TMPRSS6 iRNA COMPOSITIONS AND METHODS OF USE THEREOF

Related Applications

This application claims the benefit of priority to U.S. Provisional Patent Application No. 61/826,178, filed on May 22, 2013 and U.S. Provisional Patent Application No. 61/912,988, filed on December 6, 2013. This application is related to U.S. Provisional Application No. 61/561,710, filed on November 18, 2011, and PCT/US2012/065601, filed on November 16, 2012. The entire contents of each of the foregoing applications are hereby incorporated herein by reference.

Sequence Listing

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 21, 2014, is named 121301-00720_SL.txt and is 449,620 bytes in size.

Background of the Invention

TMPRSS6 (Transmembrane Protease, Serine 6) gene encodes TMPRSS6, also known as matriptase-2, a type II serine protease. It is primarily expressed in the liver, although high levels of TMPRSS6 mRNA are also found in the kidney, with lower levels in the uterus and much smaller amounts detected in many other tissues (Ramsay et al., *Haematologica* (2009), 94(6), 840-849). TMPRSS6 plays a role in iron homeostatis by binding and proteolytically degrading the hepcidin activator and BMP co-receptor HJV (hemojuvelin), which causes down-regulation of hepcidin levels.

TMPRSS6 consists of a short N-terminal intracytoplasmic tail, a type II transmembrane domain, a stem region composed of two extracellular CUB (complement factor Cls/Clr, urchin embryonic growth factor and BMP (bone morphogenetic protein)) domains, three LDLR (low-density-lipoprotein receptor class A) domains, and a C-terminal trypsin- like serine protease domain. There are also consensus sites for N-glycosylation in the extracellular domain, and a potential phosphorylation site in the intracytoplasmic tail region.

Numberous disorders can be associated with iron overload, a condition characterized by increased levels of iron. Iron overload can result in excess iron deposition in various tissues and can lead to tissue and organ damage. Accordingly, methods for effective treatment of disorders associated with iron overload are currently needed.

Summary of the Invention

The present disclosure provides compositions comprising RNAi agents, *e.g.*, double-stranded iRNA agents, targeting TMPRSS6. The present disclosure also provides methods using the compositions of the disclosure for inhibiting TMPRSS6 expression and for treating TMPRSS6

associated disorders, *e.g.*, iron overload associated disorders, such as thalassemia, *e.g.*, β-thalassemia, or hemochromatosis.

In one aspect, provided herein is a double stranded RNAi agent for inhibiting expression of TMPRSS6 in a cell, comprising a sense strand and an antisense strand forming a double-stranded region, wherein the antisense strand comprises at least 15 contiguous nucleotides which differ by no more than three nucleotides from the nucleotide sequence AGAAUGAACCAGAAGAAGCAGGU (SEQ ID NO:187), wherein each strand is independently 15 to 30 nucleotides in length, wherein at least one nucleotide is a modified nucleotide, and wherein at least one strand is conjugated to a ligand.

In one aspect, provided herein is a pharmaceutical composition for inhibiting expression of a TMPRSS6 gene comprising the double stranded RNAi agent as described herein.

In one aspect, provided herein is an *in vitro* method of inhibiting TMPRSS6 expression in a cell, the method comprising: (a) contacting the cell *in vitro* with the double stranded RNAi agent of any one of claims 1-23, or the pharmaceutical composition of claim 24; and (b) maintaining the cell produced in step (a) *in vitro* for a time sufficient to obtain degradation of the mRNA transcript of a TMPRSS6 gene, thereby inhibiting expression of the TMPRSS6 gene in the cell.

In one aspect, provided herein is a use of a therapeutically effective amount of the double stranded RNAi agent as described herein, or the pharmaceutical composition as described herein, in the manufacture of a medicament for treating a subject having a TMPRSS6 associated disorder.

In one aspect, provided herein is the double stranded RNAi agent as described herein, or the pharmaceutical composition as described herein for use in therapy.

Accordingly, in one example, the present disclosure provides RNAi agents, *e.g.*, double-stranded RNAi agents, capable of inhibiting the expression of TMPRSS6 (matriptase-2) in a cell, wherein the double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences of SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10,

wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides, and

wherein the sense strand is conjugated to a ligand attached at the 3'-terminus.

In one example, all of the nucleotides of said sense strand and all of the nucleotides of said antisense strand are modified nucleotides.

In one example, the sense strand and the antisense strand comprise a region of complementarity which comprises at least 15 contiguous nucleotides differing by no more than 3

nucleotides from any one of the antisense sequences listed in any one of Tables 1, 2, 4, 5, 8, 10, and 12.

In one example, at least one of the modified nucleotides is selected from the group consisting of a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a nucleotide comprising a 5'-phosphorothioate group, a nucleotide comprising a 5' phosphate or 5' phosphate mimic (see, *e.g.,* PCT Publication No. WO 2011/005860), and a terminal nucleotide linked to a cholesteryl derivative or a dodecanoic acid bisdecylamide group.

In one example, at least one strand comprises a 3' overhang of at least 1 nucleotide. In another example, at least one strand comprises a 3' overhang of at least 2 nucleotides. In another example, the present disclosure provides RNAi agents, *e.g.*, double-stranded RNAi agents, capable of inhibiting the expression of TMPRSS6 (matriptase-2) in a cell, wherein the double stranded RNAi agent comprises a sense strand complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding TMPRSS6, wherein each strand is about 14 to about 30 nucleotides in length, wherein the double stranded RNAi agent is represented by formula (III):

sense: $5'\ n_p\text{-}N_a\text{-}(X\ X\ X)_i\text{-}N_b\text{-}Y\ Y\ Y\text{-}N_b\text{-}(Z\ Z\ Z)_j\text{-}N_a\text{-}n_q\ 3'$

antisense: $3'\ n_p'\text{-}N_a'\text{-}(X'X'X')_k\text{-}N_b'\text{-}Y'Y'Y'\text{-}N_b'\text{-}(Z'Z'Z')_l\text{-}N_a'\text{-}n_q'\ 5'$ (III)

wherein:

i, j, k, and l are each independently 0 or 1;

p, p', q, and q′ are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

each n_p, n_p', n_q, and n_q', each of which may or may not be present, independently represents an overhang nucleotide;

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides;

modifications on N_b differ from the modification on Y and modifications on N_b' differ from the modification on Y′; and

wherein the sense strand is conjugated to at least one ligand.

In one example, i is 0; j is 0; i is 1; j is 1; both i and j are 0; or both i and j are 1. In another example, k is 0; l is 0; k is 1; l is 1; both k and l are 0; or both k and l are 1.

In one example, XXX is complementary to X′X′X′, YYY is complementary to Y′Y′Y′, and ZZZ is complementary to Z′Z′Z′.

In one example, YYY motif occurs at or near the cleavage site of the sense strand.

In one example, Y′Y′Y′ motif occurs at the 11, 12 and 13 positions of the antisense strand from the 5'-end.

In one example, Y′ is 2′-O-methyl.

In one example, formula (III) is represented by formula (IIIa):

sense: 5' n_p -N_a -Y Y Y -N_a - n_q 3'

antisense: 3' $n_{p'}$-$N_{a'}$- Y′Y′Y′- $N_{a'}$- $n_{q'}$ 5' (IIIa).

In another example, formula (III) is represented by formula (IIIb):

sense: 5' n_p -N_a -Y Y Y -N_b -Z Z Z -N_a - n_q 3'

antisense: 3' $n_{p'}$-$N_{a'}$- Y′Y′Y′-$N_{b'}$-Z′Z′Z′- $N_{a'}$- $n_{q'}$ 5' (IIIb)

wherein each N_b and N_b′ independently represents an oligonucleotide sequence comprising 1-5 modified nucleotides.

In yet another example, formula (III) is represented by formula (IIIc):

sense: 5' n_p -N_a –X X X -N_b -Y Y Y -N_a - n_q 3'

antisense: 3' $n_{p'}$-$N_{a'}$- X′X′X′-$N_{b'}$- Y′Y′Y′- $N_{a'}$- $n_{q'}$ 5' (IIIc)

wherein each N_b and N_b′ independently represents an oligonucleotide sequence comprising 1-5 modified nucleotides.

In one example, formula (III) is represented by formula (IIId):

sense: 5' n_p -N_a –X X X- N_b -Y Y Y -N_b -Z Z Z -N_a - n_q 3'

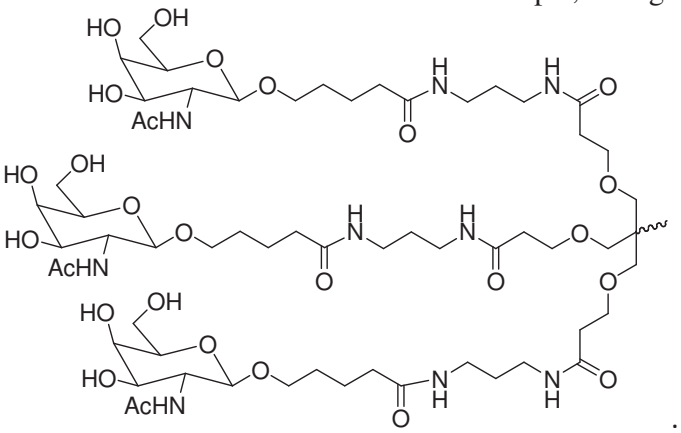
antisense: 3' $n_{p'}$-$N_{a'}$- X′X′X′- $N_{b'}$-Y′Y′Y′-$N_{b'}$-Z′Z′Z′- $N_{a'}$- $n_{q'}$ 5' (IIId)

wherein each N_b and N_b′ independently represents an oligonucleotide sequence comprising 1-5 modified nucleotides and each N_a and N_a′ independently represents an oligonucleotide sequence comprising 2-10 modified nucleotides.

In one example, the double-stranded region is 15-30 nucleotide pairs in length. In another example, the double-stranded region is 17-23 nucleotide pairs in length. In yet another example, the double-stranded region is 17-25 nucleotide pairs in length. In one example, the double-stranded region is 23-27 nucleotide pairs in length. In another example, the double-stranded region is 19-21 nucleotide pairs in length. In another example, the double-stranded region is 21-23 nucleotide pairs in length. In one example, each strand has 15-30 nucleotides. In another example, each strand has 19-30 nucleotides.

In one example, the modifications on the nucleotides are selected from the group consisting of LNA, HNA, CeNA, 2′-methoxyethyl, 2′-O-alkyl, 2′-O-allyl, 2′-C- allyl, 2′-fluoro, 2′-deoxy, 2'-hydroxyl, and combinations thereof. In another example, the modifications on the nucleotides are 2′-O-methyl or 2′-fluoro modifications.

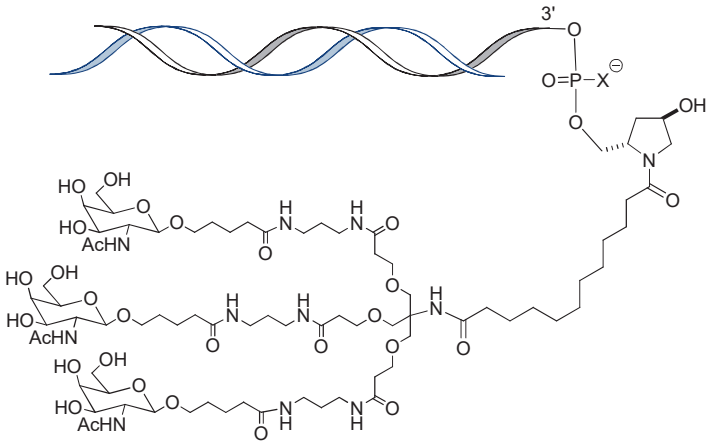
In one example, the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. In another example, the ligand is



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In one example, the ligand is attached to the 3′ end of the sense strand.

In one example, the RNAi agent is conjugated to the ligand as shown in the following schematic



wherein X is O or S. In a specific example, X is O.

In one example, the agent further comprises at least one phosphorothioate or methylphosphonate internucleotide linkage.

In one example, the phosphorothioate or methylphosphonate internucleotide linkage is at the 3'-terminus of one strand. In one example, the strand is the antisense strand. In another example, the strand is the sense strand.

In one example, the phosphorothioate or methylphosphonate internucleotide linkage is at the 5'-terminus of one strand. In one example, the strand is the antisense strand. In another example, the strand is the sense strand.

In one example, the phosphorothioate or methylphosphonate internucleotide linkage is at the both the 5'- and 3'-terminus of one strand. In one example, the strand is the antisense strand.

In one example, the RNAi agent comprises 6-8 phosphorothioate internucleotide linkages.

In one example, the antisense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus and two phosphorothioate internucleotide linkages at the 3'-terminus, and the sense strand comprises at least two phosphorothioate internucleotide linkages at either the 5'-terminus or the 3'-terminus.

In one example, the base pair at the 1 position of the 5′-end of the antisense strand of the duplex is an AU base pair.

In one example, the Y nucleotides contain a 2′-fluoro modification.

In one example, the Y′ nucleotides contain a 2′-O-methyl modification.

In one example, p′>0. In another example, p′=2.

In one example, q'=0, p=0, q=0, and p' overhang nucleotides are complementary to the target mRNA. In another example, q'=0, p=0, q=0, and p' overhang nucleotides are non-complementary to the target mRNA.

In one example, the sense strand has a total of 21 nucleotides and the antisense strand has a total of 23 nucleotides.

In one example, at least one n_p' is linked to a neighboring nucleotide via a phosphorothioate linkage.

In one example, all n_p' are linked to neighboring nucleotides via phosphorothioate linkages.

In one example, the RNAi agent is selected from the group of RNAi agents listed in any one of Tables 1, 2, 4, 5, 8, 10, and 12.

In one example, the RNAi agent is AD-59743. In another example, the RNAi agent is AD-60940.

In one example, the present disclosure provides double stranded RNAi agents for inhibiting expression of TMPRSS6 in a cell,

wherein the double stranded RNAi agent comprises a sense strand and an antisense strand forming a double stranded region,

wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences of SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10,

wherein substantially all of the nucleotides of the sense strand comprise a modification selected from the group consisting of a 2'-O-methyl modification and a 2'-fluoro modification,

wherein the sense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus,

wherein substantially all of the nucleotides of the antisense strand comprise a modification selected from the group consisting of a 2'-O-methyl modification and a 2'-fluoro modification,

wherein the antisense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus and two phosphorothioate internucleotide linkages at the 3'-terminus, and

wherein the sense strand is conjugated to one or more GalNAc derivatives attached through a branched bivalent or trivalent linker at the 3'-terminus.

In one example, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

In another example, the present disclosure provides RNAi agents, *e.g.*, double stranded RNAi agents, capable of inhibiting the expression of TMPRSS6 (matriptase-2) in a cell, wherein the double stranded RNAi agent comprises a sense strand complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding TMPRSS6, wherein each strand is about 14 to about 30 nucleotides in length, wherein the double stranded RNAi agent is represented by formula (III):

$$\begin{array}{lll} \text{sense:} & 5'\ n_p\text{-}N_a\text{-}(X\ X\ X)_i\text{-}N_b\text{-}Y\ Y\ Y\text{-}N_b\text{-}(Z\ Z\ Z)_j\text{-}N_a\text{-}n_q\ 3' & \\ \text{antisense:} & 3'\ n_p'\text{-}N_a'\text{-}(X'X'X')_k\text{-}N_b'\text{-}Y'Y'Y'\text{-}N_b'\text{-}(Z'Z'Z')_l\text{-}N_a'\text{-}n_q'\ 5' & \text{(III)} \end{array}$$

wherein:

i, j, k, and l are each independently 0 or 1;

p, p', q, and q′ are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

each n_p, n_p', n_q, and n_q', each of which may or may not be present independently represents an overhang nucleotide;

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2′-O-methyl or 2′-fluoro modifications;

modifications on N_b differ from the modification on Y and modifications on N_b' differ from the modification on Y′; and

wherein the sense strand is conjugated to at least one ligand.

In yet another example, the present disclosure provides RNAi agents, *e.g.*, double stranded RNAi agents, capable of inhibiting the expression of TMPRSS6 (matriptase-2) in a cell, wherein the double stranded RNAi agent comprises a sense strand complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding TMPRSS6, wherein each strand is about 14 to about 30 nucleotides in length, wherein the double stranded RNAi agent is represented by formula (III):

sense: 5' n_p -N_a -(X X X) $_i$-N_b -Y Y Y -N_b -(Z Z Z)$_j$ -N_a - n_q 3'

antisense: 3' n_p′-N_a′-(X′X′X′)$_k$-N_b′-Y′Y′Y′-N_b′-(Z′Z′Z′)$_l$-N_a′- n_q′ 5' (III)

wherein:

i, j, k, and l are each independently 0 or 1;

each n_p, n_q, and n_q′, each of which may or may not be present, independently represents an overhang nucleotide;

p, q, and q′ are each independently 0-6;

n_p′ >0 and at least one n_p′ is linked to a neighboring nucleotide via a phosphorothioate linkage;

each N_a and N_a′ independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b′ independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2′-O-methyl or 2′-fluoro modifications;

modifications on N_b differ from the modification on Y and modifications on N_b′ differ from the modification on Y′; and

wherein the sense strand is conjugated to at least one ligand.

In a further example, the present disclosure provides RNAi agents, *e.g.*, double stranded RNAi agents, capable of inhibiting the expression of TMPRSS6 (matriptase-2) in a cell, wherein the double stranded RNAi agent comprises a sense strand complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding TMPRSS6, wherein each strand is about 14 to about 30 nucleotides in length, wherein the double stranded RNAi agent is represented by formula (III):

sense: 5' n_p -N_a -(X X X) $_i$-N_b -Y Y Y -N_b -(Z Z Z)$_j$ -N_a - n_q 3'

antisense: 3' n_p′-N_a′-(X′X′X′)$_k$-N_b′-Y′Y′Y′-N_b′-(Z′Z′Z′)$_l$-N_a′- n_q′ 5' (III)

wherein:

i, j, k, and l are each independently 0 or 1;

each n_p, n_q, and n_q', each of which may or may not be present, independently represents an overhang nucleotide;

p, q, and q′ are each independently 0-6;

$n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via a phosphorothioate linkage;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2′-O-methyl or 2′-fluoro modifications;

modifications on N_b differ from the modification on Y and modifications on N_b' differ from the modification on Y′; and

wherein the sense strand is conjugated to at least one ligand, wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In another example, the present disclosure provides RNAi agents, *e.g.*, double stranded RNAi agents capable of inhibiting the expression of TMPRSS6 (matriptase-2) in a cell, wherein the double stranded RNAi agent comprises a sense strand complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding TMPRSS6, wherein each strand is about 14 to about 30 nucleotides in length, wherein the double stranded RNAi agent is represented by formula (III):

sense: 5' n_p -N_a -(X X X) $_i$-N_b -Y Y Y -N_b -(Z Z Z)$_j$ -N_a - n_q 3'

antisense: 3' n_p'-N_a'-(X′X′X′)$_k$-N_b'-Y′Y′Y′-N_b'-(Z′Z′Z′)$_l$-N_a'- n_q' 5' (III)

wherein:

i, j, k, and l are each independently 0 or 1;

each n_p, n_q, and n_q', each of which may or may not be present, independently represents an overhang nucleotide;

p, q, and q′ are each independently 0-6;

$n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via a phosphorothioate linkage;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2′-O-methyl or 2′-fluoro modifications;

modifications on N_b differ from the modification on Y and modifications on N_b' differ from the modification on Y′;

wherein the sense strand comprises at least one phosphorothioate linkage; and

wherein the sense strand is conjugated to at least one ligand, wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In yet another example, the present disclosure provides RNAi agents, *e.g.*, double stranded RNAi agents, capable of inhibiting the expression of TMPRSS6 (matriptase-2) in a cell, wherein the double stranded RNAi agent comprises a sense strand complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding TMPRSS6, wherein each strand is about 14 to about 30 nucleotides in length, wherein the double stranded RNAi agent is represented by formula (III):

sense: 5' n_p -N_a -Y Y Y - N_a - n_q 3'

antisense: 3' n_p'-N_a'- Y′Y′Y′- N_a'- n_q' 5' (IIIa)

wherein:

each n_p, n_q, and n_q', each of which may or may not be present, independently represents an overhang nucleotide;

p, q, and q′ are each independently 0-6;

$n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via a phosphorothioate linkage;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides;

YYY and Y′Y′Y′ each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2′-O-methyl or 2′-fluoro modifications;

wherein the sense strand comprises at least one phosphorothioate linkage; and

wherein the sense strand is conjugated to at least one ligand, wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one example, the present disclosure provides RNAi agent selected from the group of RNAi agents listed in any one of Tables 1, 2, 4, 5, 8, 19, and 12.

In one example, the present disclosure provides compositions comprising a modified antisense polynucleotide agent, wherein the agent is capable of inhibiting the expression of TMPRSS6 in a cell, and comprises a sequence complementary to a sense sequence selected from the group of the sequences listed in any one of Tables 1, 2, 4, 5, 8, 10, and 12, wherein the polynucleotide is about 14 to about 30 nucleotides in length.

The present disclosure also provides cells, vectors, host cells, and pharmaceutical compositions comprising, *e.g.,* the double stranded RNAi agents of the disclosure.

In some examples, the RNAi agent is administered using a pharmaceutical composition.

In preferred examples, the RNAi agent is administered in a solution. In some such examples, the siRNA is administered in an unbuffered solution. In one example, the siRNA is administered in water. In other examples, the siRNA is administered with a buffer solution, such as an acetate buffer, a citrate buffer, a prolamine buffer, a carbonate buffer, or a phosphate buffer or any combination thereof. In some examples, the buffer solution is phosphate buffered saline (PBS).

In one example, the pharmaceutical compositions further comprise a lipid formulation. In one example, the lipid formulation comprises a LNP, or XTC. In another example, the lipid formulation comprises a MC3.

In one example, the present disclosure provides methods of inhibiting TMPRSS6 expression in a cell. The methods include contacting the cell with an RNAi agent, *e.g.*, a double stranded RNAi agent, or a modified antisense polynucleotide agent of the disclosure, or vector of the disclosure, or a pharmaceutical composition of the disclosure; and maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a TMPRSS6 gene, thereby inhibiting expression of the TMPRSS6 gene in the cell.

In one example, the cell is within a subject.

In one example, the subject is a human.

In one example, the TMPRSS6 expression is inhibited by at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 100%.

In another example, hepcidin gene expression is increased by at least about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, or about 5-fold.

In yet another example, serum hepcidin concentration is increased by at least about 10%, about 25%, about 50%, about 100%, about 150%, about 200%, about 250%, or about 300%.

In one example, serum iron concentration is decreased by at least about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%.

In another example,a percent transferrin saturation is decreased by at least about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%.

In another example, the present disclosure provides methods of treating a subject having a disorder mediated by, or associated with, TMPRSS6 expression. The methods include administering to the subject a therapeutically effective amount of an RNAi agent, *e.g.*, a double stranded RNAi agent, of the disclosure, or a modified antisense polynucleotide agent of the disclosure, or a vector of the disclosure, or a pharmaceutical composition of the disclosure, thereby treating the subject.

In one example, the present disclosure provides methods of treating a subject having a TMPRSS6-associated disorder. The methods include subcutaneously administering to the subject a therapeutically effective amount of a double stranded RNAi agent,

wherein the double stranded RNAi agent comprises a sense strand and an antisense strand forming a double stranded region,

wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences of SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10,

wherein substantially all of the nucleotides of the antisense strand comprise a modification selected from the group consisting of a 2'-O-methyl modification and a 2'-fluoro modification,

wherein the antisense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus and two phosphorothioate internucleotide linkages at the 3'-terminus,

wherein substantially all of the nucleotides of the sense strand comprise a modification selected from the group consisting of a 2'-O-methyl modification and a 2'-fluoro modification,

wherein the sense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus and,

wherein the sense strand is conjugated to one or more GalNAc derivatives attached through a branched bivalent or trivalent linker at the 3'-terminus, thereby treating the subject.

In one example, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

In one example, the subject is a human.

In one example, the subject has a disorder associated with iron overload, *e.g.*, hereditary hemochromatosis, β-thalassemia (*e.g.,* β-thalassemia major and β-thalassemia intermiedia) erythropoietic porphyria, Parkinson's Disease, Alzheimer's Disease or Friedreich's Ataxia.

In one example, the RNAi agent, *e.g.*, double stranded RNAi agent, is administered at a dose of about 0.01 mg/kg to about 10 mg/kg, about 1 mg/kg to about 10 mg/kg, about 2 mg/kg to about 10 mg/kg, about 3 mg/kg to about 10 mg/kg, about 4 mg/kg to about 10 mg/kg, about 5 mg/kg to about 15 mg/kg, about 6 mg/kg to about 15 mg/kg, about 7 mg/kg to about 15 mg/kg, about 8 mg/kg to about 15 mg/kg, about 9 mg/kg to about 15 mg/kg, about 10 mg/kg to about 20 mg/kg, about 12

mg/kg to about 20 mg/kg, about 13 mg/kg to about 20 mg/kg, about 14 mg/kg to about 20 mg/kg, about 15 mg/kg to about 20 mg/kg, about 16 mg/kg to about 20 mg/kg or about 18 mg/kg to about 20 mg/kg. In particular examples, the double stranded RNAi agent is administered at a dose of about 0.1 mg/kg, about 1.0 mg/kg, or about 3.0 mg/kg.

In one example, the RNAi agent, *e.g.*, double stranded RNAi agent, is administered subcutaneously or intravenously.

In one example, the RNAi agent is administered in two or more doses. In a specific example, the RNAi agent is administered at intervals selected from the group consisting of once every about 12 hours, once every about 24 hours, once every about 48 hours, once every about 72 hours, once every about 96 hours, once about every 7 days, or once about every 14 days. In particular examples, the RNAi agent is administered once a week for up to 2 weeks, up to 3 weeks, up to 4 weeks, up to 5 weeks, or longer.

In yet another example, the present disclosure provides methods of treating an iron overload associated disorder in a subject. The methods include administering to the subject a therapeutically effective amount of an RNAi agent, *e.g.*, a double stranded RNAi agent, or the vector of the disclosure, thereby treating the subject.

In one example, the iron overload associated disorder is hemochromatosis. In another example, the iron overload associated disorder is a thalassemia, *e.g.*, β-thalassemia (*e.g.,* β-thalassemia major and β-thalassemia intermiedia), or erythropoietic porphyria. In yet another example, the iron overload associated disorder is a neurological disease, *e.g.*, Parkinson's Diasease, Alzheimer's Disease or Friedreich's Ataxia.

In one example, the subject is a primate or rodent. In another example, the subject is a human.

In one example, the RNAi agent, *e.g.*, double stranded RNAi agent, is administered at a dose of about 0.01 mg/kg to about 10 mg/kg, about 0.5 mg/kg to about 50 mg/kg, about 10 mg/kg to about 30 mg/kg, about 10 mg/kg to about 20 mg/kg, about 15 mg/kg to about 20 mg/kg, about 15 mg/kg to about 25 mg/kg, about 15 mg/kg to about 30 mg/kg, or about 20 mg/kg to about 30 mg/kg.

In one example, the RNAi agent, *e.g.*, double stranded RNAi agent, is administered subcutaneously or intravenously.

In one example, the RNAi agent is administered in two or more doses. In a specific example, the RNAi agent is administered at intervals selected from the group consisting of once every about 12 hours, once every about 24 hours, once every about 48 hours, once every about 72 hours, once every about 96 hours, once about every 7 days, or once about every 14 days.

In one example, administering results in a decrease in iron levels, ferritin level and/or transferrin saturation level in the subject.

In one example, the methods further comprise determining the iron level in the subject.

In one example, the methods of the disclosure which include administering an iRNA agent of the disclosure (or pharmaceutical composition of the disclosure) to a subject are practiced in combination with administration of additional pharmaceuticals and/or other therapeutic methods. In one example, the methods of the disclosure further comprise administering an iron chelator, *e.g.,* deferiprone, deferoxamine, and deferasirox, to a subject.

The present disclosure is further illustrated by the following detailed description and drawings.

Brief Description of the Drawings

Figure 1 is a graph showing relative levels of TMPRSS6 mRNA in the liver of wild-type mice following administration of a single dose of 1 mg/kg, 3 mg/kg or 10 mg/kg of the iRNA agent AD-59743.

Figure 2 is a graph showing relative levels of hepcidin mRNA in the liver of wild-type mice following administration of a single dose of 1 mg/kg, 3 mg/kg or 10 mg/kg of the iRNA agent AD-59743.

Figures 3A-3E show the levels of hepatic TMPRSS6 mRNA (Figure 3A), hepatic hepcidin mRNA (Figure 3B), serum hepcidin (Figure 3C), total serum iron (Figure 3D), and percent transferrin saturation (Figure 3E) in C57BL/6 mice at various time points following a

CLAIMS

1. A double stranded RNAi agent for inhibiting expression of TMPRSS6 in a cell, comprising a sense strand and an antisense strand forming a double-stranded region, wherein the antisense strand comprises at least 15 contiguous nucleotides which differ by no more than three nucleotides from the nucleotide sequence AGAAUGAACCAGAAGAAGCAGGU (SEQ ID NO:187),

wherein each strand is independently 15 to 30 nucleotides in length,

wherein at least one nucleotide is a modified nucleotide, and

wherein at least one strand is conjugated to a ligand.

2. The double stranded RNAi agent of claim 1, wherein the antisense strand comprises at least 16 contiguous nucleotides which differ by no more than three nucleotides from the nucleotide sequence AGAAUGAACCAGAAGAAGCAGGU (SEQ ID NO:187).

3. The double stranded RNAi agent of claim 1, wherein the antisense strand comprises at least 17 contiguous nucleotides which differ by no more than three nucleotides from the nucleotide sequence AGAAUGAACCAGAAGAAGCAGGU (SEQ ID NO:187).

4. The double stranded RNAi agent of anyone of claims 1-3, wherein each strand is independently 17-25 nucleotides in length.

5. The double stranded RNAi agent of anyone of claims 1-3, wherein each strand is independently 19-25 nucleotides in length.

6. The double stranded RNAi agent of anyone of claims 1-3, wherein each strand is independently 19-23 nucleotides in length.

7. The double stranded RNAi agent of anyone of claims 1-3, wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides.

8. The double stranded RNAi agent of anyone of claims 1-3, wherein all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand are modified nucleotides.

9. The double stranded RNAi agent of anyone of claims 1-3, wherein the at least one modified nucleotide is selected from the group consisting of a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a nucleotide comprising a 5'-phosphorothioate group, a nucleotide comprising a 5' phosphate or 5' phosphate mimic, and a terminal nucleotide linked to a cholesteryl derivative or a dodecanoic acid bisdecylamide group.

10. The double stranded RNAi agent of anyone of claims 1-3, wherein the at least one modified nucleotide is selected from the group consisting of a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, an abasic nucleotide, and a 2'-amino modified nucleotide.

11. The double stranded RNAi agent of anyone of claims 1-3, wherein at least one strand comprises an overhang of at least 1 nucleotide.

12. The double stranded RNAi agent of any one of claims 1-3, wherein the agent further comprises at least one phosphorothioate or methylphosphonate internucleotide linkage.

13 The double stranded RNAi agent of claim 12, wherein the phosphorothioate or methylphosphonate internucleotide linkage is at the 3'-terminus of one strand.

14. The double stranded RNAi agent of claim 13, wherein the strand is the antisense strand.

15. The double stranded RNAi agent of claim 13, wherein the strand is the sense strand.

16. The double stranded RNAi agent of claim 12, wherein the phosphorothioate or methylphosphonate internucleotide linkage is at the 5'-terminus of one strand.

17. The double stranded RNAi agent of claim 16, wherein the strand is the antisense strand.

18. The double stranded RNAi agent of claim 12, wherein the phosphorothioate or methylphosphonate internucleotide linkage is at both the 5'- and 3'-terminus of one strand.

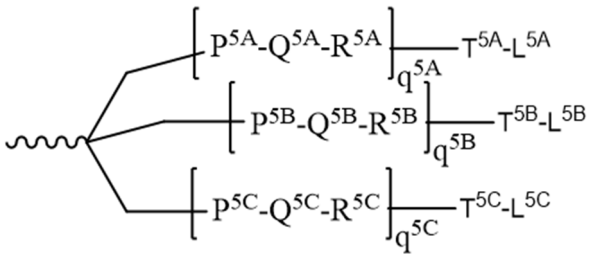
19. The double stranded RNAi agent of claim 18, wherein the strand is the antisense strand.

20. The double stranded RNAi agent of claim 12, wherein the double stranded RNAi agent comprises 6-8 phosphorothioate internucleotide linkages.

21. The double stranded RNAi agent of anyone of claims 1-3, wherein the ligand comprises an N-acetylgalactosamine (GalNAc) derivative.

22. The double stranded RNAi agent of claim 21, wherein the N-acetylgalactosamine (GalNAc) derivative ligand is attached through a trivalent branched linker.

23. The double stranded RNAi agent of claim 22, wherein the trivalent branched linker comprises the structure shown in Formula (VII):



Formula (VII)

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24. A pharmaceutical composition for inhibiting expression of a TMPRSS6 gene comprising the double stranded RNAi agent of any one of claims 1-23.

25. An *in vitro* method of inhibiting TMPRSS6 expression in a cell, the method comprising:

(a) contacting the cell *in vitro* with the double stranded RNAi agent of any one of claims 1-23, or the pharmaceutical composition of claim 24; and

(b) maintaining the cell produced in step (a) *in vitro* for a time sufficient to obtain degradation of the mRNA transcript of a TMPRSS6 gene, thereby inhibiting expression of the TMPRSS6 gene in the cell.

26. The method of claim 25, wherein the TMPRSS6 expression is inhibited by at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100%; serum hepcidin concentration is increased by at least 10%, 25%, 50%, 100%, 150%, 200%, 250%, or 300%; serum iron concentration is decreased by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100%; or percent transferrin saturation is decreased by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100%.

27. Use of a therapeutically effective amount of the double stranded RNAi agent of any one of claims 1-23, or the pharmaceutical composition of claim 24, in the manufacture of a medicament for treating a subject having a TMPRSS6 associated disorder.

28. The use of claim 27, wherein the subject is a human.

29. The use of claim 27 or 28, wherein the TMPRSS6 associated disorder is hereditary hemochromatosis, β-thalassemia, erythropoietic porphyria, or a disorder associated with iron overload.

30. The use of any one of claims 27-29, wherein the double stranded RNAi agent is to be administered at a dose of 0.01 mg/kg to 10 mg/kg or 1 mg/kg to 10 mg/kg.

31. The use of any one of claims 27-30, wherein the double stranded RNAi agent is for subcutaneous administration.

32. The use of any one of claims 27-30, wherein the double stranded RNAi agent is for intravenous administration.

33. An isolated cell containing the double stranded RNAi agent of any one of claims 1-23.

34. The double stranded RNAi agent of any one of claims 1-23, or the pharmaceutical composition of claim 24 for use in therapy.