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(54) Title: Antisense oligonucleotides or oligonucleotide analogues

(72) Inventor: DOUGLAS LESTER

(73) Patent Granted to: UNIVERSITY OF ABERTAY DUNDEE, Kidd Building Bell Street Dundee DD1 1HG, United Kingdom
Antisense Oligonucleotides or Oligonucleotide Analogues

The present invention relates to antisense oligonucleotides and oligonucleotide analogues. In particular, the present invention is directed to antisense oligonucleotides and oligonucleotide analogues which target the site of incorrect splicing in pre-mRNA in GNB3 mRNA associated with the GNB3 825C>T polymorphism. The invention also relates to therapeutic compositions and methods of using said compositions in therapy.

The common GNB3 825C>T polymorphism has been shown, in worldwide studies, to be significantly associated with hypertension, especially in obese patients. Moreover, the GNB3 825T allele has also been associated with increased cell proliferation and metastasis, in a subset of cancers. It has also shown to be associated with predisposition to erectile dysfunction. In addition, this same GNB3 allele has been shown to act as a pharmacogenetic marker for an increased favourable response to both beta blockers and serotonin uptake inhibitors, in both heart and depressed patients respectively.

The c.825C>T polymorphism is situated in exon 10 of the GNB3 gene and paradoxically causes the introduction of a cryptic splice site in the middle of exon 9 in half of all transcripts. In both GNB3 825T/825T homozygote and 825T/825C heterozygote individuals the resultant partial induction of the exon 9 cryptic splice site leads to the expression of both a normal length (GNB3n) and a 123bp smaller length transcript (GNB3s). In GNB3 825T/825T homozygote individuals this results in the ubiquitous expression, in most cell types and tissues in the human body, of ~50% normal Gβ3 protein and ~50% of the smaller and more active Gβ3s protein. The expression of the Gβ3s protein appears to have a dominant effect over the Gβ3 protein as 825T/825C heterozygote individuals, who only express ~25% GNB3s transcript are also predisposed to hypertension, albeit to a lesser extent than 825T/825T individuals.

The inventors have designed a sequence specific morpholino oligonucleotide to the GNB3s cryptic splice site in exon 9 of the GNB3 gene. The result of using this morpholino in human GNB3 825T/825T and 825T/825C lymphoblast cell lines had a surprising and more favourable effect than the expected mere exon skipping effect, which has been observed in prior use of morpholinos, for example in dystrophin morpholinos in the treatment of Duchenne muscular dystrophy (DMD) patients.

Instead of producing an expected “exon-skipped” smaller GNB3s transcript, the GNB3 exon 9 cryptic splice site morpholino appears to both block the production of the aberrant GNB3s
transcript and, remarkably, to increase the expression to >50% of the normal GNB3n transcript.

The inventors have previously shown that a complete absence of functional GNB3, in the homozygous del153D chicken, produces both renal and retinal abnormalities. In addition they have also shown that chickens which express 50% normal GNB3 appear to be asymptomatic.

All of these results taken together demonstrate that the use of GNB3s, cryptic splice site, exon 9, specific antisense oligonucleotides (or related molecules) are unlikely to produce any unwanted renal or retinal side effects, in the successful treatment of 825T patients, suffering from a variety of disorders.

**Statements of the Invention**

According to the present invention there is provided an oligonucleotide or oligonucleotide analogue which is adapted to bind at or near the site of aberrant splicing in a pre-mRNA which encodes GNB3s.

The site of aberrant splicing is a cryptic splice site in exon 9 of the GNB3 gene lying at position 620 of the cDNA of normal GNB3n transcript.

Preferably the oligonucleotide or oligonucleotide analogue at least partially restores expression of full length GNB3 (GNB3n). Thus, the present invention can be characterised by the fact that the oligonucleotide or oligonucleotide analogue does not simply result in destruction of pre-mRNA to which it binds, but rather in that it corrects to some extent the aberrant splicing phenomenon which results in production of GNB3s thereby restoring expression of GNB3n. For example, the oligonucleotide or oligonucleotide analogue can decrease aberrant splicing events by 20% or more, preferably by 50% of more, more preferably by 75% or more, and even by 90% or more. Quantitative measurement of splicing events can be made by techniques well known to the skilled person, such as quantitative RT-PCR.

This represents a major and surprising advantage of the present invention. The fact that an antisense oligonucleotide can be used to restore normal expression is unexpected, and provides the ability to restore the levels of functional GNB3n to normal or near normal levels, rather than simply eliminate GNB3s. Clearly this has physiological benefits as it means a
subject has the correct, or closer to correct, levels of GNB3n, rather than simply having GNB3s eliminated.

The site of aberrant splicing in the GNB3 pre-mRNA has the following sequence:

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gugggacacacgggagacugcaugagcgcugugucuccugacucaucucuuucuggccccugugacaguuccagUGCCAAGCUCUGGAUGUGCGAGGGGACCUCGCGUCAG (SEQ ID NO 1)
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The bases in lower case represent sequence which is removed during the aberrant splicing event, and the bases in upper case represent sequence which is retained in the spliced mRNA. Thus the point of change from lower to upper case corresponds to the ‘cryptic splice boundary’.

It is preferred that the oligonucleotide or oligonucleotide analogue binds at a site which within 50 bases of the cryptic splice boundary. That is to say, at least one base within 50 bases of the cryptic splice boundary will be base-paired to at least one base on the oligonucleotide or oligonucleotide analogue (this is considered to be the upper limit of binding ‘near’ to the cryptic splice site). Preferably the oligonucleotide or oligonucleotide analogue binds at a site which is within 25 bases of the cryptic splice boundary, more preferably 10 bases, and yet more preferably at the cryptic splice boundary.

The invention thus includes any oligonucleotide or oligonucleotide analogue which binds to the pre-mRNA and results in a correction of the aberrant splicing event, i.e. that results in a full length GNB3 mRNA. As will be known to the skilled person, splicing of mRNA is performed by an RNA and protein complex known as the spliceosome, containing, inter alia, snRNPs designated U1, U2, U4, U5, and U6. This complex is relatively large and involves interactions with RNA both at and near the actual site of splicing. Accordingly, it is reasonable to assume that oligonucleotides which bind near to, but not actually at, the cryptic splice boundary would be able to perform this function. Following the various techniques described below and other techniques known in the art, it would be routine to screen any putative oligonucleotide or oligonucleotide analogue for the desired function.

It is, however, typically preferred that the oligonucleotide or oligonucleotide analogue binds to bases at, or slightly upstream (i.e. less than 10 bases), of the cryptic splice boundary.
Such oligonucleotide or oligonucleotide analogue would directly block the point at which the pre-mRNA is split during an aberrant splicing event.

In a preferred embodiment of the invention the oligonucleotide or oligonucleotide analogue binds to the target sequence GGGGGCCCUGUGAUGC (SEQ ID NO 2). More preferably the oligonucleotide or oligonucleotide analogue binds to the target sequence AUUUCGGGGGCCUGUGAUGCAGUG (SEQ ID NO 3). The oligonucleotide or oligonucleotide analogue may, of course, bind to additional sequences which flank this core sequence.

It is not possible to define specifically the binding affinity required. However, clearly, high affinity to the sequence is preferred to avoid any off-site targeting events. Accordingly, it is typically preferred that the oligonucleotide or oligonucleotide analogue binds to the target sequence is exactly complementary to the target sequence over a length of at least 15 bases, more preferably at least 20 bases, and most preferably 25 bases or more. However, oligonucleotide or oligonucleotide analogues with sequence variations (such as substitutions, additions or deletions), especially sequence variations outside of the core binding region, can work adequately well. It would be a routine task for the person skilled in the art to test any particular oligonucleotide or oligonucleotide analogue using their normal skills and the various experiments described below.

For example an oligonucleotide or oligonucleotide analogue which has a core binding portion and one or more additional portions (e.g. capable of forming secondary structures) might be highly suitable for the intended purpose.

In a preferred embodiment the oligonucleotide or oligonucleotide analogue is between 10 and 50 nucleotides in length, more preferably from 15 to 30 nucleotides in length.

In a particularly preferred embodiment, the oligonucleotide or oligonucleotide analogue comprises the sequence CACTGGCATCAGGCCCCCGAAT (SEQ ID NO 4), or a sequence which is at least 80% identical thereto, more preferably at least 85% identical thereto, yet more preferably at least 90% identical thereto or at least 95% identical thereto. Suitably the oligonucleotide or oligonucleotide analogue consists of the sequence CACTGGCATCAGGCCCCCGAAT (SEQ ID NO 4).

It should be noted that the base T has been shown in the abovementioned sequence, but it is of course possible that this could be substituted with a U, in the case of RNA-based or
other uracil containing oligonucleotides according to the present invention. Thus T as shown in SEQ ID NO 4 can represent T or U.

It will be apparent to the skilled person that other sequences could be created which differ from the specific sequences listed above, but which nonetheless bind at or near the aberrant splice site and have the effects desired in the present invention, i.e. reducing expression of GNB3s and preferably restoring to some extent expression of GNB3n. It is a routine matter to create and test the efficacy of various oligonucleotides to achieve this, for example by producing a library of suitable oligonucleotides and screening them for ability to bind the target region on GNB3 pre-mRNA. Additionally, in silico methods of predicting the affinity of an antisense oligonucleotide to a given sequence are known in the art (see, for example Biotechnol. Bioeng. 1999 Oct 5; 65(1):1-9. ‘Prediction of antisense oligonucleotide binding affinity to a structured RNA target’. Walton SP, Stephanopoulos GN, Yarmush ML, Roth CM),

Suitably the oligonucleotide or oligonucleotide analogue is able to hybridise to the relevant target sequence of the pre-mRNA (e.g. SEQ ID NO 3) under conditions of low stringency, preferably medium stringency conditions, most desirably high stringency conditions (as defined below) in a standard hybridization assay. See, e.g., J. Sambrook & Green, Molecular Cloning, A Laboratory Manual, 4th Ed., 2012 (Cold Spring Harbor Laboratory Press):

- Low stringency conditions: conditions represented by a wash stringency of 2x SSC at 37 °C;
- Medium stringency conditions: conditions represented by a wash stringency 1x SSC at 37 °C; and
- High stringency conditions: conditions represented by a wash stringency of 0.1X SSC at 37 °C.

Higher levels of stringency are preferred because they reflect that the oligonucleotide or oligonucleotide analogue has a higher degree of complementarity, and hence affinity, to the target sequence, and thus it is more likely that the oligonucleotide or oligonucleotide analogue will bind to the target sequence in the pre-mRNA, and it is less likely that there will be off target effects.

Preferably the oligonucleotide or oligonucleotide analogue is a modified oligonucleotide or a nucleotide analogue. Modified oligonucleotides and nucleotide analogues will be collectively referred to herein as ‘non-natural’ oligonucleotides.
Preferably the non-natural oligonucleotide has increased resistance to degradation compared with a conventional oligonucleotide.

The oligonucleotide or oligonucleotide analogue can be chimera, i.e. containing a portion which consists of modified or contains nucleotide analogues, and a core portion which consists of normal (i.e. phosphodiester) nucleotide. In such chimeric oligonucleotides, it is typical that the ends of the oligonucleotide are non-natural, and the central portion is natural.

Various modified nucleotides or nucleotide analogues are known in the art. For example, the following have been described:
- Methylphosphonates
- Phosphorothioates
- α-nucleosides
- 2'-O-substituted RNA
- Phosphoramidites
- Morpholinos

Any of these could be used in the present invention.

The non-natural oligonucleotide may or may not be capable of inducing RNase H-mediated antisense activity in a cell.

In certain embodiments it is preferred that the non-natural oligonucleotide is not capable of inducing RNase H-mediated antisense activity. In particular this can mean that rather than mRNA being degraded though the action of RNase H, aberrant slicing can be corrected.

In a particularly preferred embodiment, the nucleic acid analogue is a morpholino oligonucleotide (known as a 'morpholino'). Morpholinos are analogues of nucleic acids and have standard nucleic acid bases, but the bases are bound to morpholine rings instead of deoxyribose rings. These morpholine rings are linked through phosphorodiamidate groups instead of phosphates. Morpholinos are sometimes referred to as "PMO" (phosphorodiamidate morpholino oligo).

Morpholinos can be lined to an additional moiety to assist in vivo delivery. For example, it is known in the art to link morpholinos to dendrimers (see Morcos, PA; Li YF, Jiang S (2008).

In a further aspect the present invention provides the use of such an oligonucleotide or oligonucleotide analogue to modulate expression of GNB3 in a cell or organism. This can be for any purpose, e.g. for research.

In a further aspect the present invention provides a method of modulating the expression of GNB3 in a cell comprising the step of administering said oligonucleotide or oligonucleotide analogue to the cell.

In a further aspect the present invention provides the use of an oligonucleotide or oligonucleotide analogue as set out above in the treatment or prevention of a disease in a subject, especially a human.

In particular, the oligonucleotide or oligonucleotide analogue can be used to prevent or treat hypertension and/or cardiovascular disease.

In an alternative embodiment, the oligonucleotide or oligonucleotide analogue can be used to prevent or treat cancer. In particular the oligonucleotide or oligonucleotide analogue can be used to treat one or more of the following cancers in which a link to the GNB3 825T allele has been found:

- prostate cancer (Safarinejad et al. 2012),
- gastric cancer (Shibata et al. 2010),
- extrahepatic cholangiocarcinoma. (Fingas 2010),
- head and neck squamous cell carcinoma. (Lehnerdt 2010),
- oncocytic thyroid tumour (Sheu 2007), and
- adenomas and differentiated thyroid carcinomas of follicular cell origin (Sheu 2005).

In an alternative embodiment, the oligonucleotide or oligonucleotide analogue can be used to prevent or treat erectile dysfunction.

In particular, the oligonucleotide or oligonucleotide analogue can be used to treat subjects who have the GNB3 825T allele. It can be used to treat subjects who are heterozygous or homozygous for the GNB3 825T allele.
In a further aspect the present invention provides an oligonucleotide or oligonucleotide analogue as set out above for use in a method of preventing or treating a disease. Such oligonucleotides or oligonucleotide analogues are, of course, especially useful for treating or preventing any one of the conditions set out above.

In a further aspect the present invention provides a nucleic acid construct which is capable of being transcribed in a cell to provide an oligonucleotide as set out above. Typically such a construct will comprise a sequence encoding the desired oligonucleotide associated with suitable transcription control elements. Such expression constructs are well known in the art and it is within the normal and routine skills of the skilled person to design a suitable construct for a particular situation. Furthermore, there are various commercially available expression constructs.

Clearly such a construct is only suitable for creating natural oligonucleotides as it is not possible to create non-natural oligonucleotides or oligonucleotide analogues in vivo using a cells transcription system.

In a further embodiment, the present invention provides a pharmaceutical composition comprising an oligonucleotide or oligonucleotide analogue as set out above formulated for pharmaceutical use and optionally further comprising a pharmaceutically acceptable diluent, excipient and/or carrier.


In a further aspect, the present invention provides a method of treating or preventing a disease in a subject, the method comprising administering a therapeutically effective amount of a pharmaceutical composition comprising an oligonucleotide or oligonucleotide analogue as set out above.

The method of administration can be any suitable technique known to the art. However, typically the pharmaceutical composition comprising an oligonucleotide or oligonucleotide analogue is injected into a subject, e.g. intravenously. Alternatively, more tissue specific delivery methods may be appropriate in certain circumstances.

Systemic delivery into many cells in adult organisms can be accomplished by using covalent conjugates of morpholino oligos with cell-penetrating peptides, and, while toxicity has been
associated with moderate doses of the peptide conjugates, they have been used in vivo for effective oligo delivery at doses below those causing observed toxicity. For example, an octa-guanidinium dendrimer attached to the end of a morpholino can deliver the modified oligo (called a "Vivo-Morpholino") from the blood to the cytosol. Accordingly, such covalent conjugates form a preferred embodiment of the present invention.

The invention therefore includes pharmaceutical compositions which may include, in addition to active oligonucleotide or oligonucleotide analogue, a pharmaceutically acceptable diluent, excipient and/or carrier. Such compositions may be used in the methods of the present invention. Additionally or alternatively, pharmaceutical compositions may include a buffer, stabiliser and/or other material well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the oligonucleotide or oligonucleotide analogue. The precise nature of the carrier or other material will depend on the route of administration, which may be any suitable route, for example by a parenteral route and particularly by infusion or injection (with or without a needle).

The route of administration is preferably intravenous injection or infusion. The route of administration may alternatively be subcutaneous injection.

For parenteral administration, the oligonucleotide or oligonucleotide analogue can be formulated as a solution, suspension, emulsion or lyophilized powder in association, or separately provided, with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 1-10% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives).

The compositions is preferably sterilised, e.g. by known or suitable techniques.

Formulations for parenteral administration can contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. Aqueous or oily suspensions for injection can be prepared by using an appropriate emulsifier or humidifier and a suspending agent, according to known methods. Vehicles for injection can be a non-toxic diluting agent such as aqueous solution or a sterile injectable liquid. As the usable vehicle or solvent, water, Ringer's solution, isotonic saline, etc. are allowed; as a solvent or suspending liquid, a sterile non-volatile oil can be used. For these purposes, any kind of non-volatile oil may be used,
including natural or synthetic or semisynthetic fatty oils or fatty acids; natural or synthetic or semisynthetic mono- or di- or tri-glycerides.

The compositions for administration, therefore, can include a solution of the oligonucleotide or oligonucleotide analogue dissolved in a pharmaceutically acceptable carrier, such as an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilised by conventional, well known sterilisation techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride and/or sodium lactate. The concentration of active in these compositions can vary widely, and may be selected based on fluid volumes, viscosities and/or body weight in accordance with the particular mode of administration selected and the subject’s needs.

Amounts effective for therapeutic use, which may be a prophylactic use, will depend upon the severity of the disease and the general state of the patient’s health. A therapeutically effective amount of the oligonucleotide or oligonucleotide analogue is that which provides either subjective relief of a symptom(s) or an objectively identifiable improvement as noted by the clinician or other qualified observer.

An oligonucleotide or oligonucleotide analogue of the disclosure may be administered in conjunction with another active agent, whether simultaneously, separately or sequentially. The other active agent may be a second active agent of the invention or an active agent falling outside the invention.

Single or multiple administrations of the compositions of the disclosure are administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the oligonucleotide or oligonucleotide analogue disclosed herein to effectively treat the patient, bearing in mind though that it may not be possible to achieve effective treatment in every instance. The dosage can be administered once but may be applied periodically until either a therapeutic result is achieved or until side effects warrant discontinuation of treatment. The dose may be sufficient to treat or ameliorate symptoms or signs of disease without producing unacceptable toxicity to the patient.
Specific embodiments of the invention will now be described, by way of example only, with reference to the accompanying drawings. These specific examples should in no way be considered to be limiting of the scope of the invention.

5 **Brief Description of the Figures**

- Figure 1 – An illustration of G protein signalling. Heterotrimeric G proteins are activated in response to stimulation of G protein-coupled receptors by light, small, numerous hormones, neurotransmitters, chemokines, pharmacological agents, and other substances. Upon activation, the heterotrimeric G protein dissociates into its α- and βγ-subunits, which transduce signals to various effectors in the cell illustrated here for the different subunit groups (diagram adapted from Melien 2007).

- Figure 2 – A schematic of the structure of GNB3. The gene encoding the β3 subunit of heterotrimeric G proteins consists of 11 exons, the start codon ATG being located in exon 3 and the stop codon TGA in exon 11. The C825T polymorphism is located within exon 10. Both a regularly but also an alternatively spliced Gβ3 mRNA is observed in 825T allele carriers. The C>T transition in exon 10 supports alternative splicing, approximately half of the time, in a new cryptic splice site, in the middle exon 9 (cDNA position 620). The alternatively spliced mRNA displays a deletion of 123 nucleotides corresponding to 41 amino acids Diagram adapted from Siffert 2000.

- Figure 3 – The proposed structures of both the Gβ3 (GNB3n) and the Gβ3s (GNB3s) protein subunits. Gβ-subunits form a propeller-like structure with seven so-called propeller blades (top). The deletion resulting in the generation of Gβ3s is indicated (hatched background; top). This results in a novel Gβ3 protein with only 6 propeller blades (bottom) Diagram adapted from Siffert et al. 2000.

- Figure 4 – Systolic blood pressure (SBP) distribution according to GNB3 genotype and obesity status; anova P values were adjusted for age and ethnicity. Diagram taken from Danoviz et al. 2006.

- Figure 5 – Global distribution of the chromosomal frequency of the GNB3 825T allele. Diagram taken from (Siffert, Rosskopf and Erbel 2000).

- Figure 6 – Arrangement of 6-multiwell plates.

- Figure 7 – Lanes 2-7 GNB3 semi-quantitative RT-PCR results using total RNA extracted from β3s-blocker Morpholino (M), GNB3 anti-sense (AS) unmodified oligonucleotide (SEQ ID NO 5), and mock treated human EBV transformed cell lines. The two treated cell lines were a GNB3 825T homozygote (TT) and a GNB3 825T/825C heterozygote (CT), from normal Nigerian Yorba tribe EBV transformed lymphoblasts.
commercially available from Cornell University USA. These results show a clear
decrease in the expression of the smaller 8-9b GNB3 (367bp) transcript that translates
the aberrant Gβ3s protein, following transfection treatment with the β3s-blocker
Morpholino (M), see lanes 3 and 6. There also appears to be a clear increase in the
amount of normal 8-9a (490bp) transcript following treatment with this morpholino (M).
No significant differences in either 8-9a or 8-9b transcripts were observed using either an
antisense oligo (AS) or a mock treatment. Control Lanes 8-13 shows that actin
expression was unaltered by any of the three treatments. Lane 1 is a Hyperladder II
(Bioline) size marker.

Figure 8 - Organisation of Exons 8, 9a, 9b and 10 of the GNB3 gene. This diagram
(adapted from Siffert et al. 1998) shows the proposed mechanism of action of the β3s-
blocker. In homozygote c.825C individuals splicing occurs only from exon8 to exon9a.
Exon 9a is coloured red in the lower part of the diagram and only exons 8, 9a/9b and 10
are shown in the upper part of the diagram. Whereas in individuals carrying the c.825T
allele exon 8 splicing occurs half of the time with exon9a and half of the time with
Exon9b. The exon 8-9a transcripts are translated to produce "normal" Gβ3 protein. The
exon 8-9b transcripts, however, are translated to produce the smaller aberrant Gβ3s
protein. The β3s-blocker is an antisense morpholino that is only complementary to the 9b
splicing site and was designed as an exon "skipping" morpholino using the "Genetools"
bioinformatic software. If this morpholino were to work like a DMD specific "exon
skipping" morpholino, Then you would expect the morpholino to block the splicing of
exon 8 to exon 9b and catalyse the splicing of exon 8 to exon10.

Figure 9 - Structures of conventional DNA and morpholino oligonucleotides. (A)
Conventional DNA oligonucleotide. (B) Morpholino oligonucleotide. Note the six-
membered morpholino ring in B and the non-ionic phosphorodiamidate link between the
two rings (Diagram adapted from Eisen and Smith 2008).

Figure 10 - Lack of ERK2 and AKT phosphorylation in D153del GNB3 chickens.
Phospho immuno reactivity of ERK2 (MAPK) and AKT in GNB3 D153del affected retina,
brain, heart liver and kidney. Mutant rge chicken protein extracts showed significant
changes in relative phosphorylation activity when compared to normal age matched
controls that was confirmed by semi quantitative densitometry analyses. Experiments
represent a total of n=3 independent experiment. Statistical significance is shown as *
P<0.05, **P<0.01, ***P<0.001. (adapted from Tummala et al. 2011)

Figure 11 - Tissue specific regulation of cAMP levels are, shown by a significant fold
increase in GNB3 D153del affected retina and brain samples and decreased in D153del
affected heart, liver and kidney, when compared to levels of normal controls. Data are
the mean with standard error (bars) of n=3 independent experiments performed in octuplets. Statistical significance is shown as * P<0.05, **P<0.01, ***P<0.001. (adapted from Tummala et al. 2011)

Figure 12 - Hypothetical interaction between the G protein β3 subunit C825T polymorphism and ACE I/D polymorphism leading to increased activity of sodium–proton exchanger in erythrocytes. R = Angiotensin II receptor, which activates the bound heterotrimeric protein consisting of Gai, Gβ3 or Gβ3s (c.825T allele) and a γ subunit. Diagram adapted from Kedzierska et al 2006

Figure 13 - Model for the pleiotropic regulatory action of aldosterone via Gβ3 and Gβ3s. Aldosterone controls the expression of K-Ras and SGK in target epithelia. Thereby, it controls the level of efficiency of signalling pathways, which regulate ENaC function and/or surface expression. Similarly, aldosterone has a potentiating activity on antidiuretic hormone-induced cAMP production and stimulatory action on ENaC activity by inducing the expression of as yet not determined elements of this pathway (stippled arrows point to putative induced proteins). Abbreviations are: Ins, insulin; aldo, aldosterone; ADH, antidiuretic hormone; V2, vasopressin (antidiuretic hormone) receptor V2; Gs, trimeric G protein s subunit; AC, adenylyl cyclase; PKA, protein kinase A; IR, insulin receptor; PI3K, phosphatidylinositol 3-kinase; PInsP3, phophatidylinositol 3-phosphate; PDK1, 3-phosphoinositide-dependent protein kinase 1; sgk, serum and glucocorticoid-induced kinase. Diagram adapted from Verrey et al. 2001.

Specific Description of the Invention

The vertebrate body contains, in its different multi differentiated cell types, over 1,500 different, G protein coupled receptors (GPCR), which when activated by different ligands e.g. hormones, light and smell, alter their shape and in turn stimulate a bound hetero-trimeric G protein complex. This hetero-trimeric complex comprises of a single alpha, a single beta and a single gamma subunit. There are 22 different known alpha subunits, 11 different gamma subunits, but only 6 different beta subunits, namely GNB1, GNB2, GNB3n, GNB3s, GNB4 and GNB5 (Hamm 1998, Milligan and Kostenis 2006, Siffert et al. 1998).

Following GPCR stimulation the alpha subunit of the heterotrimeric G protein is further activated by GTP (G) and subsequently splits from the residual beta gamma dimer. Both the G activated alpha subunit and the beta gamma dimers then independently bind and activate a plethora of different downstream signalling proteins (see figure 1) e.g. map kinases, phosho kinases, phosphodiesterases and phospholipaseses, which in turn control a plethora of cellular processes e.g. growth, light and smell detection (Bando et al. 2006, Clapham and
In the majority of humans the β subunit can be one of 6 different subunits namely:

- GNB1 (Gβ1),
- GNB2 (Gβ2),
- GNB3* (Gβ3 normal transcript, also known as GNB3n),
- GNB3s* (Gβ3s variant transcript),
- GNB4 (Gβ4),
- GNB5 (Gβ5),

The different structural shapes of the Gβ subunits determine which γ and α subunits they preferentially bind to in a particular cell type and also the level of activation for each particular pathway.

* The same GNB3 gene encodes both the Gβ3 and the common variant Gβ3s protein. Only humans carrying the more common c.825T variant express the Gβ3s protein.

Most heterotrimeric G proteins are generally ubiquitously expressed, with the notable exception of some retinal specific alpha subunits, e.g. GNAT1 (rod specific) and GNAT2 (cone specific) (Deeb 2004, Fu and Yau 2007). In contrast GNB3 is ubiquitously expressed in almost all tissues and cell types examined, e.g. retinal cones, kidney, heart, brain, (Sun et al. 2005), with the notable exception of retinal rods (Peng et al. 1992). It is therefore not surprising that my research group identified significant changes in phosphor-ERK, GRK2, cAMP, cGMP in several different tissue types in a naturally occurring recessively inherited deactivating GNB3 chicken mutant (del153D) (Tummala et al. 2011). These chickens were initially only diagnosed with a retinopathy globe enlarged (rge) phenotype (Montiani-Ferreira et al. 2005, Montiani-Ferreira et al. 2003, Montiani-Ferreira et al. 2007, Tummala et al. 2008). We have recently also shown that these chickens also suffer from an early onset renal pathology with severely enlarged glomeruli and macrophage invasion (Tummala et al. 2011). Recently a homozygous recessive GNB3 knockout mouse model has been created in the USA (Dhingra et al. 2012), which also exhibit neural retinal signalling defects but without any apparent renal defects (Prof Stewart Fleming, Dundee University personal communication).
The human GNB3 825C>T common variant that produces the GNB3s subunit

To date no human mutant has been identified with anything resembling an rge/abnormal renal pathology phenotype and/or a severe deactivating GNB3 mutation. Approximately half of all humans, however, are carriers of the common synonymous base pair change 825C>T in exon 10 of the GNB3 gene (reviewed by (Sifert 2000). The GNB3 825T allele in exon 10 paradoxically leads to the induction, in half of all mRNA transcripts, of an alternative splice site in the middle of the “normal” exon 9 (Sifert et al. 1998). This leads to the deletion of 123bp of mRNA sequence, which when translated results in a 41 amino acid smaller protein named GNB3s (see figures 2 and 3). The GNB3s protein is highly stable and appears to enhance downstream signalling processes (Kedzierska et al. 2006, Pietruck et al. 1996, Sifert et al. 1995, Sifert et al. 1998, Virchow et al. 1999, Lindemann et al. 2001, Rosskopf et al. 2003).

Link between the GNB3 825T allele and hypertension

In 1998 Sifert et al. first demonstrated, in a small German population study, that there is a significant link between the common GNB3 825T allele and a predisposition to essential hypertension (Sifert et al. 1998). Subsequent larger individual studies and meta-analysis, in many different population groups ((Andersen et al. 2006, Bagos et al. 2007, Klenke, Kussmann and Sifert 2011, Lu et al. 2012, Marques et al. 2010, Niu and Qi 2011, Takeuchi et al. 2012), have, with a few notable exceptions, appear to largely replicate Sifert et al’s (1998) initial results. The correlation of the GNB3 825T allele’s predisposition to hypertension, does however, appear to be generally stronger in individuals who are living in the higher latitudes of the world e.g. Europe, North America and Asia (Bagos et al. 2007, Niu and Qi 2011, Young et al. 2005). Globally, however, there also appears to be also a high correlation between the GNB3 825T allele, and increased blood pressure and symptoms of obesity and/or metabolic syndrome (e.g. diabetes, insulin resistance, autonomic nervous changes and dyslipidemia) in individuals regardless of ethnicity (Benjafeld et al. 2001, Chang et al. 2012, Danoviz et al. 2006, Dong et al. 2004, Dzida et al. 2002, Grove et al. 2007, Hauner, Rohrig and Sifert 2002, Rosskopf, Manthey and Sifert 2002, Hsiao et al. 2012, Andersen et al. 2006, Bozkurt et al. 2009, Chistiakov et al. 2009, Daimon et al. 2008, Fernandez-Real et al. 2003, Kiani et al. 2005, Lin et al. 2009, Ohshiro et al. 2001, Pollex et al. 2006, Shcherbak and Schwartz 2001). For example a study involving Brazilians of different ethnic backgrounds demonstrated a strong, dose dependant, correlation of higher
blood pressure and the GNB3 825T allele (see figure 4), in Obese, but not in non-obese individuals (Danoviz et al. 2006)

Danoviz et al. (2006) clearly show that obese homozygote 825T/825T individuals have significantly higher blood pressure than heterozygote 825T/825C obese individuals, who in turn have a significantly higher blood pressure than obese homozygote 825C/825C individuals (see figure 4). These results would suggest that the successful genetic inhibition of the GNB3s specific transcript, would be likely to significantly reduce the blood pressure in hypertensive patients that carry the 825T allele.


**Global distribution of the GNB3 825T allele**

The 825T allele is particularly prevalent in people of Sub-Saharan African descent (>65% in all populations studied including Afro Americans and West Indians) and in Australian Aborigines (see figure 5). Its allele frequency is also > than 40% in East Asia and North Africa with a maximum allele frequency of 56% in an Egyptian population study. In the rest of Asia and in people of European descent the allele frequencies are lower ranging from 21-36% (Pemberton et al. 2008, Rosskopf, Manthey and Siffert 2002, Vargens et al. 2008). For example the frequency in population studies in the UK have estimated the frequency to be approximately 30%. Using the Hardy Weinberg equilibrium formula (p² + 2pq + q² = 1) this equates to an estimated frequency in the British population of 9% 825T/ 825T homozygotes and 42% 825T/825C heterozygotes. This means that >50% of individuals in the UK are likely to carry at least one 825T allele. Applying the same formula in individuals of Sub-Saharan African descent, including Afro-americans >90% are likely to carry at least one 825T allele.

**Development of a novel morpholino sequence, that specifically down regulates the specific expression of the GNB3 825T transcript.**
Not surprisingly GNB3 has previously been touted as a drug target (reviewed by Klenke and Siffert (2011), but to date no such drug has been published in the public literature or patent database on this topic. Moreover any such drug should preferably be designed specifically to: a) inhibit the translation of the 825T allele leading to a reduction in the production of GNB3s protein or b) to inhibit the action of the GNB3s protein itself. Development of a non-specific GNB3 mRNA or protein inhibitors may cause renal abnormalities and/or retinopathy phenotypes which are observed in our rge chickens. This is because the normal GNB3 (and probably the GNB3s variant) protein(s) are probably essential for both normal retinal and renal function (Tummaia et al. 2011).

Interestingly heterozygous del153D/wt chickens do however, appear to be completely asymptomatic and live as long as their normal sibs (Montiani-Ferreira et al. 2005, Montiani-Ferreira et al. 2003, Montiani-Ferreira et al. 2007). This observation suggests that a normal vertebrate phenotype can be maintained with only 50% normal GNB3 protein.

Inspired by the recent success of an exon skipping, sequence specific, morpholino in the treatment of human Duchenne Muscular dystrophy patients (Kanehisa et al. 2006, Berger et al. 2011, Brolin and Shiraishi 2011, Cirak et al. 2011, Heemskerk et al. 2009, Hu et al. 2010, Jearawiriyapaisarn et al. 2010, Kinali et al. 2009, Lu and Wu 2012, Malerba, Boldrin and Dickson 2011, McClorey et al. 2006, Mendell et al. 2012, Moulton and Moulton 2010, Popplewell et al. 2010, Popplewell, Malerba and Dickson 2012, Sazani et al. 2011, Sazani, Weller and Shrewsbury 2010, Widrick et al. 2011, Wu et al. 2011a, Wu et al. 2011b, Yin et al. 2010, Yokota, Duddy and Partridge 2007, Yokota, Hoffman and Takeda 2011, Yokota et al. 2012), I therefore similarly designed a sequence specific morpholino to the abnormal splice site in exon 9 of the GNB3 gene, with the hope that only abnormally spliced GNB3s producing mRNA would be targeted. This morpholino was expected to abnormally fuse exon 8 to exon 10 in only GNB3s mRNA leading to an even smaller GNB3 protein with a premature stop. Theoretically such a protein would be likely to be unstable and rapidly degraded by the Ubiquitin proteasome system. This in theory would result in a reduction in the pathological Gβ3s affecting the levels of normal Gβ3 protein in GNB3 825T individuals.

2. Materials & Methods

2.1.1 Materials

- 0.02% EDTA Solution – Sigma-Aldrich
- 6 Multi-well Plate
- 2x My Taq – Bioline
- A549 Cells –
- Advanced RPMI 1640 – Invitrogen
- Agarose Powder – Sigma-Aldrich
- Antisense Morpholino Oligonucleotide – Gene Tools
- Chloroform – Sigma-Aldrich
- Cos-7 Cells –
- Dulbecco’s Modified Eagle Medium – Invitrogen
- Green Fluorescent Protein expressing plasmid
- GM18500 (TT) Lymphoblast Cells - Coriel University Cell repository
- GM18506 (CT) Lymphoblast Cells - Coriel University Cell repository
- HaCaT Cells
- HeLa Cells
- Hyperladder II – Bioline
- Lipofectamine™ - Invitrogen
- M-MLV RT – Invitrogen
- MCF-7 Cells – ACE Labs
- Morpholino – Gene Tools
- OPTI-MEM – Invitrogen
- Phosphate Buffer Saline – Invitrogen
- Primary Antibody (Rabbit Anti-Chicken GNB3) – Dr. Doug Lester
- Primers GNB3 exon 7-10 (25 nmol) – Sigma-Aldrich
- Primers GNB3 Full Length (45.1 nmol) – Sigma-Aldrich
- Primers Actin (31.1nmol) – Integrated DNA Technologies
- QIAzol – Qiagen
- RNeasy mini prep Kit – Qiagen
- Safeview Safe Nucleic Acid Dye – ABM
- Secondary Antibody (Anti-Rabbit IgG with Alexafluor® 568) – Invitrogen
- T-50 Culture Flasks
- Trypsin – Invitrogen
- Vectashield® (Containing DAPI) – Vectorlabs

2.1.2 Morpholino oligo design
The inventor submitted the following GNB3 aberrant for morpholino oligo design to the Gene Tools exon skipping morpholino algorithm www.gene-tools.com:

mRNA Sequence #1: cuuccacauuuuggggccugugauuggcagUGCAAGCUCUGGGGAUGUGCG (SEQ ID NO 1)

This tool produced the following morpholino design statement:

*Here is a Morpholino oligo sequence written from 5' to 3' and complementary to your splice junction target:*

CACTGGGCAATCACAGGCCCCCGAAAT (SEQ ID NO 4)

The Morpholino sequence to order is given above and the text below is provided for your information.

Brackets have been inserted around the pre-mRNA target to illustrate its position in your sequence [shown below]. Note that the brackets are placed on a sense strand.

cuuccacauuuuggggccugugauuggcagUGJCCAAGCUCUGGGGAUGUGCG (SEQ ID NO 1)

The morpholino GNB3-specific aberrant splice blocker sequence below was therefore ordered from Gene Tools, i.e. 5'- CACTGGGCAATCACAGGCCCCCGAAAT- 3' (SEQ ID NO 4)

This sequence is the reverse compliment which crosses the aberrant splice site, this is shown below in the coding cDNA which starts within exon 3 (the dotted underlining alternates on and off differentiating between adjacent exons):

1 ATGGGGAGATGAGCAACTGCTAGGAAGCAGCCAGCTCAAGAAGCAGATTCGAGAT
61 GCCAGGAAGCCTGTGCTGACGTTTACCTGCTGCCGAGACTGGTGTCTGGCTAGAGGTGTG
121 GGACGAGTCCAGATGCCGGACGCGCCGACGTTAAGGGCGACACCTCGCCAAGATTTACGCC
181 ATGCACTGCGCCACTGATTCTAAGCCTGTGGTAAAGCTCGGACCAAGATGGGAGGCTGATC
241 GTGTGGGACACGCTACACACCAACAAAGGTGCCATGCAATCCCCACTGCCGCTGCTTCCGCTG
301 ATGACCTGTGCTATGCCACATCGGAACCTTGGTCATGTTGGGGCTGGGACAACTATG
361 TGTTCCATCTCAAACACTCAGATGCCGAGCTGGGCAATGTCAAGGTTCAAGGCGCCGCGAATTTCT
421 GCTCAACACAGGTATCTCTCCCTGCTGCCGCTTTCGATGACAAACATATTGTGACCCAGC
481 TCGGGGGCCCTGTGATGCCagTGCAAGCTCTCCGTGGATGAGACTGGGGCAGCAAGATGCTGATT e9
541 GTGGGACACCGCGTGACTGCTGATGCCCTGCTGCTCTCTCCTGACTCTMATCTTCCATT
601 TCGGGGGCCCTGTGATGCCaGCGCTCAGCTGCTGAGAGGAGAGGAGCAGCTGCGCCTCAG
661 ACTTTCACGCGACGATCGGCRCTCAACGGACATCTGTTCCTCCCAATGGAGGAGCC e10
721 ATCTGGACGCGCTGCTGATGCGGGTCTCTGGCGGCTGTTGAGCCCTGGGCGGAGACCAGAGG
781 CTGATCTGACTCTCTCCACGAGACCATCATCTCGGCGCACTACGTCyGTGCGCTTCTCCCTC
841 AGTGSGCGCCCTACTATATTGCCTGCTACGACGACCTTCAACTGTCATGCTGGGACTCCAAT
5 901 AAGCTGACGCAGCTGGGACTCCCTTCTCTGGCCACGATAACAGGGTTGACGTGCCTGGGAGTC
961 ACGATGCGACGGATGGCGCTGTGGGCGCAACAGGTTCCTGGGACAGCTTCTCCAAAATCTGGGAC
(SEQ ID NO 6)

Key:
10 – Bold & Solid Underlined = Homologous site of Morpholino in Exon 9
– Aberrant Acceptor Site in Exon 9
– y = GNB3 825C>T polymorphic site

When the Antisense Morpholino binds to the homologous sequence in exon 9 (e9) on the mRNA, in theory this should block the aberrant acceptor site causing the next acceptor site to be used instead which is the ‘ag’ sequence before exon 10 (e10).

2.1.2 Antisense Oligonucleotide design

20 This molecule was designed by targeting the GNB3s variant which has had the 123bp spliced from exon 9. The antisense oligonucleotide is complementary to the last 10 bases in exon 8 and the first 10 bases after the aberrant ‘AG’ splice site in exon 9. The reverse complement sequence for the GNB3s “preferential” antisense oligonucleotide is as shown below:

5’GAGCTTGGCACACGCTGTG3’ (SEQ ID NO 5)

In theory this oligonucleotide will bind preferentially to the GNB3s mRNA transcript and less, or not at all, to the normal GNB3 transcript.

30 Normal GNB3:

GUAUCUCUCUCUCUCGUCCGUCCGUCCGUUCUGGGAGACAAAAUUUGUGACCGGGGCACACCCAGUG
GGUGGGAACUUGAGACUCCGACCAAGACACUGUUAUUUGUGGGACACACGGGGUGACUGGCAUGAG
35 CCUGGUGCUGUGCUGUCCUCACUUACUUCUACAUUCGCGGGGCUGUGAUGCCAGUCCGCAAGUG
GUGCGAGAGGGAGCAGGCAGGCAUCUCAGUGCGGACACGUGGAAUCAACGCCAUUCUGU
(SEQ ID NO 7)
GNB3s Slice Variant:

GUUAAUCUCUCCUCCGCGCCUUCCUGGAUGACAACAAUAUUUGUGACCAGUCGGGGAACACCACGUGU
GCCAAGCUCUGGAAGUGCCGAGGGACCUGCUCGUCAGACUUUCACUGGCACCAGGACACUCAAGCGC
CCAUUCGU (SEQ ID NO 8)

Key:
- **Bold** = Binding site(s) of Antisense oligonucleotide
- Exon 8 is shown by dotted underlining

2.2.2 Design of GNB3 Exon 7 – Primers

These primers were designed around exon 9, so that both the normal GNB3 and the GNB3s splice variant can produce bands that only differ due to the deletion of the 123bp. A sequence was selected and using primer3 (Primer3) the primers were designed to produce the desired PCR product.

e7  GAGCTTTTCTGCTCACACAGTTAT
20  CTTCTCCTGCTGCCCTTCCTGGATGACACAAATATATTGTGACCAGCTCAGGAGACACCAG

Key:
- 123bp deleted portion
- **Splice sites involved with 123bp deletion**
- y = C825T Site
- **Forward primer target**
Reverse primer target

The forward primer starts at the end of exon 7 and into the beginning of exon 8. The reverse primer is located close to the end of exon 10, the C825T (cDNA) polymorphism site has been included so that if possible with other cells the band can be removed and put through a gel extraction process and sequenced to check for the genotype. Therefore using these primers, if the normal GNB3 gene is being expressed there should be a band of 490bp, and if the GNB3s variant is being expressed there will be a band of 367b (123bp smaller than the GNB3 normal product). If the morpholino is successful in causing the skipping of exon 8 completely then you would expect a band of 278bp (202bp smaller than the GNB3 normal product).

The following sequence summarises the significant sequences associated with the GNB3 coding sequence, including the morpholino and primer target sequences. The sequence in upper case is the common sequence between GNB3 (normal) and the GNB3s transcript. Lower case sequence represents GNB3s 'intronic' sequence, which is spliced out GNB3s transcript only. Also shown are the

GNB3 coding cDNA sequence with PCR primer locations:

```
1 ATGGGGGAGATGGGACACTGCTAGGAAGCGAGCGCAGCTCAAGAAGCAGATTGCAGAT
61 GCCAGAAAGCCCTGTGCTGACGTATCTCTGCGAGAGCTGCTGCTGCGCTAGGCTGTT
121 GAGCGAGTCGCTAGTGCGGACGACGCAGCGCTCGTTAAGGGGCAACCAGCTGGCAAGATTTACGCC
181 ATGGACCTTGGCGCACTGATTCTAAAACTGTGCGCTGAGTGTGAGTGGGAAGCTGAT
241 GTGTGGGACACTCACCAACAAACAGGTTCGACCCATCCCACCTGGTGATGAAATTTTCCTCGGCTGATCC
301 ATGGCCCTTGCTCTATGCCCCATCAGGGAAACTTGTGGCAGTGTGGGGCTGGGACACATG
361 TGGTCCTACATCTCAAATGTCCCCCTGAGGGGAAATGTCAAAGTGCAGCCGCGGGAGCCTTCTCT
421 GCTCACACAGGTCTATCTCTTCGTTCCCTCTGGATGACAAACATATTGTGACCAGC
481 TCGGGGACACCACGGTGGTGTCTGTGGGAGAGCGGAGCGAAGGACACTgtatttt
541 gttggacacacaggtgtgctgtgactggtacgctggtctgctgctctcattttcatt
601 tcgggggctgtgtgacgcTGGCCAAGCTCTGGGATGTCGAGAGGGACCTGGCGCTGAG
661 ACTTTACGTGACCAGCTGGCAACATCAACGCCATCCTGCTTCTCTCTCAATGAGGAGGCC
721 ATCTGACACGGGCTGCGATGCTGACCTGCGCTCTGGTGTGACCTGGGGAGCGACAGCAAGGAG
781 CTGATCTGCTCTCTCAACGAGAGCAGCAGTTCTGCGGCACTCAGTGTGCTGCGCTTCTCCCTC
35 841 AGTGGCGCCCTACTATTCGCTGCGTCAGGACTTCAACTGCAAAGCTCGGACTTATG
```
5 Forward PCR primer – **GAGCTTTCTGCTCACACAGG** (SEQ ID NO 11)
Reverse PCR primer location - **CAATGTCTGGGACTCCATGA** (SEQ ID NO 12)
Actual Reverse primer (reverse complement) – **TCATGGAGTCCCAGACATTG** (SEQ ID NO 13)
Actual Morpholino sequence – **Cactggcatcagcccccgaaat** (SEQ ID NO 4)
10 Reverse complement Morpholino – **attcggggcctgtatgcgcagTG** (SEQ ID NO 14)

### 2.2 Cell Culture

The GM18500 (TT) and GM18506 (CT) Nigerian Yorba tribe, transformed, lymphoblastoid cells, obtained from Coriell Cell Repositories (http://ccr.coriell.org/Default.aspx), were collected from liquid nitrogen storage, and the screw cap vials were added into a thermomixer at 37°C to thaw for 1 – 2min. Before the vials were added to the Class 2 fume cupboard they were sprayed with 70% alcohol. For each cell line, 30ml of RPMI 1640 culture medium was added to a T-50 culture flask (50ml), and the cells were added drop by drop into the medium and then agitated to ensure even spreading. Each of the flasks were incubated upright at 37°C, for 24-48hours. The cells were removed after their incubation and their confluence was checked under the microscope and by assessing the colour change of the media. Once confluent, the cells were sub-cultured, so that the same cell line could be used in subsequent experiments.

### 2.3 Cell Line Sub-Culturing

The two cell culture flasks of Lymphoblastoid cells were removed from the incubator, and were examined to ensure there was no contamination and that the cells were confluent. The two flasks were added to the Class 2 fume cupboard, the cells were re-suspended by gently swirling the flask until evenly spread. For both cell lines 30ml of the cell suspension was aseptically removed and added to a falcon tube, then placed in the centrifuge 5804R for 10mins at 720g. Once it finished the supernatant was removed and the pellets were checked for. The pellets were re-suspended in 2ml of sterile Phosphate-Buffered Saline (PBS). Into 4 T-50 culture flasks, 20ml of RPMI 1640 was added, and then into the 2 flasks 1ml of the re-suspended GM18500 (TT) lymphoblastoid cells were added, and into the other 2 flasks

23
1ml of the re-suspended GM18506 (CT) cells was added into each. The flasks were incubated for 24 hours at 37°C, until fully confluent.

2.4 Transfection (Lipofectamine™)

Two cell culture flasks, one containing GM18500 (TT) and the other GM18506 (CT) Lymphoblastoid cells were removed from the incubator, and placed in the culture hood. The cells re-suspended and added to a 10ml falcon tube, and pelleted in the 5804R Centrifuge for 10 min at 720g, afterwards the supernatant was removed, leaving the pellet. The pellets were gently re-suspended in 1000µl of Advanced RPMI 1640 (Reduced serum medium). Two tissue culture 6-multiwell plates were placed in the culture hood and both labelled as shown in Figure 6.

One of the two plates was for RNA analysis and the other for Immunocytochemistry (ICC). 100µl of the cell suspension was added to each corresponding well along with 1000µl of Advanced RPMI 1640 media into each of the 12 wells. Four 1.5ml eppendorf tubes were labelled, and the following was added to each as below in Table 1 below:

<table>
<thead>
<tr>
<th>Labelled Tube</th>
<th>Solution</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Morpholino DNA&quot;</td>
<td>4µl Morpholino (1mM)</td>
<td>200µl OPTI-MEM</td>
</tr>
<tr>
<td>&quot;Morpholino Lipo&quot;</td>
<td>12µl Lipofectamine™</td>
<td>200µl OPTI-MEM</td>
</tr>
<tr>
<td>&quot;Antisense DNA&quot;</td>
<td>4µl Antisense Oligo</td>
<td>200µl OPTI-MEM</td>
</tr>
<tr>
<td>&quot;Antisense Lipo&quot;</td>
<td>12µl Lipofectamine™</td>
<td>200µl OPTI-MEM</td>
</tr>
</tbody>
</table>

20 The 4 eppendorf tubes were incubated for 10 min at room temperature. After incubation, the 2 tubes labelled GNB3s specific Morpholino (Gene Tools) were mixed together and the 2 tubes labelled Antisense were mixed together along with 3µl of Green Fluorescent Protein (GFP) Plasmid. The 2 mixed tubes were then incubated for a further 20 min, allowing the lipofectamine™ (Sigma) to form a complex with the Morpholino and Antisense Oligonucleotide. 100µl from the Morpholino/Lipofectamine tube was added to the 4 Morpholino wells, and 100µl from the Antisense/Lipofectamine tube was added to the 4 Antisense wells. For the 4 mock samples 12µl Lipofectamine™ was mixed with 200µl OPTI-MEM, incubated for 10min, then 3µl of GFP was added and allowed to incubate at room temp for 20min before being added to the mock cells. The multi-well plate was shaken gently back and forth to insure distribution of the lipofectamine complex. Another 1000µl of Advanced RPMI 1640 was added to each of the 12 wells. These were placed in the incubator at 37°C overnight (around 24 hours). Afterwards the multi-well plates were
removed from incubation and to each of the 12 wells, 3ml of DMEM was added. The 2 6 multi-well plates were then incubated for a further 48 hours at 37°C.

2.5 RNA Extraction

After incubation the 6 multi-well plate for RNA analysis containing Morpholino and Antisense transfected GM18500 (TT) and GM18506 (CT) Lymphoblastoid cells and un-treated mocks for each, were removed from the incubator and placed in the culture hood. The cells were removed from the surface by trypsinization with 500 µl Trypsin, and incubated at 37°C. Once cells had detached, 1ml of DMEM was added, the cells from each were added to labelled 1.5 ml eppendorf tubes and added to the Eppendorf Centrifuge 5814R and centrifuged at 424.8 g for 10 min. Afterwards the supernatant was removed and only the pellet remained. The eppendorf tubes were placed on ice and to each 100 µl of QIAzol (Qiagen) was added and mixed by pipetting until the cell pellet was fully re-suspended, the tubes were then incubated at room temperature for 5min, before being placed back on ice. To each eppendorf tube 20 µl of chloroform was added and vortexed for 10sec, then incubated for 3min at room temperature, before being placed back on ice. The eppendorf tubes were then centrifuged at 23,264 g for 15min at 3 °C. After this each eppendorf tube presented 3 phases: a top colourless aqueous phase, a middle white interphase and a lower red phenol-chloroform phase. The upper aqueous phase containing the RNA was removed from each eppendorf tube without disturbing the 2 lower phases and added to a new labelled 1.5ml eppendorf tube. The RNA was precipitated by adding 50 µl of isopropanol and incubated for 10min at room temperature. The 6 eppendorf tubes were centrifuged at 23,264 g for 10min at 3°C. The supernatant was removed and only a gel pellet of RNA remained in each. The pellets were washed by mixing with 100µl of 75% ethanol, and being centrifuged at 239 g for 10min at 3°C, the supernatant was removed and this wash step was repeated. Afterwards the RNA pellet was left for the ethanol to evaporate for 7min. The RNA pellet was then re-suspended in 50 µl of DNase free water and the eppendorf tubes were stored at -80 °C.

2.6 RNA Purification

To remove any DNA contamination that may be present within the RNA samples, the RNeasy Mini prep kit from QIAGEN was used to purify the RNA. The 6 eppendorf tubes were removed from the -80°C freezer and thawed and placed on ice. To each tube the following was added:
5 The 58 μl reactions were incubated at 37°C for 30min. After incubation, 2μl of 1mM EDTA was added (as it is an exonuclease inhibitor it will inhibit DNase I) and incubated at 85°C for 5min to inactivate the reaction. Each tube had its volume adjusted to 100μl by adding 40μl of RNase free water. To each 100μl reaction, 350μl of RLT buffer was added and mixed by pipetting, and then 250μl of 100% ethanol was added and mixed by pipetting. Each of the 700μl reactions were then added to RNeasy spin columns in a 2ml collection tube and centrifuged at 10,621g for 15sec, and the flow through from the collection tubes was discarded (without touching the tip of the column). 500μl of buffer RPE was added to each of the spin columns (closing the lid gently) and centrifuged at 10,621g for 15sec, and the flow through was discarded. Another 500μl of buffer RPE was added to the spin columns and they were centrifuged at 10,621g for 2min. The flow through was discarded and the spin columns were centrifuged again at 10,621g for 1min. The collection tubes were discarded and replaced with 1.5ml eppendorf tubes, and 50μl of RNase free water was added to each spin column and the columns were centrifuged at 12,851g for 1min. The 1.5ml eppendorf tubes containing the 50μl of purified RNA were removed from the spin columns, labelled appropriately (M-TT, M-CT, AS-TT, AS-CT, Mock-TT & Mock CT) and stored at -80°C.

2.7 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

25 2.7.1 Stage 1 (cDNA Synthesis)
The cDNA synthesis was undertaken using M-MLV Reverse Transcriptase (RT) (Invitrogen). The purified RNA was thawed and placed on ice, 6 new 1.5ml eppendorf tubes were labelled (M-TT, M-CT, AS-TT, AS-CT, Mock-TT & Mock CT) and into each, 5μl of the corresponding RNA was added and the tube placed on ice, and the other 45μl of RNA was stored back into the -80°C freezer. To each of the eppendorf tubes the following was added:

- 1μl OligoDT
- 1μl Mixed dNTP (10 mM each of dATP, dCTP, dGTP & dTTP)
- 12μl DNase free water
The eppendorf tubes containing the 19μl reaction were incubated at 65°C for 5min, and then chilled quickly on ice. After incubation, the eppendorf tubes were placed on ice and the following was added to each:

- 4μl 5x First Strand Buffer
- 2μl 0.1M DTT
- 0.2μl RNase Inhibitor
- 0.8μl DNase free water

The eppendorf tubes containing the 26μl reaction were incubated at 37°C for 2min then placed back onto ice. A dilution of the M-MLV RT was made, by adding 1μl of M-MLV RT (200units) to 23μl of DNase free water, 4μl of the diluted M-MLV RT was added to each eppendorf tube, bringing each of the reactions to 30μl. The eppendorf tubes were incubated at 37°C for 50min, and afterwards the reaction was deactivated at 70°C for 15min, and the eppendorf tubes containing the cDNA were placed on ice.

2.7.2 Stage 2 (PCR)

The necessary Vials were thawed: MyTaq™ Red Mix, DNase free water,

- GNB3 exon 7 – 10 Forward primer (5’GAGCTTTTCTGCTCACACAGG’3) (SEQ ID NO 11),
- GNB3 exon 7 – 10 Reverse primer (5’T CATGGAGTCCCAGACATTG’3) (SEQ ID NO 13),
- GNB3 Full Length (HINDGNB) Forward primer (5’GCAAGCTTGCCATGGGGGAGATGGAGC’3) (SEQ ID NO 15),
- GNB3 Full Length (xhol) Reverse primer (5’TCGAGTCAGTCCAGATTTTGGAGGAAGCTG’3) (SEQ ID NO 16),
- control Actin Forward primer (5’GCAAGACCTGTACGCCAAC’3) (SEQ ID NO 17),
- control Actin Reverse primer (5’GTCGACTACTCCTGCTTGCTG’3) (SEQ ID NO 18).

Each of the primers arrived at a different concentration, and then they were diluted by adding 5μl of the primer to 95 μl of DNase free water.

18 PCR tubes were labelled and into each were added as shown in Table 2 below.
Table 2 - This table shows what was added to the 18 PCR tubes before being placed in the thermo cycler.

<table>
<thead>
<tr>
<th>Tube</th>
<th>cDNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>MyTaq™ Red Mix</th>
<th>DNase free water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1µl TT Mock</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>2</td>
<td>1µl TT Morpholino</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>3</td>
<td>1µl TT Antisense</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>4</td>
<td>1µl CT Mock</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>5</td>
<td>1µl CT Morpholino</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>6</td>
<td>1µl CT Antisense</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>7</td>
<td>1µl TT Mock</td>
<td>1µl Exon 7-10</td>
<td>1µl Exon 7-10</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>8</td>
<td>1µl TT Morpholino</td>
<td>1µl Exon 7-10</td>
<td>1µl Exon 7-10</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>9</td>
<td>1µl TT Antisense</td>
<td>1µl Exon 7-10</td>
<td>1µl Exon 7-10</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>10</td>
<td>1µl CT Mock</td>
<td>1µl Exon 7-10</td>
<td>1µl Exon 7-10</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>11</td>
<td>1µl CT Morpholino</td>
<td>1µl Exon 7-10</td>
<td>1µl Exon 7-10</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>12</td>
<td>1µl CT Antisense</td>
<td>1µl Exon 7-10</td>
<td>1µl Exon 7-10</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>13</td>
<td>1µl TT Mock</td>
<td>1µl Actin Control</td>
<td>1µl Actin Control</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>14</td>
<td>1µl TT Morpholino</td>
<td>1µl Actin Control</td>
<td>1µl Actin Control</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>15</td>
<td>1µl TT Antisense</td>
<td>1µl Actin Control</td>
<td>1µl Actin Control</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>16</td>
<td>1µl CT Mock</td>
<td>1µl Actin Control</td>
<td>1µl Actin Control</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>17</td>
<td>1µl CT Morpholino</td>
<td>1µl Actin Control</td>
<td>1µl Actin Control</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>18</td>
<td>1µl CT Antisense</td>
<td>1µl Actin Control</td>
<td>1µl Actin Control</td>
<td>25µl</td>
<td>22µl</td>
</tr>
</tbody>
</table>

Each Reaction was a total of 50 µl, and they were all placed inside a thermo cycler and the following cycle was run:

Table 3. This table shows the programme which the samples from table above where run on in the thermo cycler.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95</td>
<td>5 min</td>
</tr>
<tr>
<td>35</td>
<td>Denaturation</td>
<td>94</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>45</td>
<td>45 sec</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
</tr>
<tr>
<td>1</td>
<td>Final Extension</td>
<td>72</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Once the cycle was finished the PCR samples were placed onto Ice and a 2% agarose gel was made.

2.8 Electrophoresis
To run the PCR samples a 2% gel was made up by; adding 4g of agarose powder to 200ml of x1 TAE buffer (*), mixing and adding to the microwave for 3min until dissolved, the mixture was left to cool for several minutes. Once cooled 10µl of Safe View™ was added and gently mixed in. The agarose mixture was added to the cassette and the comb allowing for 20 wells was placed, and was left for 30min to set. Once set the comb was removed and the cassette was placed inside the electrophoresis bath of x1 TAE buffer, and the 18 50µl PCR samples were loaded in the following order:

Table 4. This table shows the order in which the PCR products where loaded into the wells in the 2% agarose electrophoresis gel, in a left to right order.

<table>
<thead>
<tr>
<th>Well</th>
<th>Sample</th>
<th>Well</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bioline Hyperladder II</td>
<td>11</td>
<td>Exon 7-10 Primers - CT Mock</td>
</tr>
<tr>
<td>2</td>
<td>Full Length Primers - TT Mock</td>
<td>12</td>
<td>Exon 7-10 Primers - CT Morpholino</td>
</tr>
<tr>
<td>3</td>
<td>Full Length Primers - TT Morpholino</td>
<td>13</td>
<td>Exon 7-10 Primers - CT Antisense</td>
</tr>
<tr>
<td>4</td>
<td>Full Length Primers - TT Antisense</td>
<td>14</td>
<td>Actin Control Primers - TT Mock</td>
</tr>
<tr>
<td>5</td>
<td>Full Length Primers - CT Mock</td>
<td>15</td>
<td>Actin Control Primers TT Morpholino</td>
</tr>
<tr>
<td>6</td>
<td>Full Length Primers - CT Morpholino</td>
<td>16</td>
<td>Actin Control Primers TT Antisense</td>
</tr>
<tr>
<td>7</td>
<td>Full Length Primers - CT Antisense</td>
<td>17</td>
<td>Actin Control Primers CT Mock</td>
</tr>
<tr>
<td>8</td>
<td>Exon 7-10 Primers - TT Mock</td>
<td>18</td>
<td>Actin Control Primers CT Morpholino</td>
</tr>
<tr>
<td>9</td>
<td>Exon 7-10 Primers - TT Morpholino</td>
<td>19</td>
<td>Actin Control Primers CT Antisense</td>
</tr>
<tr>
<td>10</td>
<td>Exon 7-10 Primers - TT Antisense</td>
<td>20</td>
<td>Empty</td>
</tr>
</tbody>
</table>

The gel was run at 150V for 1 hour 30min. Once finished the gel was removed and an image was taken whilst examined under UV light.

* For a litre of 50x TAE Stock Solution, 242g Tris Base (MW=121.1, therefore 2M) is mixed with 600ml of ddH₂O using a stir bar. Once dissolved, 57.1ml Glacial Acetic Acid and 100ml 0.5M EDTA is added and the final volume is then brought up to 1L using ddH₂O and is stored at room temperature. For 1x working solution 20ml of the 50x solution is added to 980ml of ddH₂O (Tris = 0.04M, EDTA = 0.001M).

2.9 Immunocytochemistry

After incubation the 6 multi-well plate for ICC analysis containing Morpholino and Antisense transfected GM18500 (TT) andGM18506 (CT) Lymphoblastoid cells and un-transfected mocks for each, were removed from the incubator and placed in the culture hood. The cells were detached and pelleted as for RNA Analysis, the cells were then re-suspended in 1ml of
and centrifuged at 424.8g for 5min, the supernatant was removed and the pellet re-
suspended in 200µl of ddH2O. A Smear of the re-suspended cells were placed in the middle of a microscope slide and left to dry. Once the cells were dry, the cells were fixed to the slide by adding 3% paraformaldehyde (PFA) onto the cell smear and leaving to incubate for 1 hour. The slides were washed with PBS (+0.1% Tween-20) 3 times and each wash step was for 5min. To block non-specific antibody binding, a blocking solution (10% BSA + 5% FBS in PBS) was added to the cells and left to incubate for 1 hour. After incubation another 3 5min wash steps with PBS (+0.1% Tween-20). The primary antibody, anti-GNB3 (Anti-
chicken GNB3 antibody raised in rabbit), 1µl was added to 500µl of blocking solution, this was added to the cells and left to incubate for 1 hour. After incubation the slides were washed with PBSx1 3 times for 5min each. 1µl of the secondary antibody (containing Fluoresceinisothiocyanate-FITC) was diluted in 500µl of blocking solution, this was added to the cells and left to incubate for 1 hour in the dark. After incubation the slides were washed with PBSx1 3 times for 5min each and left to drying the dark. Once dry a drop of Vectashield® mounting media (containing DAPI) was added to the cells and a cover-slip was placed over it, bubbles were removed and the cover slip was sealed by adding nail polish around the edges, to prevent drying and movement under the microscope. The slides were left for the nail polish to dry and then analysed under a fluorescent microscope.

2.10 Other Cell Lines

Other cell lines were tested to see if these cells produced the GNB3s splice variant, by analysing their RNA. The cells lines which were used were: HeLa, Cos-7, A549, MCF-7 and HaCaT. These cell lines were cultured and sub-cultured in T50 culture flasks using the same methods as for the Lymphoblastoid cells above.

2.11 RNA Extraction & Purification

After incubation of the 5 cell lines in T50 culture flasks, were removed from the incubator, their confluence checked and placed in the culture hood. The cells were removed from the surface by trypsinization with 2ml Trypsin, and incubated at 37°C. Once cells had detached, 5ml of DMEM was added, the cells from each cell line were added to labelled 10ml falcon tubes and centrifuged in the Eppendorf Centrifuge 5804R for 10mins at 720g. Afterwards the supernatant was removed and only the pellet remained. The falcon tubes were placed on ice and to each 1ml of QIAzol was added and mixed by pipetting until the cell pellet was fully re-suspended. The cell suspensions were then added to homogenizers and placed in liquid nitrogen for 10sec, removed and homogenized on ice, this was repeated 3 times for
each cell line. The homogenized cells were added to corresponding labelled 1.5ml eppendorf tubes and incubated at room temperature for 5min, before being placed back on ice. To each eppendorf tube 200µl of chloroform was added and vortexed for 10sec, then incubated for 3min at room temperature, before being placed back on ice. The eppendorf tubes were then centrifuged at 23,264g for 15min at 3°C. As before the upper aqueous phase containing the RNA was removed from each eppendorf tube without disturbing the 2 lower phases and added to a new appropriately labelled 1.5ml eppendorf tube. The RNA was precipitated by adding 500µl of isopropanol and incubated for 10min at room temperature. The 5eppendorf tubes were centrifuged at 23,264g for 10min at 3°C. The supernatant was removed and only a gel pellet of RNA remained in each. The pellets were washed by mixing with 1ml of 75% ethanol, and being centrifuged at 239g for 10min at 3°C, the supernatant was removed and this wash step was repeated. Afterwards the RNA pellet was left for the ethanol to evaporate for 7min. The RNA pellet was then re-suspended in 50µl of DNase free water and the eppendorf tubes were stored at -80°C. The RNA samples were purified using the same RNasey Mini prep kit from QIAGEN and method used for the lymphoblastoid cells.

2.12 RT-PCR & Electrophoresis

2.12.1 Stage 1 (cDNA synthesis)
The same method for cDNA synthesis of the lymphoblastoid cells was used for the cDNA synthesis of the HeLa, Cos-7, A549, MCF-7 and HaCaT cell lines.

2.12.2 Stage 2 (PCR)
The same primers were used for these cell lines as for the lymphoblastoid cells, GNB3 exon 7 – 10 and Full Length GNB3. The following was added into the 15PCR tubes were labelled:

Table 5. This table shows what was added to the 10 PCR tubes before being placed in the thermo cycler.

<table>
<thead>
<tr>
<th>Tube</th>
<th>cDNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>MyTaq™ Red Mix</th>
<th>DNase free water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1µl HeLa</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>2</td>
<td>1µl Cos-7</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>3</td>
<td>1µl A549</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>4</td>
<td>1µl MCF-7</td>
<td>1µl Full Length</td>
<td>1µl Full Length</td>
<td>25µl</td>
<td>22µl</td>
</tr>
<tr>
<td>Cycles</td>
<td>Step</td>
<td>Temperature (°C)</td>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------</td>
<td>------------------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95</td>
<td>5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Denaturation</td>
<td>94</td>
<td>30 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>45</td>
<td>45 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Final Extension</td>
<td>72</td>
<td>5 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Once the cycle was finished the PCR samples were placed onto ice and a 2% agarose gel was made using the same method used for the lymphoblastoid cells. The PCR samples were loaded as shown below:

<table>
<thead>
<tr>
<th>Well</th>
<th>Sample</th>
<th>Well</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bioline Hyperladder II</td>
<td>11</td>
<td>Exon 7-10 Primers - HaCaT</td>
</tr>
<tr>
<td>2</td>
<td>Full Length Primers - HeLa</td>
<td>12</td>
<td>Empty</td>
</tr>
<tr>
<td>3</td>
<td>Full Length Primers - Cos-7</td>
<td>13</td>
<td>Empty</td>
</tr>
<tr>
<td>4</td>
<td>Full Length Primers - A549</td>
<td>14</td>
<td>Empty</td>
</tr>
<tr>
<td>5</td>
<td>Full Length Primers - MCF-7</td>
<td>15</td>
<td>Empty</td>
</tr>
<tr>
<td>6</td>
<td>Full Length Primers - HaCaT</td>
<td>16</td>
<td>Empty</td>
</tr>
<tr>
<td>7</td>
<td>Exon 7-10 Primers - HeLa</td>
<td>17</td>
<td>Empty</td>
</tr>
<tr>
<td>8</td>
<td>Exon 7-10 Primers - Cos-7</td>
<td>18</td>
<td>Empty</td>
</tr>
<tr>
<td>9</td>
<td>Exon 7-10 Primers - A549</td>
<td>19</td>
<td>Empty</td>
</tr>
<tr>
<td>10</td>
<td>Exon 7-10 Primers - MCF-7</td>
<td>20</td>
<td>Empty</td>
</tr>
</tbody>
</table>

The gel was run at 150V for 1 hour 30 min. Once finished the gel was removed and an image was taken whilst examined under UV light.
3. Results and Discussion

Mechanism of Action of GNB3s transcript specific morpholino inhibitor (β3s-blocker)

From semi-quantitative RT-PCR results (see figure 6), only a band of ~490bp (GNB3 normal transcript) and a band at 367bp (GNB3s normal transcript) occurred and no smaller band at 278bp (the theoretical size of exon 9 skipped PCR product) was present. In contrast the “Allele Specific” (AS) unmodified oligonucleotide appeared to have little or no effect in reducing the expression of the GNB3s transcript (see Figure 6). These results clearly suggest that the morpholino splice blocker appears to be much more effective in inhibiting the GNB3s transcript than the ineffective unmodified oligonucleotide. This is almost certainly due to both the different mechanisms of actions and the structures of these two antisense molecules. In particular the extra larger Nitrogen atoms in the modified antisense morpholino gives increased steric hindrance and an increased half life compared to a conventional unmodified oligonucleotide (see Figure 8). Moreover the morpholino blocking molecule is designed to target all of the GNB3 heterogenous nuclear RNA (pre-mRNA) before it is subject to splicing by the spliceosome proteins (reviewed by Stanley and Guthrie 1998). In contrast the allele specific antisense oligonucleotide was designed to bind to only the GNB3s post spliced mRNA transcript and not to the normal GNB3 transcript.

Moreover from these results the morpholino 3s-blocker appears only to inhibit the aberrant splicing of Exon8 to Exon9b in pre-messenger ribonucleic acid (mRNA) transcripts of the GNB3 gene (Figure 6 and 7). This in turn should in theory reduce the amount of available transcripts to be translated into the aberrant Gβ3s protein. The β3s-blocker morpholino, does not however, appear to at all inhibit the normal splicing of exon 8 to exon 9a. If anything this mechanism appears to be enhanced, following treatment, as there appears to be an increase in the amount of this normal transcript (see figure 6). It should be noted that morpholino β3s-blocker does not constitute a gene therapy. The β3s-blocker does not alter the GNB3 gene, but alters the splicing of the pre-mRNA transcripts in probably a dose- and time-dependent fashion.

Possible therapeutic value of the β3s-blocker in the treatment of Hypertension

We have previously shown that the complete absence of functional GNB3, leads to the pleiotropic phenotype consisting of: renal, retinal and eye globe abnormalities, in the rge chicken (Tummala et al., 2011). This result would strongly suggest that the ubiquitously
expressed GNB3 gene plays a vital role in the homeostatic maintenance of the normal kidney. It is therefore also likely that the human GNB3 825T allele, that produces the GNB3s subunit, is likely to be predisposing such individuals to hypertension, by its probable involvement in signalling pathways in the kidney. Moreover using del153D/del153D homozygote whole kidney lysates we observed a decrease in cAMP levels as well as a reduction in ERK (MAPK) and AKT phosphorylation levels (see figure 10 and Tummala et al 2011). These results, however, should be treated with caution as only whole kidney was used in these experiments, instead of individual cell types or groups of cells e.g. cortical collecting duct cells or whole extracted nephrons.

As the chicken del153D GNB3 mutation is almost certainly an inactivating mutation due to its high instability (Tummala et al. 2011), it is likely to have the opposite effect to the hyperactivating Gß3s subunit, expressed in GNB3 c.825T carrying humans. Using this inverse extrapolation between the GNB3 del153D and c.825T alleles, the decreased ERK (MAPK) results in the del153D homozygous chickens, appear to be consistent with the hypothetical pathway (see Figure 11), proposed by Kedzierska et al. (2006) for the aberrant action of the Gß3s subunit, where an increase in ERK is observed in erythrocyte cells, stimulated via the Renin angiotensin pathway (figure 10). This increase in ERK, in GNB3 825T cells there is due to a generalised increase in both the Goi and Gßy signalling pathways, due to the presence of the Gß3s subunit. This has the effect of up-regulating the Sodium channel NHE, which is independent of angiotensin converting enzyme (ACE) alleles (Kedzierska et al. 2006). A similar effect in the kidney cells would therefore help explain the predisposition of individuals to hypertension carrying the GNB3 825T alleles. These results would also suggest that the morpholino specific for the inhibition of GNB3s subunit, should in theory decrease the activity of the NHE ion channel also independently of an individual’s ACE genotype. This also suggests that the GNB3s morpholino would act independently and/or synergistically with any morpholino Renin inhibitor. Further weight to the NHE up-regulation hypothesis comes from the significant association of the 825T allele with both an increased plasma sodium (Martin et al. 2005) and renal perfusion (Zeltner et al. 2001).

The decrease in levels of cAMP, in the del153D chicken kidney, are however not consistent with the NHE up-regulation model shown in figure 10, but more consistent with a vasopressin regulated pathway (Figure 12), in the kidney cortical collecting duct cell (Verrey et al. 2001). Interestingly the levels of cAMP and an increase in ERK (MAPK) phosphorylation have both previously been shown to be critical in regulating the salt controlling channel ENAC in the kidney (e.g. Goin et al. 2001, Hallows et al. 2009, Michlig 2004). This would suggest that the pathological action of Gß3s subunit could be through
both the Renin/angiotensin and the Vasopressin system. The Gβ3s specific steric morpholino inhibitor should therefore in theory reverse the pathological effect of both HNE and ENAC sodium transport ion channels by the Gβ3s subunit, in GNB3 c.825T humans. The Gβ3s specific morpholino inhibitor should therefore be more likely to have a larger therapeutic effect, than only inhibiting the Renin/angiotensin pathway, in such individuals.

Target hypertension market for the Gβ3s specific morpholino inhibitor

The Gβ3s specific morpholino is probably most likely to lower patient's blood pressure if they are both hypertensive and obese. This is because of the study of Danoviz et al. (2006) in an ethnically diverse Brazilian population, where they found that the correlation between GNB3 genotype and blood pressure has been found to be most pronounced (Figure 4). For example a homozygote 825T GNB3 Brazilian obese patient, who has an average systolic blood pressure of ~140mmHg should in theory have their blood pressure significantly lowered to nearer the average blood pressure of an c.825C, homozygote, obese patient (~132.5 mmHg), following successful treatment with a GNB3s specific morpholino inhibitor.

In Conclusion

The apparent successful specific inhibition of the pathogenic GNB3s transcript, by a splice site specific, antisense morpholino, has profound clinical implications. As the presence of the Gβ3s subunit has a dominant effect over the normal Gβ subunit any significant reduction in its expression should in theory ameliorate the pathogenic effect on the many pathways it is involved in. In particular as morpholino's have previously been shown to be highly diffusible from the blood stream to the muscle in Duchenne Muscular Dystrophy (DMD) patients (Cirak et al. 2011), it is likely that our Gβ3s-blocker morpholino will be able to diffuse from the blood stream into a variety of tissues. In particular the kidney should be particularly amenable to morpholino diffusion from the blood stream, especially through the glomeruli. As the predisposition to hypertension in GNB3 825T individuals is likely to be through the physiological action of the Gβ3s subunit in this tissue (Tummala et al. 2011), the Gβ3s-blocker morpholino is very likely to significantly reduce the blood pressure in such patients, especially if they are obese (Danoviz 2006). It is also reasonable to assume that other non-natural oligonucleotides (and some natural oligonucleotides) may be able to provide the same effects as the morpholino.

The Gβ3s-blocker may also be useful in ameliorating the effects of obesity and diabetes in obese 825T patients (e.g. Hsiao et al. 2012). Certain metastatic cancers may also have their
proliferation curbed by treatment with the Gβ3s-blocker (e.g. Lehnerdt et al. 2008). Finally men suffering from erectile dysfunction may also benefit from treatment with the Gβ3s-blocker (e.g. Safarinejad et al. 2012).

5 References


Claims

1. A morpholino oligonucleotide consisting of the sequence
   CACTGGCATCACAGGCCGGGAAAT (SEQ ID NO 4).


3. A pharmaceutical composition comprising the morpholino oligonucleotide of claim 1
   and a pharmaceutically acceptable carrier or excipient.
Figure 1

Figure 2

123 bp = 41 aa
Figure 3
Figure 4

Figure 5
Figure 9

A

Base

Base

B

Base

N

N

P

Figure 10

cAMP Analysis

pmol/10 µg of protein

Retina wt
Retina rge
Brain wt
Brain rge
Heart wt
Heart rge
Liver wt
Liver rge
Kidney wt
Kidney rge

***

**

*
Figure 11
Figure 12

Figure 13