Mirus LT1 reagent (Mirus Bio). Total RNA was isolated after 48 hours using TRI reagent (MRC) and cDNA was synthesized using a *GH-1*-specific RT primer with

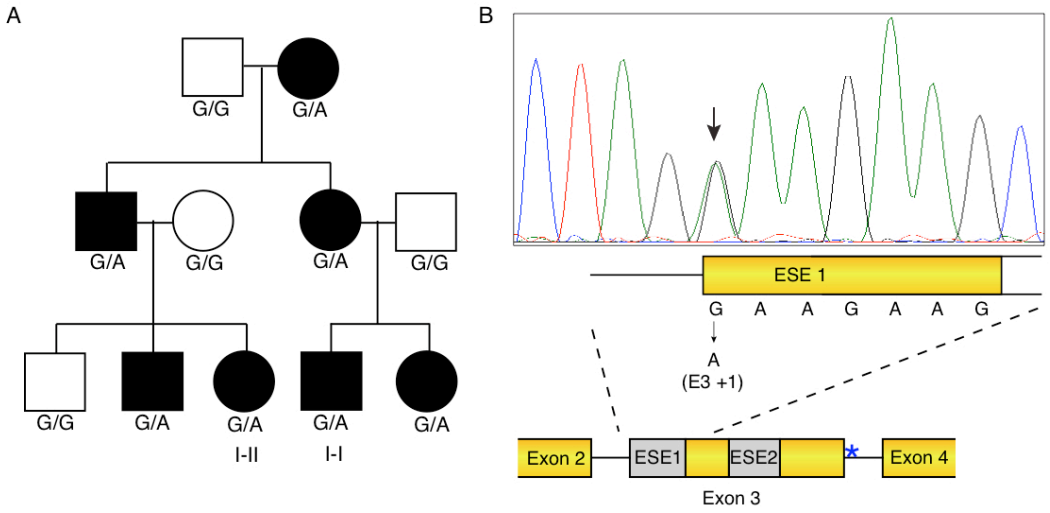

Figure 10. Identification of a heterozygous mutation in *GH-1* that causes IGHD II. (A) Genetic pedigree showing inheritance of a mutation at the first position of exon 3 (E3 +1) in the human growth hormone gene (*GH-1*). The genotype for the two alleles at this position is indicated as either homozygous (G/G) or heterozygous (G/A). Lymphoblastoid cell lines were generated from patients I-I and I-II. ■, affected males; ●, affected females; □ unaffected males; ○ unaffected females. (B) *GH-1* sequencing data from an individual containing a heterozygous G→A transition at the first base of exon 3. Blue asterisk denotes the IVS3+1 G→A mutation used in Figure 11.

M-MLV reverse transcriptase (Promega) as previously described (Ryther et al., 2004). Splicing patterns were determined by RT-PCR as above.

In Vitro Splicing Analysis

Wild-type or mutant ESE1 sequences were cloned into exon 2 of an enhancer dependent splicing construct derived from the Drosophila melanogaster doublesex gene (*DSX*; a kind gift from Dr. B. Graveley) by reverse PCR (Coolidge and Patton, 1995). Primers with either adenine, thymine or cytosine in place of the first base of ESE1 were used to generate the mutant ESE1 *DSX* constructs. The *DSX* constructs were linearized with MluI and in vitro transcribed in the presence of α-32P-ATP and a cap analogue with T7 RNA Polymerase (NEB). Labeled transcripts were incubated for 2 hours at 30°C in 60% HeLa nuclear extract. Splicing products were separated on 8% denaturing polyacrylamide gels and exposed to phosphorimager analysis.

Electroporation of siRNAs

150,000 LCLs were centrifuged at 2,000xg for five minutes, resuspended in 75µl siPORT electroporation buffer (Ambion) and electroporated with 5μg of siRNA-17.5 or siRNA-GFP (Dharmacon) in a 1mm cuvette under the following conditions: single square wave pulse, 325V, 13ms. After electroporation, cells were incubated for 10 minutes at 37°C before being plated in pre-warmed media. After 48 hours, all cells were re-electroporated under the same conditions. For

Table 2. Clinical characteristics of subject I-II [a]Before therapy; [b]After therapy; [c]Highest GH peak after stimulation; IGF BP-III, IGF-1 binding protein; TSH, thyroid stimulating hormone.

	I-I
Sex	M
Age (yrs)	2 5/12[a] 4 9/12[b]
Height (cm)	75.7[a] 104[b]
SD score	-4.62[a] -0.95[b]
Weight (kg)	9.5kg[a] 16.6[b]
BMI (kg/m2)	15.7[a] 15.32[b]
GH ng/ml	0.91
	(0.43-2.4)
Provocation tests	Chlonidine: 1.12[c]
(GH ng/ml)	Arginine: 1.14[c]
IGF-1 ng/ml	28.8
	(51-303)
IGF BP-III (ug/ml)	1.2
	(0.8-3.9)
TSH (ulU/ml)	1.18
	(0.36-7.6)

mock electroporations, LCLs were electroporated in the absence of siRNAs. Total RNA was harvested after a further 48 hours.

Results

GH-1 sequences from affected individuals showed a heterozygous single guanine to adenine transition in the first nucleotide of exon 3 (E3+1 G→A) (Figure 10b). This change results in a glutamic acid to lysine (E32K) change in the amino acid sequence of GH for all mutant transcripts that include exon 3. Despite the missense mutation, we hypothesized that the E3+1 G→A mutation causes disease through a different mechanism for two reasons. First, affected individuals are heterozygous for the mutation and haploinsufficiency does not typically cause IGHD II. Second, the majority of mutations that cause IGHD II do so by inducing skipping of exon 3 to produce the dominant negative 17.5-kDa isoform. There are at least two possible ways splicing could be affected. First, the mutation weakens the 3' splice site of exon 3 to AG|A (Figure 10b). The consensus sequence for 3' splice sites is AG|G where the last guanine is the preferred nucleotide as the first base of exons. For the major class of introns, the AG dinucleotide at the end of introns is absolutely conserved whereas the G at the first position of exons is found in only ~52.5% of cases (A is 22.5%; C and U both 12.5%) (Burge et al., 1999). A second, but not mutually exclusive possibility, is that the E3+1 G→A mutation disrupts ESE 1 (Figure 10b).

A Weak 3' Splice Site Increases Exon Skipping

To determine if the E3+1 G→A mutation actually alters splicing, we transfected rat somatotroph (GH3) cells with a vector expressing wild type GH or a construct containing the E3+1 G→A mutation. To analyze the effects of base changes in the first nucleotide of exon 3, we also created constructs containing E3+1 G→T and E3+1 G→C. The E3+1 G→T mutation has previously been reported to cause IGHD II (Takahashi et al., 2002). Cells were transfected with these constructs, RNA was isolated 48 hours later, and splicing patterns were analyzed by RT-PCR. As expected, the wild-type sequence resulted in predominant production of transcripts encoding the 22-kDa isoform with only ~6% exon 3 skipping (Figure 11a). All three base changes resulted in an increase in exon 3 skipping (Δ3 transcripts) with the E3+1 G→A mutation causing less skipping (39%) than either of the other mutants, G→T or G→C, 78% and 65%, respectively (Figure 11a). The E3+1 G→T mutant introduces an in-frame premature termination codon (GAA→UAA; PTC) and, consistent with nonsense-mediated mRNA decay (NMD), no wild-type transcripts were detectable when this construct was expressed (Figure 11a, lane 2). The E3+1 G→C causes a glutamic acid to glutamine amino acid change upon inclusion of exon 3 which should not cause NMD. Nevertheless, this mutation led to only minimal production of correctly spliced transcripts and significantly increased levels of transcripts encoding the 17.5-kDa isoform. Comparing the wild type, A, and C constructs, it is apparent that there is a preference for a G at position 1 of exon 3 followed by A and then C. The effect of a T cannot be determined under these

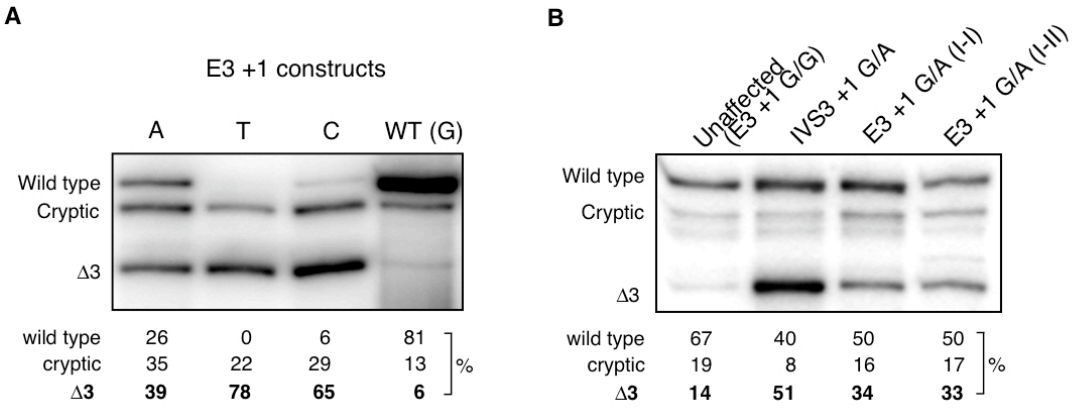

Figure 11. E3+1 G→A causes an increase in skipping of exon 3. (A) Rat GH3 cells were transfected with human *GH-1* E3+1 constructs in which the nucleotide at the first position of exon 3 is as indicated above. The wild type sequence contains a G. RNA was isolated and RT/PCR was performed using *GH-1*-specific primers in exon 2 and exon 5. Bands corresponding to the different spliced products are indicated to the left and the percentages of these products are listed below based on ratios within each lane from three independent experiments. (B) Patient derived lymphoblastoid cell lines were generated from patients as described in Fig. 1. *GH-1* splicing patterns were determined by isolation of RNA from individual cell lines and RT/PCR as above. IVS3+1 G→A was derived from a patient heterozygous for a mutation in the first base of intron 3.

conditions due to NMD. Despite that, the results are consistent with the percent that each of these bases is found in consensus 3' splice sites.

Splicing Analysis in Patient-Derived Lymphoblastoid Cell Lines

The experiments shown in Figure 11a were performed in cultured GH3 cells, which mimic a homozygous mutant background. To confirm that the splicing patterns observed under these conditions accurately reflect splicing patterns that occur in heterozygous, patient derived cell lines, we created LCLs from both affected and unaffected individuals. Although GH is normally expressed in anterior pituitary somatotrophs, we sought to determine whether we could detect limited amounts of *GH-1* transcripts in LCLs derived from IGHD II patients rather than after transfection into heterologous GH3 cells. RNA was isolated from these cell lines and endogenous *GH-1* splicing patterns were examined by RT-PCR using specific primers. Interestingly, as shown in Figure 11b, we could readily detect *GH-1* transcripts in LCLs derived from both normal and GH-deficient patients. The overall pattern of RT-PCR products is slightly different from that observed in transfected GH3 cells due to some additional unknown faint bands migrating close to the band corresponding to transcripts encoding the 20-kDa isoform. While this may slightly alter the percentage of each transcript, it is clear that there is a dramatic increase in transcripts encoding the 17.5-kDa isoform in GH-deficient LCLs. When we compared the levels of $\Delta 3$ transcripts in three individuals with GH deficiency, the levels of the dominant negative 17.5-kDa isoform correlated with disease severity. For the two

individuals with heterozygous E3+1 G→A mutations, the levels of Δ3 transcripts were lower (Figure 11b, lanes 3 and 4) compared to an individual containing a mutation at the 5' splice site of intron 3 (IVS3+1 G→A), in agreement with clinical observations for both types of mutations (Binder et al., 2001; Millar et al., 2003; Moseley et al., 2002; Mullis et al., 2005).

Disruption of ESE 1 and Exon 3 Skipping

While the 3' splice site consensus sequence is altered in patients containing the E3+1 G→A mutation, there are many functional 3' splice sites that contain an adenine as the first base of the exon. Thus, as far as IGHD II is concerned, this mutation may be deleterious because it not only makes the 3' splice site weaker, but it also alters ESE 1. To determine whether the E3+1 G→A mutation causes skipping of exon 3 due to disruption of ESE1, we created a series of constructs designed to test enhancer activity. Single copies of wild type and mutant ESE1 were cloned into a construct derived from the *D. melanogaster doublesex* (*DSX*) gene where splicing is enhancer dependent (Figure 12a) (Caputi et al., 2002; Graveley et al., 1998; Tian and Maniatis, 1992; Tian and Maniatis, 1994). RNA transcripts from these constructs were produced by in vitro transcription and then spliced in HeLa cell nuclear extracts. Previous work has shown that multimers of small, purine-rich sequences can act as enhancer elements in this setting (Graveley et al., 1998). Therefore a single G→A mutation at the start of ESE 1 might not alter enhancer activity if inserted as a single change amidst a multimer of ESE1 elements. As a result, we chose to insert

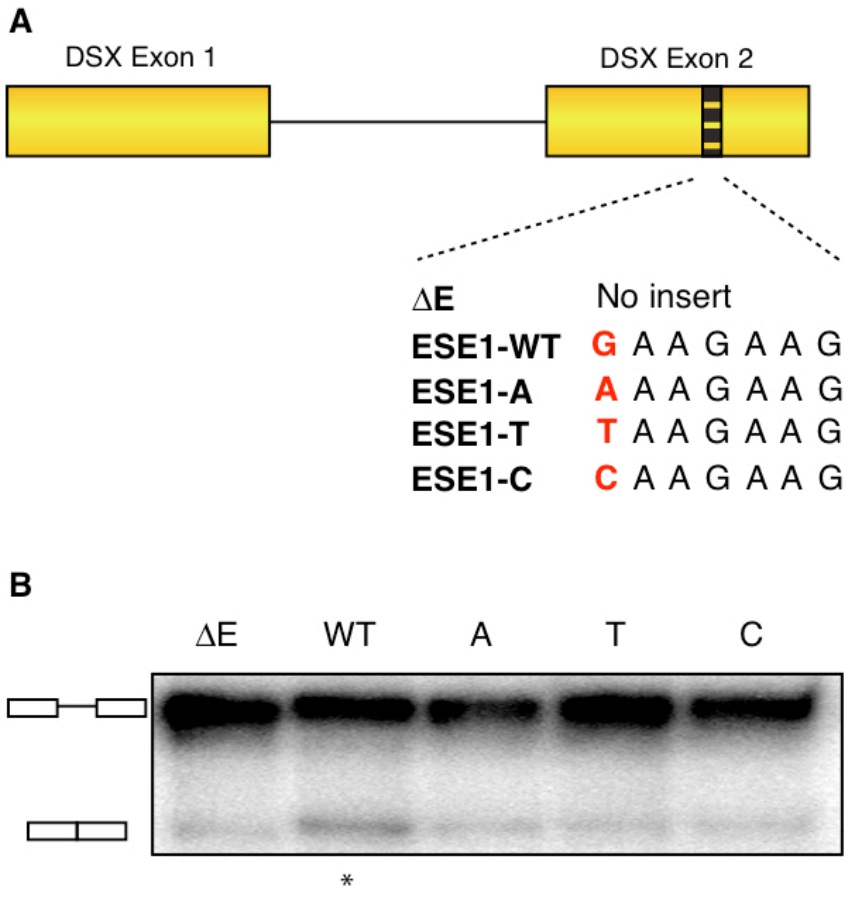

Figure 12. E3+1 G→A disrupts enhancer function. (A) Schematic of the doublesex (*DSX*) minigene construct showing two exons (boxes) and an intron (line). Wild type and mutant ESE1 sequences were cloned into exon 2 (striped box). The first base of ESE1 (red, bold) was mutated to A, T or C. (B) RNAs derived from the constructs above were prepared by in vitro transcription followed by splicing in HeLa nuclear extracts. Splicing reactions were subjected to denaturing polyacrylamide electrophoresis with the precursor and products as depicted. *Activation of splicing by wild-type ESE1 is significantly greater than the other four constructs (p-value < 0.0001; n = six independent experiments).

only a single enhancer element even though splicing activation was expected to be less than robust. Nevertheless, we were able to detect splicing activity and therefore enhancer activity upon insertion of a single wild type ESE 1 (Figure 12b). In contrast, all three mutant ESE 1 constructs (*DSX*-ESE1-A, *DSX*-ESE1-T and *DSX*-ESE1-C) were unable to rescue splicing. This suggests that altering the first base of ESE 1 destroys its ability to function as an enhancer. Thus, the E3+1 G→A mutation causes aberrant skipping of exon 3 by both altering the 3' splice site and by disrupting ESE1.

RNAi in Lymphoblastoid Cell Lines

Transcripts lacking exon 3 possess a unique sequence at the boundary of exon 2 and exon 4 that is not present in any other spliced *GH-1* products. As a result, the dominant negative Δ3 transcripts present an ideal target for knock down by RNAi using siRNAs complementary to this unique sequence (Figure 13a). In chapter III we show that RNAi can be used successfully *in vivo* to rescue IGHD II phenotypes in a mouse model (Shariat et al., 2007). To see if we could reduce levels of the Δ3 transcript in heterozygous patient-derived cells, we electroporated LCLs with siRNAs directed against the unique exon 2-4 junction found only in transcripts encoding the 17.5-kDa isoform (siRNA-17.5). RT-PCR analysis showed specific knock down of mutant transcripts in three patient lines whereas an siRNA control (siRNA-GFP) had no effect (Figure 13b). We observed no apparent defects in the rates of cell growth or other obvious

phenotypes in the siRNA treated cell lines suggesting that off target effects are minimal (Jackson et al., 2006b). This is consistent with similar findings using RNAi to rescue a murine model of IGHD II supporting the idea that RNAi could be useful to treat IGHD II in humans (Shariat et al., 2007).

Discussion

The *GH-1* gene contains multiple weak splice sites and even in normal individuals, exhibits low levels of aberrant splicing resulting in the skipping of different exons (Procter et al., 1998). To maintain fidelity, multiple cis-acting regulatory enhancers have been identified (Ryther et al., 2004). The great majority of mutations in *GH-1* that cause IGHD II occur in and around exon 3 and cause skipping of this exon (Mullis, 2007). These mutations include splice site mutations as well as disruption of splicing enhancer elements that are necessary to promote constitutive splicing. In this study, we have identified and characterized a new mutation, E3+1 G→A, which causes IGHD II due to increased exon 3 skipping. Another GH-1 missense mutation, E32A, was recently characterized and also shown to increase exon 3 skipping due to disruption of ESE 1 due to an A→C transversion (Petkovic et al., 2007). Since this mutation occurs at the second nucleotide of exon 3, it would not be expected to affect the 3' splice site.

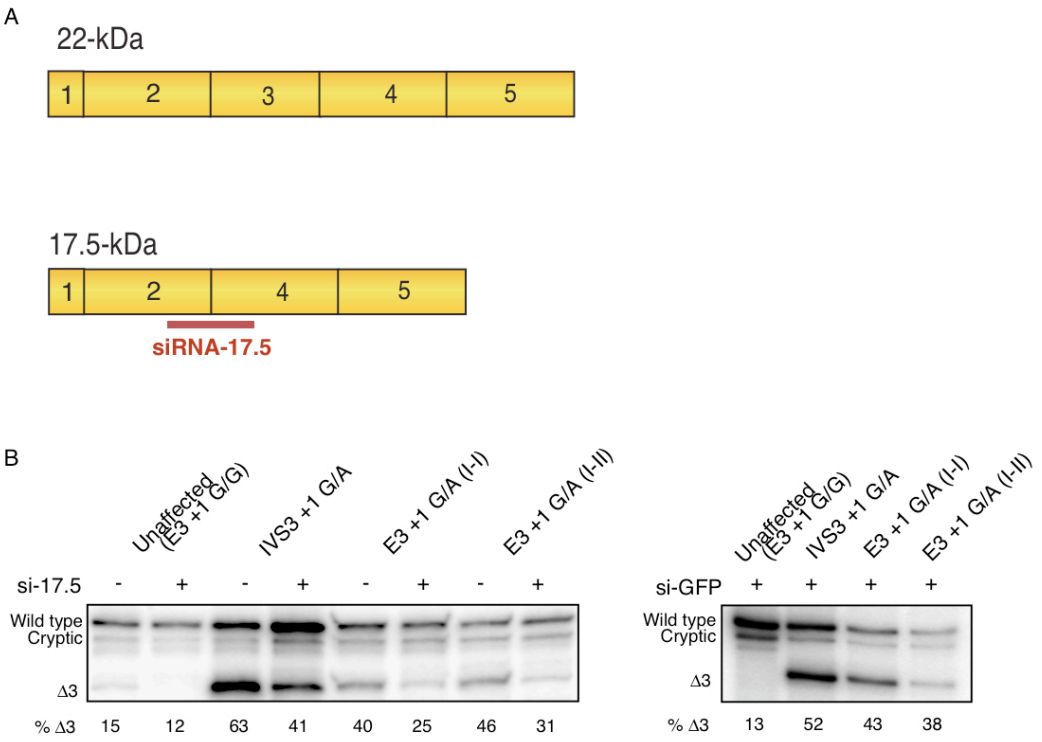

Figure 13. Specific targeting of Δ3 transcripts in patient lymphoblast cell lines by RNAi. (A) siRNA-17.5 is complimentary to the unique exon 2-exon 4 junction in Δ3 transcripts that enode the 17.5-kDa isoform. (B) Lymphoblastoid cell lines from affected and unaffected individuals as in Figures 10 and 11 were electroporated with siRNA-17.5, siRNA-GFP or mock electroporated. Total RNA was isolated and spliced products amplified using *GH-1*-specific primers. The percentages of Δ3 transcripts are shown below and represent three independent experiments.

Exon 3 Skipping and Disease Severity

The 17.5-kDa isoform represents 1–5% of wild type *GH-1* transcripts even in normal human pituitaries (Procter et al., 1998). Variability in the age of onset and severity of IGHD II among patients is thought to be due to increasing amounts of the 17.5-kDa isoform relative to the full length, 22-kDa protein (Hayashi et al., 1999b; McGuinness et al., 2003; Millar et al., 2003). Patients with IGHD II caused by mutations in either of the first two bases of intron 3 (IVS3 +1/+2) have a more severe phenotype with earlier onset than those with mutations within ESE1 due to increased production of the 17.5-kDa isoform. We show here that this difference in expression is observed in RNA obtained from patient derived LCLs (Figure 11b). IVS3 +1/+2 corresponds to the conserved GU dinucleotide at the 5' splice site that pairs with U1 snRNP binding in the initial stages of spliceosome assembly and exon definition. In this study, we show that patients with the E3+1 G→A mutation do not exhibit as extreme a phenotype as patients with mutations at IVS3 +1/+2, most likely due to reduced exon 3 skipping and therefore less production of the dominant negative 17.5-kDa isoform. These phenotypic differences may reflect a threshold and dose dependency of the amount of 17.5-kDa isoform above which pituitary defects are triggered (Mullis et al., 2005). In transgenic mouse models of IGHD II, lines expressing high copy numbers of human *GH-1* alleles containing mutations that result in exclusive exon 3 skipping have a more severe phenotype with much reduced growth and severe anterior pituitary hypoplasia than mice with lower copy numbers (McGuinness et al., 2003; Shariat et al., 2007).

ESE1 ensures proper exon 3 definition by activating the 3' splice site in intron 2 while concurrently preventing activation of the cryptic splice site in exon 3 (Ryther et al., 2003). For ESE1 mutations, the effects of the G→A and G→T mutations correlate with the severity of IGHD II. Both mutations cause an increase in Δ3 transcripts encoding the dominant negative 17.5-kDa isoform. The E3+1 G→T mutation produces a PTC that invokes NMD such that any transcripts containing the PTC are destroyed so the ratio of spliced products is biased toward Δ3 transcripts that lack PTCs. In contrast, readily detectable levels of completely spliced products are detectable with the E3+1 G→A mutation. Nevertheless, for both mutations and indeed for the future discovery of the E3+1 G→C mutation, disease severity will correlate with the levels of exon skipping.

Code Within a Code Within a Code

The experiments shown here provide an excellent illustration of the information contained within RNA. Prior to the realization of the multiple effects that mutations can have on RNA processing, it is likely that the effect of the E3+1 G→A mutation would be assumed solely due to the E32K missense mutation that is expected to accompany this change. However, since the mutation alters the first base of exon 3 and the first base of ESE 1, its effects, especially related to IGHD II, are due to aberrant splicing rather than production of a missense form of hGH. The position of the E3+1 G→A creates a change in the 3' splice site from the favored guanine residue. The initial stages of spliceosome assembly involve

the binding of the small subunit of U2 Auxiliary Factor (U2AF35) to the 3' splice site with a preference for G at the first base of the exon (Merendino et al., 1999; Wu et al., 1999; Zorio and Blumenthal, 1999). Even though all four bases can function as the first base of exons, the preference for guanine by U2AF35 weakens sites that contain other nucleotides. For *GH-1*, any such weakening is further compounded by the fact that ESE 1 begins with the first base of exon 3 so that the E3+1 G→A mutation creates double indemnity. For IGHD II, production of the dominant negative 17.5-kDa isoform is the key so whether the E32K missense mutation actually affects GH function is not known nor particularly relevant. However, for other mutations, should the change affect protein production, triple indemnity could ensue for a given mutation. A pertinent example is that of germline mutations in the human breast cancer susceptibility gene, *Brca-1*, which are responsible for approximately half of all familial hereditary breast cancer cases and have been shown to confer increased risk of ovarian, colon or prostate cancer (Nathanson et al., 2001). Some mutations in *Brca-1* that were initially annotated as missense mutations were later shown to cause aberrant splicing instead (Yang et al., 2003).

Potential Therapeutic Role of RNAi in IGHD II

Current treatment for IGHD II involves subcutaneous injections of recombinant GH (rGH) (Drake et al., 2001). While this enables patients to attain near normal stature, it does not replicate the normal, pulsatile pattern of GH secretion nor does it prevent anterior pituitary hypoplasia, which can lead to pan-

pituitary defects (McGuinness et al., 2003; Mullis et al., 2005; Romijn et al., 2003). In addition, there are side affects associated with rGH therapy (Monson, 2003). RNAi provides a potential, attractive therapeutic strategy to specifically target transcripts encoding the 17.5-kDa isoform for degradation (Ryther et al., 2004; Shariat et al., 2007). While we have shown that genetic delivery of shRNAs can rescue a mouse model of IGHD II, we have not attempted to use RNAi to target *GH-1* transcripts in human cells. Here, we have delivered siRNAs directly to patient-derived LCLs and shown specific decreases in $\Delta 3$ transcript levels. Fortunately, no observable phenotypic effects attributable to potential off target effects were observed suggesting that RNAi might be a viable therapeutic strategy for IGHD II.

Acknowledgments

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CHAPTER III

RESCUE OF PITUITARY FUNCTION IN A MOUSE MODEL OF ISOLATED GROWTH HORMONE DEFICIENCY TYPE II BY RNAI.

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Abstract

Splicing mutations in the human growth hormone (hGH) gene (*GH-1*) that cause skipping of exon 3 result in a form of GH deficiency termed Isolated Growth Hormone Deficiency type II (IGHD II) (Figure 9). The *GH-1* gene contains 5 exons; constitutive splicing produces the wild type 22-kDa hormone while skipping of exon 3 results in transcripts encoding a 17.5-kDa isoform that acts as a dominant negative to block secretion of the wild type hormone. Common characteristics of IGHD II include short stature due to impaired bone elongation growth and, in severe cases, anterior pituitary hypoplasia. Typically, IGHD II is treated by subcutaneous delivery of hGH which can rescue stature but, unfortunately, does not inhibit pituitary hypoplasia. Direct destruction of transcripts encoding the dominant negative 17.5-kDa isoform should both rescue stature and prevent hypoplasia. Here, we have used delivery of short hairpin RNAs (shRNAs) to rescue a murine model of IGHD II by specifically targeting transcripts encoding the 17.5-kDa isoform using RNA interference. To our knowledge, this is the first example where an shRNA has been expressed to specifically degrade an incorrectly spliced transcript and rescue a dominant negative disease phenotype in vivo.

Introduction

The great majority of mutations that lead to IGHD II occur in and around exon 3 and lead to aberrant splicing (Mullis, 2007). These mutations include splice site mutations as well as disruption of splicing enhancer elements that are

necessary to promote constitutive splicing (Figure 8) (Ryther et al., 2004). *GH-1* comprises five exons that are constitutively spliced to encode the wild type 22-kDa isoform. Transcripts lacking exon 3 encode a 17.5-kDa dominant negative isoform that prevents secretion of wild type protein from somatotrophs (Hayashi et al., 1999b; Lee et al., 2000a). Previously, we reported the creation of mouse lines expressing a human *GH-1* transgene containing a G to A transition at the 5' splice site of intron 3 (IVS3+1) that leads to exclusive production of the 17.5-kDa isoform (McGuinness et al., 2003). This transgene is expressed from a cosmid containing the entire *GH-1* locus control region including upstream DNA elements required for somatotroph-specific expression. The IVS3+1 mutant exerts a dominant negative effect on wild type mouse GH and generates a transgene dose-dependent IGHD II phenocopy. Even in the presence of two wild type mGH alleles, high copy transgenic mice exhibit IGHD II with concomitant reduced weight, severely reduced pituitary GH content, and progressive anterior pituitary hypoplasia (McGuinness et al., 2003; Ryther et al., 2003). Overproduction of the 17.5-kDa isoform triggers not only somatotroph death but also destruction of neighboring cells by macrophage invasion, leading to severe hypoplasia and additional anterior pituitary hormone deficiencies (McGuinness et al., 2003). The 17.5-kDa isoform arises because the normal exon 3 splice sites are relatively weak with accurate splicing requiring the presence of at least 3 splicing enhancer elements (Ryther et al., 2004). Even in the normal pituitary, a small number of *GH-1* transcripts (<1–3%) are incorrectly spliced and encode the 17.5-kDa isoform (Procter et al., 1998). Variability in the age of onset and

severity of IGHD II among patients is thought to be due to the amount of the 17.5-kDa isoform relative to the full length, 22-kDa protein (Hayashi et al., 1999b; McGuinness et al., 2003; Millar et al., 2003). These phenotypic differences may reflect a threshold and dose dependency of the amount of the 17.5-kDa isoform produced from mutated alleles, sufficient to cause somatotroph death and trigger pituitary defects (McGuinness et al., 2003). Currently, recombinant hGH is used in replacement therapy for IGHD II to overcome short stature but is relatively expensive, can lead to unwanted side effects (Monson, 2003) and, importantly, does not prevent anterior pituitary hypoplasia and other ensuing anterior pituitary deficits (Mullis et al., 2005). Since IGHD II arises as a direct consequence of excessive production of a specific dominant negative isoform, strategies designed to decrease levels of the 17.5-kDa isoform without affecting the normal 22-kDa product could be an effective form of therapy. Here we have used RNAi to specifically target the mutant *GH-1* transcript encoding the 17.5-kDa isoform in vivo and we show rescue of IGHD II in a mouse model.

Materials and Methods

Cell Culture

Wild type and Δ3 human GH cDNAs were cloned into pcDNA 3.1(+) (Invitrogen) as described (Deladoey et al., 2001) using identical primers. pSuper-sh17.5 was cloned as described (Ryther et al., 2003). Mouse AtT20/D16v-F2 cells (EACC, UK) were grown in DMEM with 10% FBS and 1%

penicillin/streptomycin with 4.5g/L glucose. Cells were transfected with hGH constructs and/or pSuper-sh17.5 using LT-1 (Mirus) according to the manufacturer's protocol.

RT-PCR

RNA was isolated 48 hours post transfection using TRI-reagent (Molecular Research Center). For analysis of human *GH-1* splicing patterns in AtT20 cells, RT-PCR was performed as described (Ryther et al., 2003) with the exception that M-MLV reverse transcriptase (Promega) was used. For analysis of pituitary RNA, 500ng total RNA was used for first strand cDNA synthesis with the following primer that recognizes both mouse and human GH sequences 5'-CGGGGGCTGCCATCTTCCAGC-3'. The same primer was used for PCR amplification with a 32P-labeled forward primer, 5'-GCCTGCTCTGCCTGCYCTGGC-3'. PCR products were separated on 6% denaturing polyacrylamide gels and visualized on a phosphorimager. Amplifying both products with the same primers allows accurate quantitation within the same lane obviating the need for a loading control. Results are thus shown as a ratio of the two products derived from a single lane.

To assay possible interferon responses, first strand cDNA was synthesized using oligo d(T) with 2μg of total pituitary RNA. Subsequent RT-PCR amplification was done using: *OAS-1*-forward 5'-CAGCCGTCAATGTCGTGTGTGATT-3' and *OAS-1*-reverse 5'-TGTTAAGGAACACCACCAGGTCAG-3'.

Western Blots

At-T20/D16v-F2 cells were lysed in 1x Laemmli loading buffer (LLB) 48 hours post transfection. Whole pituitaries were briefly sonicated in RIPA buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) and mixed with an equal amount of 2x LLB. All samples were denatured for five minutes at 95°C before loading onto 10% SDS gels. After transfer, PVDF membranes were blocked with 5% milk and incubated with primary antibodies against hGH or α-tubulin (Abcam). After incubation with HRP-conjugated secondary antibodies (GE Healthcare), proteins were visualized by ECL (Perkin Elmer Life Sciences).

Transgenic Mice

A *Not1-Xho1* restriction fragment of pSuper-17.5 (Figure 15) was purified and used for pronuclear injection of C57BL/6 embryos. Injections and oviduct transfers were performed by the Vanderbilt Transgenic Core Facility using standard techniques in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (VU-IACUC). Transgenic mice were verified by PCR from tail DNA using primers: 5'-GCTCTAGAACTAGTGGATCC-3' (forward) and 5'-CTAGAGTCTCTTGAACTCTAGG-3' (reverse). The sh-20 and sh-25 lines contain 3 and 7 copies of the transgene, respectively (data not shown). Transgenic mice were bred with wild type C57BL/6 mice. IGHD II mice were also bred with wild type C57BL/6 mice and transgenic animals were identified by

PCR, as described (McGuinness et al., 2003). Progeny resulting from crosses of shRNA-17.5 mice and IGHD II mice were identified by PCR, using the same primers as above. Mice were weighed once a week from week 4 – 16 after weaning. All mouse work was performed according to VU-IACUC guidelines and in accordance with VU IACUC protocol number M/05/075

Pituitary Dissections

Pituitaries were dissected from eight week-old mice, fixed in 4% paraformaldehyde in phosphate buffered saline. Pituitaries were visualized with a Leica MZ16F scope and QImaging Retiga EXi camera.

Immunohistochemistry and Imaging

20μm cryostat sections from pituitaries of 10 week-old mice were subjected to fluorescent immunohistochemical staining using goat anti-mouse GH antibodies and Cy-3 conjugated anti-goat antibodies (Santa Cruz Biotechnology). Pituitary sections were mounted in 50% glycerol and imaged with a Zeiss LSM510 Meta Laser Scanning microscope. Stacks were acquired with LSM510 software, and Z-projections and contrast adjustments were made with NIH ImageJ. See supplementary information for additional information concerning image acquisition.

Electron Microscopy

Pituitaries from 10 week-old mice were fixed overnight in 2% glutaraldehyde. Following washes in 1xPBS, pituitaries were transferred to 1% OsO_4 for 1 hour before washing with dH_2O. Preparations were then stained en bloc in 1% aqueous uranyl acetate and dehydrated in an ethanol series, passed through propylene oxide, transferred to a 1:1 Spurr:propylene oxide mixture, and removed and embedded in Spurr's embedding reagent. Ultra-thin serial sections (50-60nm) were obtained on a Leica UCT Ultracut microtome, transferred to formvar-coated grids, and examined on a CM10 Transmission Electron Microscope (FEI), operating at 80k and viewed on a Phillips CM10 TEM equipped with an AMT 2 mega pixel camera (AMT).

IGF-1 Serum Analysis

Serum was obtained by tail vein bleeding at weeks 8 and 16 post weaning. Mouse IGF-1 levels were determined using a mouse IGF-1 ELISA (Immunodiagnostic Systems Inc.).

Statistical Analysis

Statistical significance between mice was determined for data points from week 16 for all growth curves and for IGF-1 serum data using a one-way ANOVA followed by a Tukey-Kramer HSD analysis (JMP, version 5.01). Normal distribution for all data was determined by a Shapiro-Wilk's test. A summary of values used to determine statistical significance is listed in Tables 4-6.

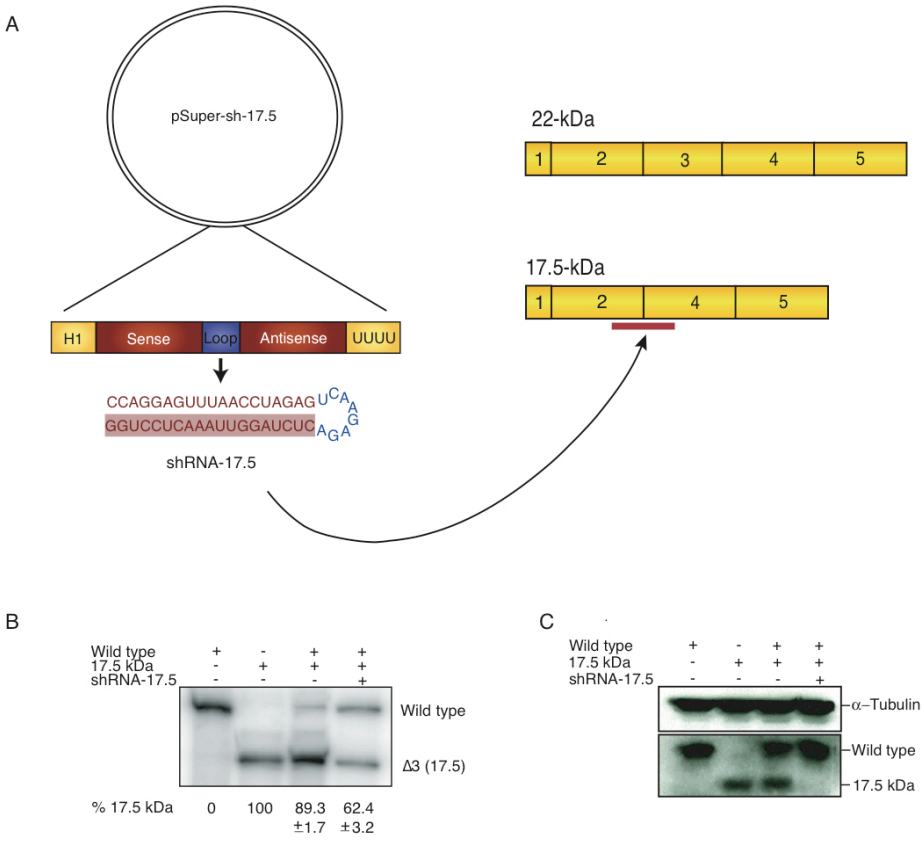

Figure 14. Allele Specific RNAi in AtT-20 cells. (A) An shRNA (shRNA-17.5) was designed complementary to the unique exon 2-exon 4 junction (red bar) and expressed from a pSuper vector. (B) AtT-20 cells were transfected with hGH cDNA constructs and pSuper-sh17.5, as indicated. RNA was isolated and the different isoforms amplified by RT-PCR using primers in exon 2 and exon 5. Bands corresponding to the wild type product (22 kDa isoform) and the exon 3 skipped product (Δ3; 17.5 kDa isoform) are as indicated. The percentage ± standard error of the Δ3 transcripts relative to wild type is shown below based on at least three independent experiments. Quantitation is based on the ratio of products within a single lane and was calculated by phosphorimager densitometry. (C) Western blots of cell lysates from the same transfections as in (B) were performed using antibodies against hGH or α-tubulin as a loading control.

Results

Allele Specific Targeting of Mutant *GH-1* Transcripts by RNAi

Skipping of exon 3 produces hGH transcripts containing a unique sequence at the junction of exon 2 and exon 4. We previously showed that shRNAs complementary to this unique sequence specifically target transcripts encoding the 17.5 kDa isoform (Δ3 transcripts) but do not alter wild type transcript levels (Ryther et al., 2004). However, we did not examine the effects of the shRNAs on protein levels. Therefore, we transfected At-T20 cells, a murine neuroendocrine cell line that does not produce endogenous growth hormone, with the vector encoding shRNA-17.5 (Figure 14) along with vectors expressing either wild type hGH or the IVS3+1 mutant that expresses only the 17.5 kDa isoform. Cells were harvested after 48 hours and analyzed for hGH protein and mRNA levels. As shown, the shRNAs effectively decreased the levels of Δ3 transcripts without affecting full-length transcripts (Figure 14b). Interestingly, despite the fact that the experiment shown in Figure 14 did not lead to complete loss of Δ3 transcripts, there was a total absence of the 17.5-kDa isoform (Figure 14c). In other experiments we do not always observe complete loss of the 17.5-kDa isoform. Despite slight experimental variability, we conclude that expression of shRNA-17.5 results in significant allele specific silencing of Δ3 transcripts and abrogation of the 17.5-kDa isoform.

Generation of shRNA-17.5 Transgenic Mice

To test if shRNA-17.5 targets the mutant *GH-1* allele in vivo, we generated mice expressing the shRNA by pronuclear microinjection of a 347 bp DNA restriction fragment of pSUPER-sh17.5 (Figure 15a,b). Ten independent lines of shRNA-positive mice were generated (Table 3). These mice show no differences in weight or lifespan as compared to wild type littermates, display no obvious phenotypes, and nine of the ten lines are fertile (Figure 15b). While overexpression of shRNAs can lead to toxicity and lethality due to oversaturation of the endogenous microRNA (miRNA) pathway or due to possible off target effects (Grimm et al., 2006; Jackson et al., 2006b), the presence of the shRNA-17.5 transgene did not induce any discernable effects in at least nine of the ten lines. Also, we did not observe any induction of interferon responses, at least as measured by levels of 2',5'-oligoadenylate synthetase (*OAS1*; Figure 15c), a key interferon responsive gene (Sledz et al., 2003).

Rescue of IGHD II *In Vivo* by RNAi

Mice expressing shRNA-17.5 were bred with the IGHD II mice to determine whether the IGHD II phenotype could be rescued in vivo (Figure 16a). Mice expressing both hGH-17.5-kDa and shRNA-17.5 showed rescue of the growth deficit compared to IGHD II littermates (Figure 16b, 17a). Of the ten founder shRNA-17.5 transgenic lines, five have thus far been crossed and all rescue growth (Table 3). Below, we more completely characterize two rescue lines. The shRNA line 20 (sh-20) completely rescued the growth deficit with no

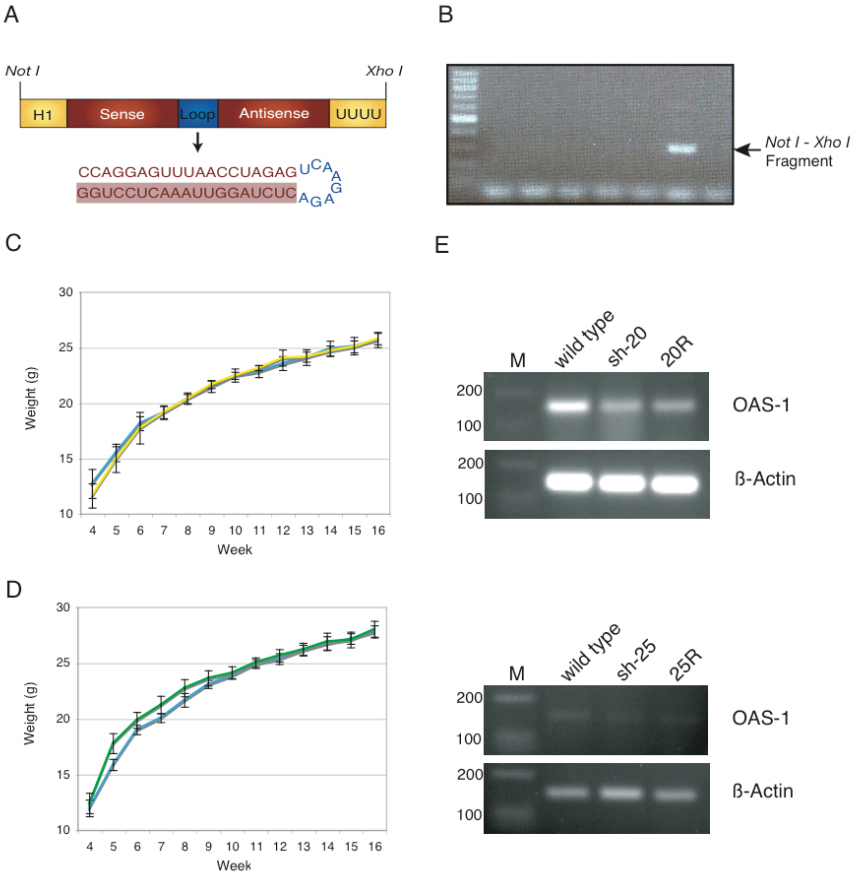

Figure 15. Generation of transgenic shRNA-17.5 mice. (A) A *Not I–Xho I* fragment of pSuper-sh17.5 containing an H1 promoter driving expression of shRNA-17.5 was used for pronuclear injections to generate transgenic mice. The guide strand is highlighted. (B) Transgenic shRNA-17.5 mice were identified by PCR. (C),(D) Growth curves of transgenic sh-20 (C) and sh-25 (D) mice. Weights of transgenic mice (sh-20 line (n=4), yellow; sh-25 line (n=6), green) and wild type littermates (n=5 for each graph) (blue) were determined between 4-16 weeks of age. Error bars are ±s.e.m. From ANOVA, p>0.7 for both data sets. Full statistical analyses are in Table 4. (E) Expression of shRNA17.5 does not elicit an interferon response. RNA was isolated from pituitaries of the indicated genotypes and RT-PCR was performed using primers against the interferon induced gene, 2', 5'-oligoadenylate synthetase (*OAS 1*) (left) and β-actin as a control (right). sh-20 mice are shown in the top panel and sh-25 in the lower panel.

Table 3. Generation of transgenic shRNA-17.5 mice. Two sets (a, b) of pronuclear injections were performed to generate shRNA-17.5 transgenic founders. Mice in bold have been shown to rescue a mouse model of IGHD II. shRNA mice 25a and 20b were selected for further analysis and were subsequently referred to as sh-25 and sh-20 respectively.

Mouse Number	shRNA transgenic	Crossed to IGHD II	Rescue
1a-20a	-		
21a	+	No	-
22a, 23a	-		
24a	**+**	**Yes**	**Yes**
25a	**+**	**Yes**	**Yes**
26a-28a	-		
1b-5b	-		
6b	**+**	**Yes**	**Yes**
7b, 8b	-		
9b	+ (infertile)		
10b-18b	-		
19b	+	No	-
20b	**+**	**Yes**	**Yes**
21b-25b	-		
26b	+	No	-
27b	**+**	**Yes**	**Yes**
28b	-		
29b	+	No	-
30b	-		

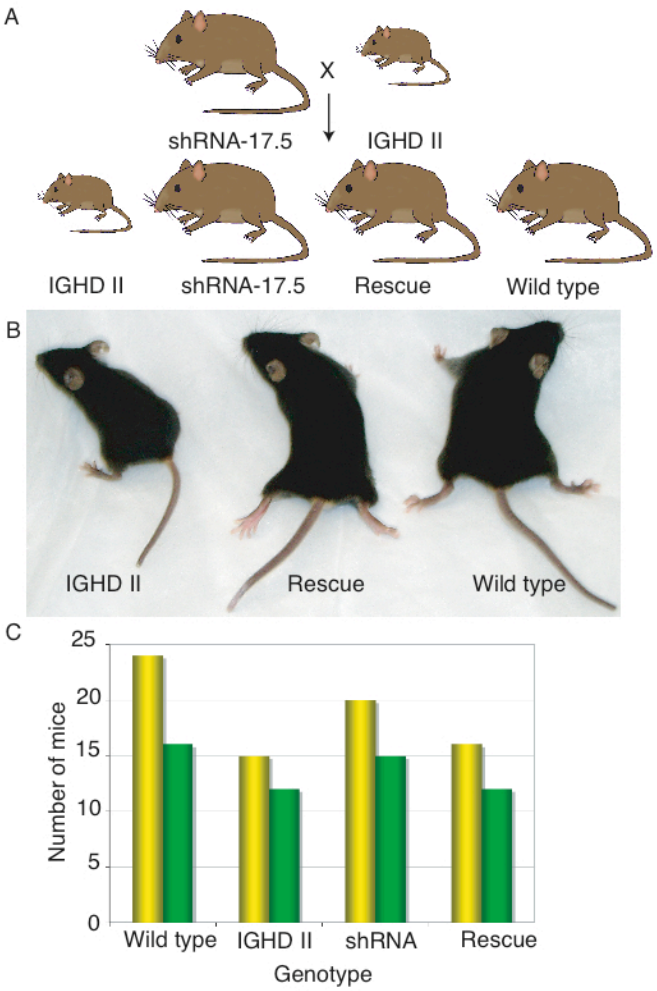

Figure 16. Mating Strategy. (A) Expected offspring from matings between IGHD II and shRNA-17.5 mice. (B) Five week-old IGHD II, rescue (20R), and wild type littermates. (C) Genetic assortment of progeny from matings of sh-20 (yellow) or sh-25 (green) with IGHD II mice.

difference in weight compared to wild type littermates. The shRNA line 25 (sh-25) significantly improved weight-gain compared to IGHD II mice but did not fully attain normal levels (Figure 17a). Both lines had lower weights at weaning but with time these increased as to be significantly greater than the IGHD II mice. For both lines, weight rescue was observed in both male and female progeny and was obvious at weaning (particularly in the sh-20 rescue line), as would be expected since this is when growth deficits become manifest in rodents. The genotypes assorted close to the predicted Mendelian ratios among the progeny for both lines (Figure 16a,c).

We next analyzed pituitary morphology and function for the sh-20 and sh-25 rescue lines (20R; 25R). Pituitary dissections revealed that the severe anterior pituitary hypoplasia observed in the IGHD II mice was not detected in either the 20R or 25R lines (Figure 17b). Pituitaries shown in Figure 17 are from 8 week old mice but the increase in pituitary size was observable at weaning (3 weeks; data not shown). Interestingly, considering the degree of weight rescue by the different lines, the anterior pituitaries from the 20R mice were smaller than the 25R pituitaries and remained so past 6 months in age. No significant differences in anterior pituitary size were observed between wild type mice and the sh-20 and sh-25 transgenic lines. As expected, the posterior pituitary was similar in size for all mice. Since the IGHD II model mice exhibit pronounced somatotroph loss, we next sectioned pituitaries and performed fluorescent immunohistochemistry using an antibody that specifically recognizes mouse GH (mGH). As shown in Figure 17c, the IGHD II mice had a severe decrease in

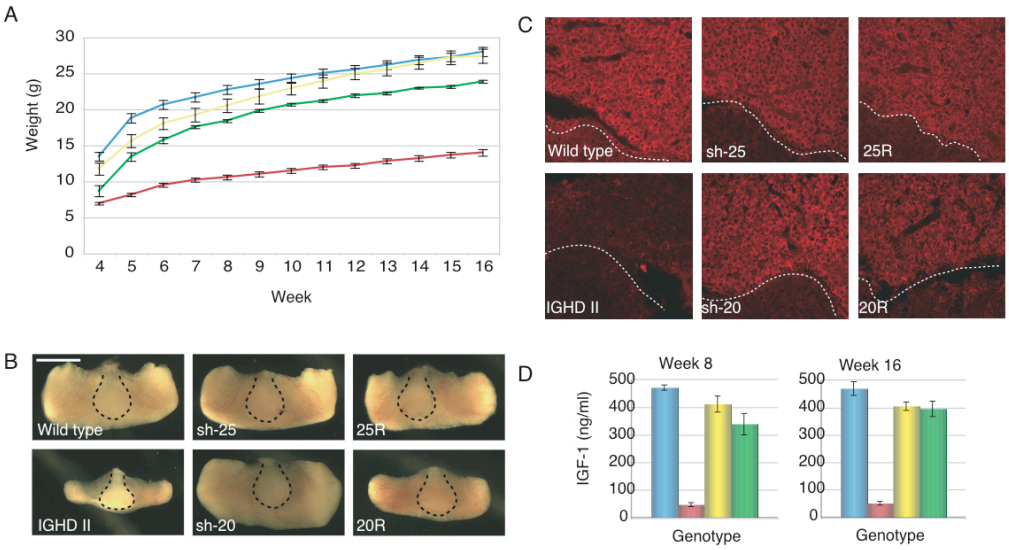

Figure 17. Rescue of IGHD II mice. (A) Growth curves of wild type (blue, n=10), IGHD II (red, n=8), 20R (yellow, n=6), and 25R (green, n=4) mice. (B) Pituitary dissections from eight week-old mice. The posterior pituitary is indicated within the dashed line. Scale bar at the upper left indicates 1mm. (C) Fluorescent immunohistochemistry staining with anti-mGH on 20μm pituitary sections taken from 10 week old mice. The posterior pituitary is indicated below the dashed line. (D) Serum IGF-1 levels were measured by ELISA from 8 and 16 week-old mice (n=3 for each genotype). Wild type, blue; IGHD II, red; 20R, yellow; 25R, green. Error bars are ±s.e.m. From ANOVA for A. and D. $p<0.0001$. For complete statistical analyses, see Tables 5 and 6.

Tables 4-6. Statistical analysis. Summary of 95% confidence intervals, comparison of both pairs by Tukey-Kramer HSD and Shapiro-Wilk's goodness of fit test for normal distribution of the values a week 16 in Figure 15c,d (Table 4),for values at week 16 in Figure 17a (Table 5) and for weeks 8 (top panel) and 16 (lower panel) for data shown in Figure 17d (Table 6). For all tables, groups with different letters are significantly different. (α = 0.001)

Genotype	95% Confidence interval (g)	Tukey-Kramer HSD	Shapiro-Wilk's Test	
			W	p value
Wild type	25.70±2.24	a	0.945401	0.7121
shRNA-20	25.78±1.63	a	0.802426	0.1029
Wild type	27.80±1.49	a	0.945633	0.7137
shRNA-25	28.04±1.84	a	0.841669	0.1257

Table 4.

Genotype	95% Confidence interval (g)	Tukey-Kramer HSD	Shapiro-Wilk's Test	
			W	p value
Wild type	27.96±1.5	a	0.951720	0.7304
IGHD II	13.96±0.99	b	0.984021	0.9806
20 Rescue	27.38±2.50	a	0.995774	0.9986
25 Rescue	23.83±0.65	a	0.840310	0.1905

Table 5.

Genotype	95% Confidence interval (ng/ml)	Tukey-Kramer HSD	Shapiro-Wilk's Test	
			W	p value
Wild type	471.8±49.1	a	0.932145	0.4967
IGHD II	45.6±36.0	b	0.947342	0.5578
20 Rescue	412.0±127.0	a	0.804221	0.1246
25 Rescue	338.9±167.0	a	0.905374	0.4028
Wild type	470.0±138.4	a	0.979934	0.7285
IGHD II	50.0±34.1	b	0.930937	0.4921
20 Rescue	405.4±65.6	a	0.968142	0.6573
25 Rescue	396.0±121.8	a	0.866832	0.2866

Table 6.

mGH production, consistent with dominant negative effects exerted by the IVS3+1 mutant. In contrast, the sh-20, sh-25, 20R and 25R lines all expressed mGH at levels indistinguishable from wild type. Quantitative measurement of serum GH in single samples is uninformative due to the pulsatile nature of GH secretion. However, long-term restoration of GH output should correct the low insulin-like growth factor 1 (IGF-1) levels observed in IGHD II. IGF-1 is secreted in the liver in response to GH stimulation and low serum IGF-1 levels indicate GH deficiency (Clemmons, 2007). Accordingly, serum IGF-1 levels were measured in groups of mice at 8 and 16 weeks of age. As shown in Figure 17d, both rescue lines showed IGF-1 levels that were similar to wild type levels and significantly higher than the IGHD II mice. At week 8, the IGF-1 serum levels in the 25R mice were slightly lower than the 20R and wild type mice but by week 16 there were no significant differences in IGF-1 levels between the three genotypes. These results are concordant with the weight trends where the 25R line rescues the IGHD II phenotype slightly less than the 20R line. Interestingly, although the 20R mice have slightly greater weights and initially higher serum IGF-1 levels than the 25R mice, the 20R pituitaries are consistently smaller than the 25R pituitaries.

Morphological Rescue of IGHD II

Growth hormone is packaged into granules, forming dense-core secretory vesicles (DCSVs). The specific mechanism responsible for the dominant negative nature of the 17.5-kDa isoform is not certain but may be due to

formation of 17.5:22-kDa dimers and oligomers which disrupt granule packaging thus preventing the secretion of either isoform from somatotrophs, ultimately triggering an unfolded protein response (McGuinness et al., 2003). These complexes apparently overwhelm the degradative capacity of the proteosome, leading to the accumulation of toxic aggregates in the cytosol, ER and Golgi apparatus. Consistent with this model, electron micrographs of anterior pituitary sections from IGHD II mice show Golgi and ER defects, loss of DCSVs and dramatically increased levels of intracellular lipid and vacuolation (McGuinness et al., 2003) (Figure 18a,b). Wild type cells show a large accumulation of mature DCSVs that are stored and released upon appropriate hypothalamic stimulation. As is evident from the electron micrographs, secretory cells from the rescue mice have abundant DCSVs, comparable to wild type, with normal morphology (Figure 18a,b). Thus, our data from gross morphology to electron micrographs indicate that genetic delivery of shRNAs against transcripts encoding the dominant negative 17.5-kDa isoform is able to rescue growth and somatotroph function in IGHD II mice.

Silencing of the 17.5 kDa Isoform

Interestingly, despite the fact that we observed rescue of IGHD II with several transgenic shRNA-17.5 lines (Table 3), we have not been able to directly detect expression of the shRNAs. We assume this is due to expression at levels below our current limit of detection and/or instability of the shRNAs. Indeed, such low levels may preclude lethality and/or toxicity as reported by Grimm *et al*.

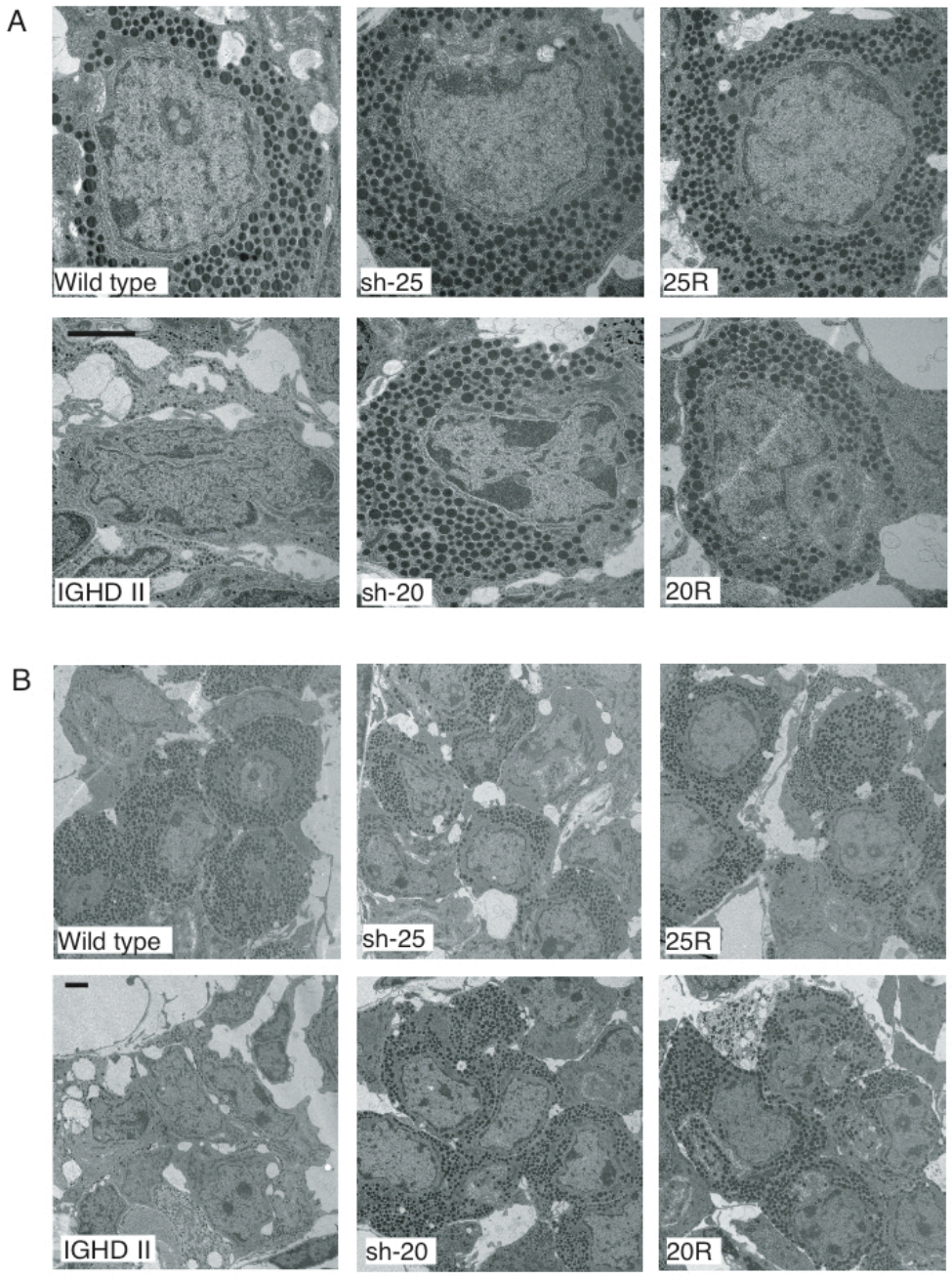

Figure 18. Morphological rescue of IGHD II. Electron micrographs of the anterior pituitaries from 10 week-old mice of the indicated genotypes. Scale bar is 2μm; magnification is (A) 10,500x and (B) 3,400x.

(Grimm et al., 2006). Nevertheless, we were able to show that the shRNAs are directly altering the levels of the 17.5-kDa isoform by examining pituitary GH RNA and protein levels in the rescue mice. First, using primers that are complementary to both human and mouse GH transcripts, we performed RT-PCR analysis to determine the relative levels of wild type, full length mGH mRNA compared to the levels of the Δ3 transcripts encoding the hGH 17.5-kDa isoform. As shown in Figure 19a, there was a dramatic change in the ratio of the two transcripts between the IGHD II mice and the rescue mice, consistent with the notion that rescue involves reducing levels of the Δ3 transcripts. Bearing in mind that IGHD II mice have severely hypoplastic anterior pituitaries, western blots using antibodies that recognize both mouse and human GH showed that what little GH was produced was mainly the 17.5-kDa isoform (Figure 19b). For the rescue mice, even though significant levels of the 17.5-kDa isoform were detected, the dramatic increase in full length, wild type mGH was apparently able to overcome any dominant negative effects exerted by the mutant, thereby rescuing growth and pituitary function (Figure 19b,c). Thus, our biochemical and genetic results are completely consistent with functional reduction of the 17.5-kDa hGH isoform to rescue the dominant negative phenotype.

Discussion

RNA interference has many potential advantages over traditional therapies, including increased specificity and versatility (Dykxhoorn et al., 2006; Kim and Rossi, 2007). Many recent advances in RNAi therapeutics have been

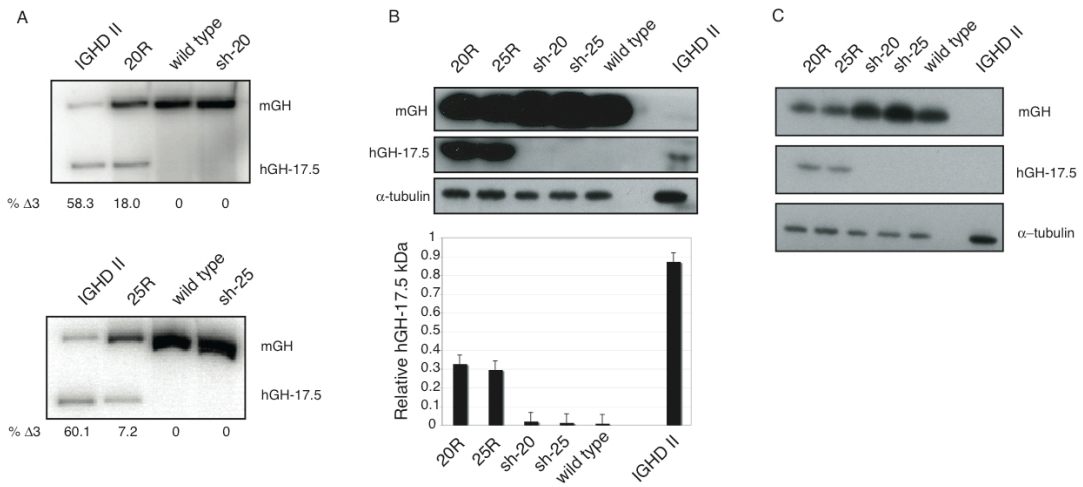

Figure 19. Silencing of the 17.5-kDa isoform. (A) Total RNA was isolated from pituitaries of the indicated genotypes and RT-PCR was performed with identical primers that distinguish between mouse and human GH. Bands corresponding to the full length spliced product from the mouse alleles (mGH) and the 17.5 kDa human transgene (hGH-17.5) are as indicated. Quantitation of the two transcripts is based on the ratio of products within a single lane and was calculated by phosphorimager densitometry. (B) Western blots of pituitary lysates from the same genotypes as in (A) were performed with an antibody that recognizes both human and mouse GH. The two upper panels were deliberately overexposed to allow visualization of the GH isoforms in IGHD II. (C) A shorter exposure (2 seconds) of the same gel shown in (B) (15 seconds).

used to successfully knock down viral genes or disease alleles though, to our knowledge, none have been used to knock out a dominant negative disease allele in an animal model. These approaches have used either small interfering RNAs (siRNAs) or shRNAs to target relatively accessible tissues such as sub-retinal injection into the eye (Reich et al., 2003), intranasal delivery to the lung (Bitko et al., 2005; Zhang et al., 2005), transfection via lipofectamine complexes to the vaginal epithelium (Palliser et al., 2006), tail vein injection to the liver (Morrissey et al., 2005b; Soutschek et al., 2004), viral delivery to the liver (Grimm and Kay, 2006) and viral delivery via intraspinal or intracranial injections (Ralph et al., 2005; Raoul et al., 2005; Xia et al., 2004). Zimmermann *et al*. used systemic delivery of siRNAs in primates targeting ApoB in the liver by saphenous vein injection (Zimmermann et al., 2006). By comparison, although it is not protected by the blood-brain barrier, the pituitary is relatively inaccessible to direct targeting due to its location. We therefore decided to deliver shRNA-17.5 via genetic means by making transgenic mice expressing the shRNAs. Using this approach, we were able to specifically reduce expression of the dominant negative 17.5-kDa hGH isoform allowing recovery of wild type GH levels to rescue an autosomal dominant mouse model of human IGHD II.

A potential caveat to our results concerns the inability to directly detect expression of shRNA-17.5 precursors or mature siRNAs. This could be used to argue that the effects we observe are indirect. However, several lines of evidence argue against such a conclusion. First, we have shown genetically that when IGHD II mice are crossed with shRNA mice, only the progeny that contain

both the shRNA transgene and the Δ3 transgene exhibit the rescue phenotype. From mating and genotyping over 400 mice, we only observed rescue in the double transgenics. Also, the extent of the rescue varied between the different shRNA lines; the 25R lines exhibit slightly lower growth rates and initial IGF-1 serum levels compared to the 20R lines (Figure 17 a,d and Table 3). Second, the transgenic shRNA lines show no overt phenotype that could be responsible for or contribute to the rescue phenotype observed in the IGHD II background. Third, the genotypes assort in the expected Mendelian ratios (Figure 16c). Thus, the genetics argue strongly against any indirect effect. Biochemically, we also showed that in the IGHD II mice the predominant isoform is the dominant negative hGH 17.5-kDa protein whereas in the rescue mice there is a switch and the major protein detected is the wild type mGH (Figure 19b). Together, these data support our conclusion that the rescue of IGHD II is a direct effect of shRNA-17.5.

Future work will be directed toward a viable therapeutic strategy using exogenous delivery of siRNAs but the results from this report show that employing RNAi provides a promising approach to treat IGHD II in humans. While GH treatment can counteract GH deficits in children and adults with Growth Hormone Deficiency (GHD), the underlying somatotroph destruction continues with additional bystander effects that can evolve to damage other pituitary hormone axes in some individuals (Salemi et al., 2005). A particularly encouraging feature of our results is the restoration of macroscopically and microscopically normal somatotroph populations in the rescue mice without any

overt phenotype in other cells. Since normalization of somatotroph function offers a selective advantage in survival and replacement during somatotroph turnover, this promotes complete functional recovery without necessarily achieving complete suppression of the aberrant allele in every cell. More generally, many human diseases result from aberrant splicing and/or dominant negative isoforms and our results show that RNAi offers a promising way to specifically degrade mutant alleles while sparing wild type, functional alleles.

Acknowledgements

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Significance

The research presented in this thesis demonstrates that RNAi can be used to effectively treat IGHD II. Importantly, RNAi treatment targets the underlying cause of the disease, overexpression of the 17.5-kDa hGH isoform. As such, this is an attractive alternative to the current treatment of subcutaneous recombinant GH injections, which does not prevent future hormonal deficiencies that can occur as a result of severe anterior pitiuitary hypoplasia. Prior to this thesis, it was demonstrated that shRNA-17.5 could be used to degrade $\Delta 3$ transcripts in tissue culture (Ryther et al., 2004). Here, as a primary step in developing shRNA-17.5 as a viable therapeutic for patients with IGHD II, we examined its ability to rescue IGHD II symptoms in a model system. To accomplish this, we used a transgenic mouse model of IGHD II where a human *GH-1* mutant allele that exclusively produces the 17.5-kDa isoform is expressed. Though expressed in concert with two copies of wild type mGH, high copy numbers of the 17.5-kDa mutant cause severe IGHD II symptoms (McGuinness et al., 2003). We made many lines of transgenic mice expressing shRNA-17.5 and bred these mice with IGHD II mice. Progeny with shRNA-17.5 in an IGHD II background exhibited rescued phenotypes that were similar to wild type phenotypes. These phenotypes include growth rates, GH expression in the

anterior pituitary and levels of serum IGF-1 (Figure 17). Rescue mice also showed normal gross and cellular morphology as determined by pituitary dissections and EMs of pituitary sections, respectively (Figure 17b, 18).

Many experimental questions abound in relation to delivery of siRNA-17.5 to the pituitary. These include time of delivery, quantity of siRNAs to administer and the number of repeated deliveries that will be required, in addition to addressing the issue of specific delivery to the pituitary. Now that we have successfully shown functional rescue of IGHD II using RNAi, we are poised to address these points and develop siRNA-17.5 as a therapeutic.

IGHD II is caused by several different *GH-1* mutations, mostly in and around exon 3 that cause exon skipping. One consequence of using siRNA-17.5 to degrade $\Delta 3$ transcripts is that it targets an aberrantly spliced isoform which can occur as a result of several different mutations, including the new mutation described in this thesis. Since the abnormally spliced product caused by the mutation is being targeted, rather than the mutation itself, siRNA-17.5 could be used to treat all cases of IGHD II caused by exon 3 skipping and over production of the dominant negative 17.5-kDa isoform, providing a wider platform for therapeutic uses.

As mentioned above, many mutations in and around exon 3 cause exon skipping. In addition to showing rescue of IGHD II using RNAi, we have also identified and characterized a new mutation in the *GH-1* gene in a family with autosomal dominant GHD. Sequencing of patient DNA identified a G→A transition at the first base of exon 3 (Figure 10). The first base of exon 3

corresponds to the 3' splice site, AG│G as well as the first base of ESE 1 and our analysis shows that this transition mediates its effects by affecting both of these elements. By disrupting ESE 1 and by weakening the 3' splice site consensus sequence, this mutation causes exon 3 skipping, resulting in an IGHD II phenotype. In elucidating the mechanisms by which this mutation functions we have further emphasized the importance of splicing fidelity and the importance of multiple splicing signals in this process.

Impact and Future Directions

Phenotypic Characterization of Rescue Mice

Pituitary Hormone Content

There is a profound loss of pituitary GH content in the mouse model of IGHD II containing high copy numbers of the Δ3-hGH transgene. Dramatic reductions in levels of PRL and TSH are also observed in these mice and transgenic males also show a loss of LH though females do not (McGuinness et al., 2003). In all cases, the loss of individual hormones is exacerbated with age. From an endocrine perspective, it will be interesting in the future to fully characterize the rescue mice. Preliminary results from five-month old mice show a significant increase in pituitary GH content in both the 20R and 25R mice compared to the IGHD II mice (Figure 20). Interestingly, the amount of GH in the rescue mice is approximately a sixth of that in wild type mice or in either of the

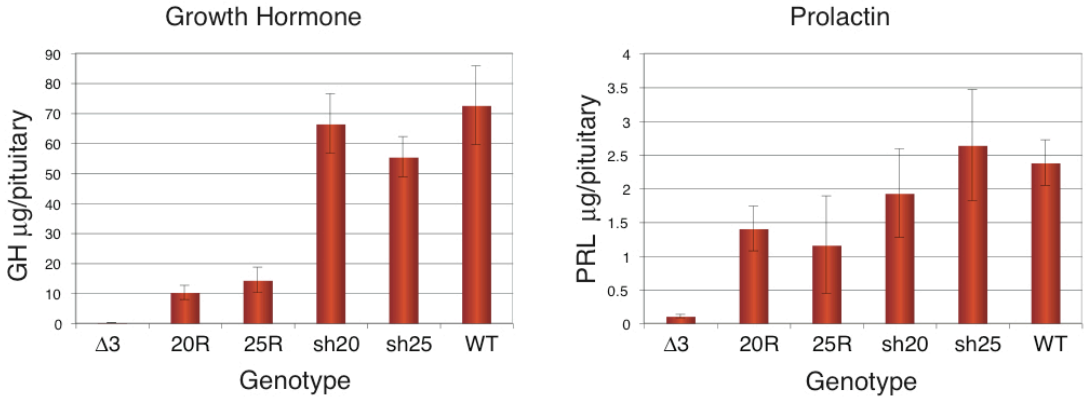

Figure 20. Analysis of pituitary hormone contents. Adult mouse pituitaries were sonicated in cold PBS and hormone contents were assayed by RIA. GH, left; PRL, right. n=3 for each genotype

two transgenic shRNA (sh-20 and sh-25) lines. This is most likely because the rescue mice are producing just enough GH to maintain normal growth such that as soon as GH is produced, it is secreted, hence the lower pituitary GH content. The Δ3 transgene contains multiple Pit-1 promoter sites and any physiological drive to increase mGH expression should also increase expression of the transgene, though it will be subsequently degraded by shRNA-17.5 in the rescue mice. Conversely, the sh-20, sh-25 and wild type mice are able to produce and store ample amounts of GH. The amount of pituitary PRL is extremely reduced in the IGHD II mice compared to wild type mice (Figure 20). Our preliminary data do not suggest any significant difference in the levels of PRL in the rescue mice compared to control mice. Apparently, although the rescue mice are not producing enough normal mGH (due to the expression of the 17.5-kDa isoform) to store abundant quantities like their wild-type counterparts, they are producing enough to prevent additional anterior pituitary hormone losses, specifically PRL. In addition to repeating this experiment, it will also be interesting to investigate the pituitary levels of TSH and LH, which have been shown to be deficient in the IGHD II model.

GH is released in pulsatile bursts and as such it is impossible to accurately quantify GH serum levels unless a stimulus is administered. This procedure is routinely done in patients with suspected GHD and agonists include chlonidine and arginine (Table 2). Subsequent serum analysis should show a peak GH level in response to the agonist. Future work to analyze GH levels in the rescue mice should use similar provocation tests in mice, taking serum

samples before and after a large dose of GHRH which causes GH release into the blood, the peak height of which is roughly proportional to the pituitary reserve (Le Tissier et al., 2005). The advantage is that this can be done in anaesthetized mice and would be a reliable serum GH assay to complement the serum IGF-1 data shown in figure 17d.

Effects on Bone Length and Mass

GH and IGF-1 have well recognized effects on bone elongation during development and the large increase in bone mass that occurs during childhood and puberty via endochondrial bone formation is stimulated by these factors (Ohlsson et al., 1998). Patients with GHD often exhibit decreased bone mineral density though the effects are significantly different depending on whether or not patients had childhood GHD or adult-onset GHD (Kaufman et al., 1992; Maheshwari et al., 2003). Mean BMD increases during long-term GH replacement therapy and eventually reaches normal levels (Saggese et al., 1996). Since GH has such effects on longitudinal growth and bone mass, it will be interesting to assay any such effects in the IGHD II mice and determine if these effects are reduced or absent in the rescue mice.

Developing siRNA-17.5 as a Therapeutic

There are at least two main considerations in developing a viable siRNA-based therapeutic to specifically target $\Delta 3$ transcripts. The first involves determining the appropriate time to deliver siRNAs in relation to the onset of

IGHD II such that pituitary damage has not progressed so far as to prevent recovery. The second consideration is to optimize specific delivery to the pituitary, which will likely prove a major obstacle in developing siRNA-17.5 therapy, indeed all RNAi based strategies.

Time of Delivery

To understand the developmental timing of pituitary damage in IGHD II mice, we sectioned pituitary tissue from newborn pups. Electron micrographs showed morphological differences in IGHD II mice as young as one day compared to wild type mice (Figure 21). Between the two, a distinct difference in the appearance of the ER was detected. Somatotrophs from the mutant mice showed a distinct lack of ribosomes in the cytosol and an abundance of protein within the ER lumen (Figure 21a,b). In contrast, the ER lumens from wild-type pituitaries were clear with readily detectable ribosomes in the cytosol. An additional difference was the location of the DCSVs. In somatotrophs from wild-type mice, they appeared dense and were located throughout the cytosol whereas DCSVs in somatotrophs from IGHD II mice seemed to congregate toward the periphery of the cell, adjacent to the plasma membrane, were less dense, and had an ovoid appearance (Figure 21c). Autophagic vesicles are also present in the mutant pituitaries and absent in wild-type samples. Although there are phenotypic differences in wild-type and IGHD II mouse anterior pituitaries even at a young age (1 day), it is promising that the extreme vacuolation and gross abnormalities caused by somatotroph loss that are visible in eight-week old

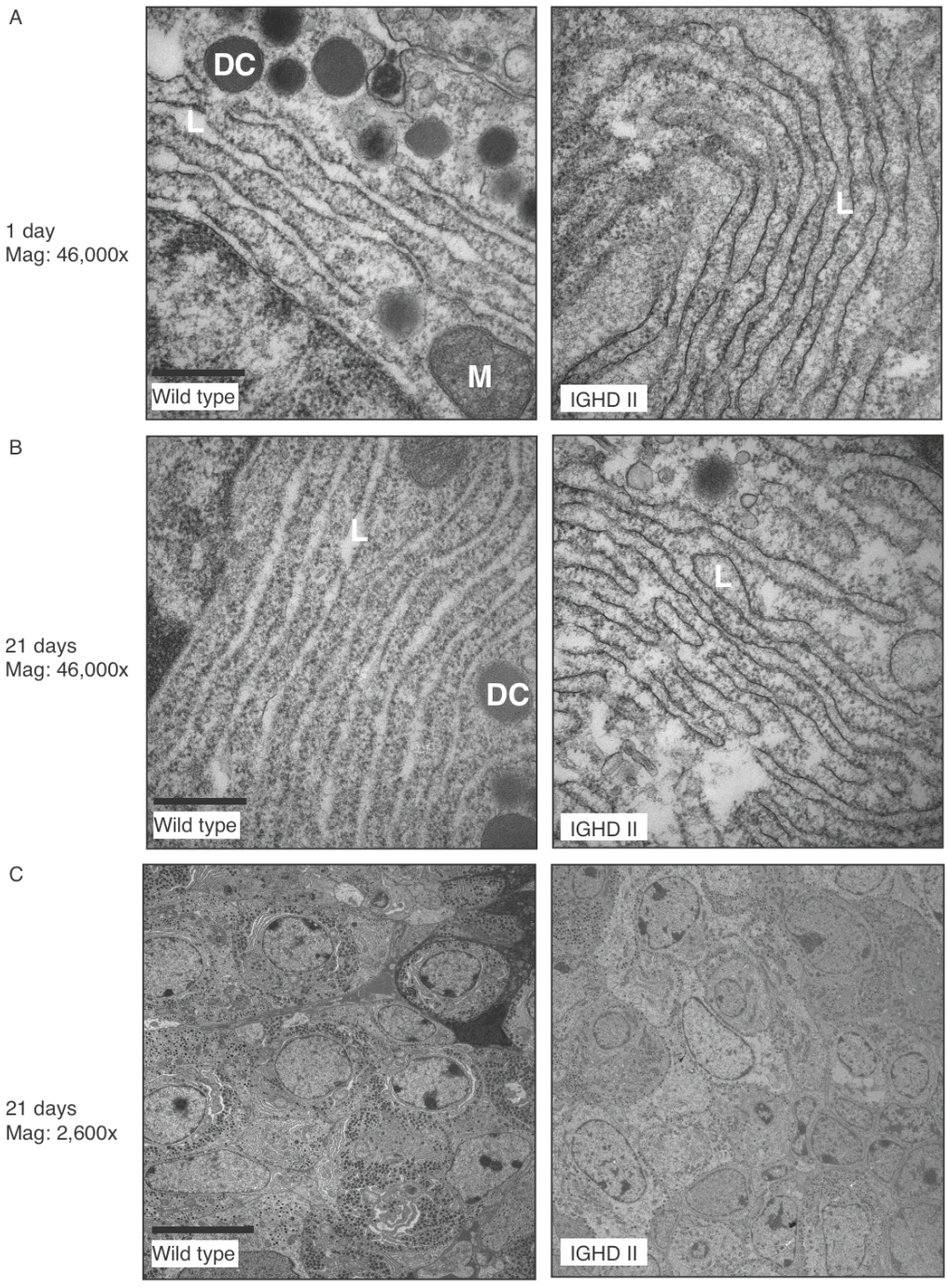

Figure 21. IGHD II mice exhibit ER defects at a young age. (A) Electron micrographs of pituitary sections from one-day old mice and (B) 21-day old mice. (C) Lower magnification of the same tissue shown in B. ER lumen, L; DCSV, DC; mitochondria, M. Scale bar in (A) and (B) is 500nm and in (C) is 10μm.

mice do not appear to manifest themselves by three weeks, the time at which mice are usually weaned. These observations suggest that it could be possible to treat IGHD II model mice with siRNAs at weaning. Mice that are handled at younger ages can often be rejected by the mother so it would be beneficial to start siRNA delivery at weaning.

In the IGHD II mouse model, there is a marked increase in GHRH expression and concurrent decrease in somatostatin expression, due to the feedback loop that regulates GH secretion (McGuinness et al., 2003). It is postulated that early treatment of IGHD II with exogenous GH replacement therapy may be important in rescuing a degree of pituitary function by providing a feedback signal to reduce the GHRH drive, thus reducing somatotroph proliferation and rate of self-destruction (McGuinness et al., 2003). In severe cases of IGHD II, such as our mouse model, the somatotroph damage leads to loss of other endocrine cells in the pituitary and their hormone production. Bearing these two aspects in mind, it would be advantageous to treat IGHD II mice with siRNA-17.5 as early as possible.

Delivery of siRNAs to the Pituitary

The pituitary is situated at the base of the brain, in the *pituitary fossa*, but is not protected by the blood brain barrier (BBB), which is only permeable to lipophilic molecules of less than 400 Da (Pardridge, 2002). This is therefore an advantage from the perspective of delivery of therapeutic molecules. However, some of the methods developed to transport nucleic acids across the BBB could

be incorporated into designing siRNAs that target the pituitary. One approach involves the use of molecular 'Trojan horses,' liposome complexes comprised of polyethylene glycol (PEG) where the nucleic acid is encapsulated within the liposome interior and thus insulated from nuclease degradation (Boado, 2007). In an elegant study targeting siRNAs to the central nervous system, ~1-2% of the PEG molecules of the nanoparticle were engineered with two different types of monoclonal antibodies. These antibodies trigger receptor-mediated endocytosis across the BBB and also target a specific cell type (Zhang et al., 2004). We could adapt this method by designing liposome complexes that contain a protein or peptide fragment that recognizes a somototroph-specific cell marker. An ideal candidate for this could involve exploiting the interaction between GHRH and its receptor. This receptor is expressed on the surface of somatotrophs and would be an attractive target in designing a selective delivery system for siRNA-17.5. We recently isolated and cultured primary mouse somatotrophs and though they were only viable for approximately five days in culture, they could be a valuable tool in designing and determining the efficacy of GHRH-conjugated siRNA-17.5 nanoparticles. For example, by supplying exogenous GHRH to the culture media, we would expect to see an up-regulation of GH, which could be quantified by assaying *GH-1* transcripts or levels of GH secreted into the media. This could be used to identify an optimum peptide fragment of GHRH that would engage with the receptor. Of note, although the GHRH receptor is mostly expressed on somatotrophs, it is also minimally expressed in tissues that exhibit autocrine GH secretion, for example breast tissue (Mukhina et al., 2004). An obvious benefit of

providing cell-specific targeting, aside from the fact that it should drastically decrease the amount of siRNA required, is that it could potentially prevent unwanted and unforeseen off target effects that may occur in other cell types.

Initial testing of delivery methods could use fluorescently labeled siRNAs such that delivery and stability can be assayed. Another alternative is to use a transgenic GFP mouse and deliver siRNAs that are complementary to GFP. This would be a relatively simple approach to assay siRNA delivery and activity by visualizing GFP silencing in multiple tissues including the pituitary. This would help address the issue of how often to administer siRNAs depending on the stability and packaging of the individual siRNA.

In the work presented above, shRNA-17.5 was expressed from birth and as such the rescue phenotype was both obvious and immediately evident. If we are delivering the siRNA to postnatal IGHD II mice, they will already have developed some, albeit mild, adverse phenotypes. How long will it take to observe a rescue phenotype upon systemic delivery? As growth is a somewhat slow indicator, perhaps serum IGF-1 levels would provide a better initial indicator of rescue. In mammals there is a burst of GH secretion at birth though the significance of this is not known (de Zegher et al., 1993; Giustina and Veldhuis, 1998). Following this spurt, GH secretion is decreased until later in development. Obviously, development in mice is much more rapid than in humans but this time lapse in GH secretion should allow us to treat mice with siRNAs at, or just prior to weaning. Additionally, the pituitary is able to proliferate and regenerate cells and in GHD subjects, there is an increase in expression of GH-releasing factor

(GRF), producing a postnatal drive to proliferate. In fact, even in mice that are 8 weeks old, the pituitary still regenerates at a rate of ~2% per day. In the IGHD II mice, as the newly proliferated somatotrophs also express the dominant negative 17.5-kDa isoform, the effect is exacerbated as there is a continual drive to produce more of the toxic protein. By supplying siRNA-17.5, it is hoped that the cells that are recovering will survive and persist, and eventually rescue GHD.

Mechanism of the Dominant Negative Function of the 17.5-kDa Isoform

The 17.5-kDa isoform acts as a dominant negative but it is not clear why this is so. Several hypotheses, most notably those of Iain Robinson and colleagues (Lee et al., 2000; McGuinness et al., 2003), suggest that the 17.5-kDa isoform forms heterodimers with wild type GH and these are inefficiently condensed when forming DCSVs, causing an accumulation of protein in the Golgi and ER. This triggers an unfolded protein response and production of the dimers ultimately exceeds the degradative capacity of the proteasome, becoming toxic to the cell (McGuinness et al., 2003). Recent work in the cultured rat somatotroph line, GH4C1, has shown that the 17.5-kDa isoform is indeed degraded by the proteosome and that proteosome inhibition leads to prominent accumulation of the mutant GH protein (Kannenberg et al., 2007).

It would be attractive to develop a system in which to study effects of the 17.5-kDa isoform that is both effective and relatively simple. One possibility is to use AtT-20 (AtT-20/D16v-F2) cells, a murine neuroendocrine cell line derived from a pituitary tumor that does not express endogenous GH but secretes ACTH

in the same fashion as GH is secreted from somatotrophs. These cells have been successfully used to visualize localization of the 17.5-kDa isoform both alone and when co-expressed with wild type GH in relation to secretory components (Salemi et al., 2006). AtT-20 cells can be stimulated with forskolin which increases cAMP levels and promotes ACTH secretion. Forskolin has also been used to stimulate GH secretion in AtT-20 cells transfected with GH constructs (Salemi et al., 2006). A disadvantage to making stable cell lines that constitutively express the 17.5-kDa isoform is that in the selection process, the cells expressing high copy numbers of the mutant will die since it is toxic and, in effect, we would be selecting for low levels of mutant expression. We are therefore developing inducible stable cell lines that express either mutant, wild type or both GH constructs under the control of the Tet-responsive element (CloneTech), allowing transcription of these transgenes to be switched on in the presence of tetracycline. These stable cell lines could be used to visualize secretory pathway defects in the ER and Golgi. These cells could also provide an ideal system to study the unfolded protein response if indeed this is responsible for cell death.

RNAi in the Pituitary

Though regulated by the hypothalamus, the pituitary itself controls secretion of the body's hormones and is often referred to as the 'master gland.' Many disease states have been characterized by decreased or absent hormone production, leading to hypopituitarism. Conversely, pituitary tumors, or pituitary

adenomas, can result in hormonal overproduction that can also have detrimental consequences. Excess of GH production can lead to acromegaly and excess PRL secretion can lead to infertility. Imbalance in hormone production from the pituitary can lead to a number of endocrine disorders. As discussed previously, there are other genetic disorders (other than mutations in GH-1) that can disrupt the GH-1 axis and any of these that function in a dominant manner are potential targets for treatment using RNAi. As an example, *Pit-1* is expressed in the pituitary and is epistatic to *GH-1*. Two dominant mutations in *Pit-1* have been characterized, P24L and R271W, that cause GHD (Ohta et al., 1992; Radovick et al., 1992), and these could be potential targets for RNAi-based therapies. When the latter mutant, R271W, was co-transfected with wild type Pit-1, it prevented transcriptional activation by the wild type protein and acted as a dominant negative (Dattani and Robinson, 2000). In IGHD II, anterior pituitary hypoplasia develops due to the dominant negative nature of the 17.5-kDa hGH isoform and its resulting toxic effects. With the exception of our study, there have not been any published reports that use RNAi to target pituitary specific mRNAs. With development of a sound and robust delivery system of siRNAs to target $\Delta 3$ transcripts in the pituitary, we could open up a new avenue in RNAi therapeutics.

Summary

In closing, the data presented in this thesis highlight the importance of maintaining splicing fidelity to prevent exon skipping and the production of deleterious protein isoforms. Where mistakes in *GH-1* splicing occur and cause

skipping of exon 3, we have shown that expression of shRNAs that specifically target aberrant transcripts can rescue a disease phenotype in a mouse model of IGHD II. Our approach involved genetic delivery of shRNAs but as work proceeds to develop systemic delivery methods, it seems clear that the ability to rescue disease using RNAi could usher in a new era in gene therapy.

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