Title: Therapeutic intervention in a genetic disease in an individual by modifying expression of an aberrantly or abnormally expressed gene.

The invention relates to the field of genetic diseases. Specifically the invention relates to the improvement of a therapy for a genetic disease by correction of a secondary effect of a genetic disease in an individual.

A genetic disease is a disease caused by abnormalities in the genetic material of an individual. The expression of a disease in an individual is not only dependent on genetic factors, environmental factors do also play a role. A possible classification for genetic disorders is a division in two different types, monogenic or polygenic. A monogenic genetic disorder is caused by a mutation that occurs in the DNA sequence of one gene. There are more than 6,000 known monogenic disorders (Human Genome Project Information). Examples are cystic fibrosis, sickle cell anemia, Marfan syndrome, Huntington's disease, and hereditary hemochromatosis. Monogenic diseases are inherited in recognizable patterns: autosomal dominant, autosomal recessive, and X-linked. A polygenic genetic disease is caused by mutations in multiple genes. Two categories that can be distinguished within the group of genetic disorders are chromosomal and mitochondrial disorders.

A chromosomal genetic disease is caused by abnormalities in chromosome structure. Abnormalities in chromosome structure as missing or extra copies or gross breaks and rejoinings (translocations), can result in disease. Some types of major chromosomal abnormalities can be detected by microscopic examination. Down syndrome or trisomy 21 is a common disorder that occurs when a person has three copies of chromosome 21.

A mitochondrial genetic disease is a relatively rare type of genetic disease. This type of disorder is caused by mutations in the nonchromosomal DNA of mitochondria. Each mitochondrion may contain 5 to 10 circular pieces

of DNA. A chromosomal as well as a mitochondrial genetic disease can be either a monogenic disease or a polygenic disease.

Identification of a gene in which a mutation has occurred offers an opportunity to develop a specific therapy. Research into the identification of genes, involved in diverse genetic diseases, has been intense for the past decade. The Human Genome Project which has identified practically all genes in human DNA had an important role in the progress of this type of research. Many of the genetic diseases are caused by a defect in crucial gene. Often, the defect results in no or a reduced amount of function of a product of the affected gene. Therapies that utilize the molecular knowledge of the underlying reasons for such genetic defects typically aim to provide the affected cells with a (partially) functional gene product. In the present invention it has been found that other genes than the affected can be deregulated in cells of said individual and that said deregulation can add to the severity and/or symptoms of the disease. Surprisingly, this deregulation (aberrant expression) was found even when the gene affected by the mutation underlying the genetic defect, is not directly responsible for expression of the aberrantly expressed gene or when the aberrant expression of the gene is not directly related to the function of the protein that is affected by the genetic defect. One object of the present invention is therefore to correct at least one of secondary, potentially disease aggravating effect of said genetic defect in said patient by modulating the expression of said aberrantly expressed gene. Thus it appears that besides a mutated gene which can be identified as a causal factor for a genetic disease there are other genes which are aberrantly expressed, although those genes seem not to be directly influenced by said mutated gene or the normal counterpart thereof. The present invention identifies several of said aberrantly expressed genes. The expression of a deregulated gene (aberrantly expressed gene) is different from the normal situation in a healthy individual. Such deregulated expression, aberrant expression is undesired. The present

observation was made in genetic diseases that are caused by a defect in differentiated cells. It has been found that genes can be deregulated already in the precursor of said differentiated cell even when the gene affected by the mutation (the cause for the genetic defect) is not normally expressed in said precursor cell. In other words, said aberrantly expressed gene is found in other cells than the differentiated cells which are generally believed to express the phenotype of said genetic disease. The invention offers new insights for the treatment of genetic disease, particular for genetic disease caused by a (partial) lack of function or absence of a gene product in differentiated cells of said individual. In the present invention, expression of said aberrantly expressed gene is modified, either in cultures of precursor cells of said differentiated cells or directly in a patient. When applied in cultures of said precursor cells, it enhances the capacity of said precursor cells to form new differentiated cells after transplantation of said precursor cells to a patient, thereby improving the success of said cell transplantation therapy. When applied directly in the patient, the invention provides a method for alleviating an aggravating symptom of a genetic disease in an individual, wherein said symptom is preferably the reduced capacity of said precursor cells to differentiate into said differentiated cells, wherein said disease is the result of a malfunctioning gene in a differentiated cell of said individual, said method comprising modifying expression of at least one aberrantly expressed gene in said precursor cell, wherein said aberrantly expressed gene is not said malfunctioning gene. The malfunctioning gene and the direct effects caused by its defect are typically referred to as the primary effect, whereas indirect effects that occur subsequently as a result of the malfunctioning of the cell comprising said genetic defect are often referred to as secondary effects. These secondary effects typically are not a direct result of the malfunctioning of the gene.

The invention further provides the use of a compound for modifying expression of at least one gene, for the manufacture of a medicament for

alleviating a symptom of a genetic disease in an individual, wherein said disease is the result of a genetic defect in a differentiated cell of said individual, wherein said gene is aberrantly expressed in a precursor of said differentiated cell and wherein said aberrant expression is not directly related to the mutation underlying said genetic defect (the malfunctioning gene).

The genetic defect typically affects at least part of the function of a gene or the product thereof. This can be caused by a great variety of mutations. For instance, a mutation can be in the coding region of the gene thereby resulting in the production of a defective protein/RNA. On the other hand, the mutation can also be in one or more of the regulatory sequence that govern the expression of the gene product. Such a mutation can also result in a loss of (partial) function of a gene product of said gene, as the level of gene product in the cell, or the timing of expression in the cell is changed. The type of mutation can also vary. The mutation can for instance be a deletion, an insertion, an inversion or a point mutation. There are also mutations that are so-called silent mutations, i.e. that do not significantly affect the health of an individual. Examples of such mutations are point mutations in codons that, due to the redundancy in the coding potential, do not change the amino acid that is incorporated into the protein. It will be clear that silent mutations are not within the scope of the present invention. A mutated gene, as a cause of a genetic disease, as used in the invention is a gene with a non-silent mutation.

Cells in the body typically have a limited life span. Many of these cells are replenished by so-called precursor cells. For instance, dead skin cells are continuously replenished from precursor (stem) cells. Cells of the intestinal lining are similarly replenished by so-called stem cells. Muscle cells are typically (re)generated by fusion with precursor cells called myoblasts; blood cells are replenished by precursor cells that ultimately originate in the bone marrow of an individual. Regeneration can only occur when the precursor cell

gives up its primitive state in a process called differentiation. Precursor cells themselves can also have a limited life span and / or activity and if so they are replaced by other precursor cells with a more extensive life span and/or regenerative potential. The end cell with limited life span and limited self-renewal potential is referred to as a differentiated cell, whereas the precursor often has a larger potential for self-renewal is called an un-differentiated cell. This latter is often due to the fact that the differentiated cells exhibit functions that are not exhibited by the precursor. In a preferred embodiment, said precursor cell is a myoblast and/or a precursor thereof. In a preferred embodiment said differentiated cell is a muscle cell.
An individual with a genetic disease can exhibit many symptoms. When reference is made to alleviating a symptom of a disease, it is meant that the severity of a symptom is at least reduced. In case of muscular wasting, for example, symptoms of the disease encompass among others, reduction of muscle strength with age, reduction in muscle mass with age, a limited life span and a decrease in the quality of life with age (for instance, the inability to walk, dependency on care and medication). In the example of muscle wasting, alleviating a symptom can be improving muscle strength, life span and/or improving quality of life in general, when compared with an untreated individual with the same prognosis as the treated individual would have had in the absence of treatment.

A gene that is aberrantly expressed in an individual suffering from said genetic disease, can either be expressed too low or too high compared to the same gene that is physiologically expressed in a healthy individual. Modifying expression in a method or use according to the invention, comprises raising expression of said aberrantly expressed gene when expressed lower than said gene in a normal state or lowering expression of said aberrantly expressed gene when expressed higher than said gene in said normal state. Raising expression when expressed too low or lowering expression when expressed too

high corrects changes secondary to the primary genetic defect and alleviates a symptom of a genetic disease. In doing so, expression of said aberrantly expressed gene is brought to physiologically more acceptable, more plausible and/or more typical levels. In a preferred embodiment of the invention expression of said aberrantly expressed gene is essentially normalized. Physiologically acceptable, more plausible and/or more typical levels will often be levels that are approximately normal levels. Normal levels are levels that can be found in a healthy individual of the same age and constitution. Physiologically acceptable, more plausible and/or more typical levels can fall outside a normal range but do still provide a sufficient function for an individual. In a preferred embodiment the invention provides a use or a method according to the invention, wherein modifying said expression comprises approximating levels of the normal state.

Modifying expression can be achieved in a number of ways. Lowering expression of an aberrantly expressed gene can for example be realized by applying anti-sense therapy or by administering a repressor protein that binds to the promoter of said aberrantly expressed gene. Raising expression of an aberrantly expressed gene is for instance effected by the addition of a transgene that expresses said aberrantly expressed gene, by the addition and / or activation of a transcription factor that stimulates the expression of said aberrantly expressed gene and/or by activating the promoter and / or enhancer-sequence of said aberrantly expressed gene. In a preferred embodiment of the invention expression is modified by providing said individual with an anti-sense sequence of said aberrantly expressed gene. There are currently many different anti-sense approaches to down-regulate production of a gene product. Anti-sense technology exploits oligonucleotide analogs to bind to target RNAs via Watson-Crick hybridisation. Once bound, the anti-sense agent either disables or induces the degradation of the target RNA. Anti-sense agents can also alter splicing. During the past decade, much

has been learned about the basic mechanisms of anti-sense , the medicinal chemistry, and the pharmacologic, pharmacokinetic, and toxicologic properties of anti-sense molecules. Anti-sense technology has proven valuable in gene functionalisation and target validation. With one drug marketed, Vitravene, and approximately 20 anti-sense drugs in clinical development anti-sense drugs are important drugs in the treatment of a wide range of diseases (for review see [1]). Non-limiting examples of some of the newer anti-sense approaches are interference RNA (RNAi), microRNA and splice interference techniques such as exon-skipping. An anti-sense sequence is preferably administered as a single stranded molecule or as part of a hairpin molecule. An anti-sense sequence can be administered directly or be produced in a cell by means of a (virally-transduced) expression cassette.

An anti-sense oligonucleotide is preferably provided to a cell as part of a gene delivery vehicle. Such a vehicle is preferably a liposome or a viral gene delivery vehicle. Liposomes are well known in the art and many variants are available for gene transfer purposes. Various viral gene delivery are currently used to transfer genes into target cells. In the present invention it is preferred to use those viral vectors that do not express their own genes but only the transferred genes. The anti-sense molecule may be present as such in the gene delivery vehicle. In a viral vector, the anti-sense molecule is preferably provided as an expression cassette wherein the expression cassette encodes a transcript comprising said anti-sense oligonucleotide. A preferred viral delivery vehicle is an adenoviral vector and more preferably an adeno-associated virus vector. The invention thus also provides such expression cassettes, vectors and gene delivery vehicles. It is within the skill of the artisan to design suitable transcripts. Preferred for the invention are PolIII driven transcripts. Preferably in the form of a fusion transcript with an U1 or U7 transcript. Such fusions may be generated as described in references [2-4].

In the present invention an oligonucleotide is a polymer of DNA or RNA nucleotides (typically between 5 and 300 nucleotides). Oligonucleotides can be synthesized in vitro or in vivo. In the latter context they are sometimes also referred to an anti-sense molecule/sequence, siRNA, miRNA and the like.

In a preferred embodiment an oligonucleotide comprises between 5-300 nucleotides, more preferably between 15-100 nucleotides, more preferably between 15-40 nucleotides and more preferably between 15 and 25 nucleotides. Said lengths are preferred for the region of complementarity of said oligonucleotide or functional equivalent thereof. An oligonucleotide or functional equivalent thereof, of between 15 and 40 nucleotides can have one or two mismatches with the region said oligonucleotide or functional equivalent is complementary to. In case of said one or two mismatches the region of complementarity preferably comprises a continuous stretch of at least 15 nucleotides. I.e. an oligonucleotide or functional equivalent thereof complementary to any stretch of 15 can have one or two mismatches. Preferably said oligonucleotide or functional equivalent thereof has one or more preferably no mismatches with the region it is complementary to. If the oligonucleotide has more than 15 nucleotides it may have up to 15% mismatches. If the calculated number of mismatches based on the percentage rule is a number between two integers, the maximum allowed number of mismatches is the higher integer number. For example, an oligonucleotide having a continuous stretch of 16 nucleotides complementarity can have 16*0.15 = 2.4 nucleotide mismatches. Thus the maximum number of mismatches allowed in this oligonucleotide is 3. In a preferred embodiment, the generated oligonucleotide is complementary to a consecutive part of between 15 and 50 nucleotides and more preferred said oligonucleotide comprises RNA and even more preferred said oligonucleotide is 2'-O-methyl RNA and has a full-length phosphorothioate backbone. 2'0-methyl RNA is a nucleic acid analogue that is characterized by good hybridization properties

that it imparts with complimentary DNA or RNA as well as, an increased stability against enzymatic degradation compared to natural nucleic acids. Most antisense oligonucleotides currently in clinical development incorporate phosphorothioate backbone modifications, to promote resistance to nucleases while preserving the ability to stimulate cleavage of the mRNA target by ribonuclease (RNase) H. The complementary oligonucleotide is preferably complementary to a consecutive part of between 13 and 50 nucleotides of said exon RNA. In another embodiment the complementary oligonucleotide is complementary to a consecutive part of between 16 and 50 nucleotides of said exon RNA. Preferably, the oligonucleotide is complementary to a consecutive part of between 13-25 nucleotides of said exon RNA. Preferably between 14 and 25 nucleotides of said exon RNA. Different types of nucleic acid may be used to generate the oligonucleotide. Preferably, the oligonucleotide comprises RNA, as RNA/RNA hybrids are very stable. Since one of the aims of the exon skipping technique is to direct splicing in subjects it is preferred that the oligonucleotide RNA comprises a modification providing the RNA with an additional property, for instance resistance to endonucleases and RNaseH, additional hybridisation strength, increased stability (for instance in a bodily fluid), increased or decreased flexibility, reduced toxicity, increased intracellular transport, tissue-specificity, etc. Preferably said modification comprises a 2'-O-methyl-phosphorothioate oligoribonucleotide modification. Preferably said modification comprises a 2'-O-methyl-phosphorothioate oligodeoxyribonucleotide modification, locked nucleic acid, PNA, or a morpholino modification or a combination thereof. In one embodiment the invention provides a hybrid oligonucleotide comprising an oligonucleotide comprising a 2'-O-methyl-phosphorothioate oligo(deoxy)ribonucleotide modification and locked nucleic acid. This particular combination comprises better sequence specificity compared to an equivalent consisting of locked nucleic acid, and comprises improved efficacy when compared with an oligonucleotide consisting of 2'-O-methyl-phosphorothioate

oligo(deoxy)ribonucleotide modification. With the advent of nucleic acid mimicking technology it has become possible to generate molecules that have a similar, preferably the same hybridisation characteristics in kind not necessarily in amount as nucleic acid itself. Such equivalents are of course also part of the invention. Examples of such mimics equivalents are peptide nucleic acid, locked nucleic acid and/or a morpholino phosphorodiamidate. Suitable but non-limiting examples of equivalents of oligonucleotides of the invention can be found in (Wahlestedt, C. et al. (2000), Elayadi, A.N. & Corey, D.R. (2001), Larsen, H.J., Bentin, T. & Nielsen, P.E. (1999), Braasch, D.A. & Corey, D.R. (2002), Summerton, J. & Weller, D. (1997). Hybrids between one or more of the equivalents among each other and/or together with nucleic acid are of course also part of the invention. In a preferred embodiment an equivalent comprises locked nucleic acid, as locked nucleic acid displays a higher target affinity and reduced toxicity and therefore shows a higher efficiency of exon skipping. An anti-sense oligonucleotide of the invention may comprise one or more nucleotide analogues. New nucleotide analogues are currently developed as a method for treatment against viral infections. These nucleotide analogues typically though not necessarily have similar binding characteristics as the nucleotide they replace. An anti-sense oligonucleotide of the invention may incorporate such a nucleotide analogue. An anti-sense oligonucleotide of the invention preferably does not comprise more than 20% of such a nucleotide analogues. Preferably an anti-sense oligonucleotide of the invention preferably does not comprise more than 10% of such a nucleotide analogues. An anti-sense oligonucleotide of the invention preferably does not comprise more than 3 of such a nucleotide analogues. An anti-sense oligonucleotide of the invention preferably does not comprise more than 1 of such a nucleotide analogues. An oligonucleotide or functional equivalent thereof may further comprise an additional entity to provide a further function to the resulting molecule. A fluorescent tag or immune regulating compound such as a CpG island may be added to said oligonucleotide or functional equivalent thereof.

The anti-sense sequence can be delivered in vivo or ex vivo, i.e. into precursor cells (I would not mention this since the antisense can also be delivered in vivo to the endogenous precursor cells; instead:). In a preffered embodiment, the precursor cells containing the antisense sequences are used for cell transplantation therapies. In a preferred embodiment said compound comprises an anti-sense molecule or a functional equivalent thereof. A functional equivalent of an anti-sense molecule of the invention has the same expression inhibiting effect in kind not necessarily in amount, as said anti-sense molecule. The anti-sense molecule or the functional equivalent thereof can be designed in various ways. Reference is made to [1] and references therein for details about the design of anti-sense molecules. This reference and the references therein are therefore herein incorporated by reference.
Lowering or down-regulating expression of an aberrantly expressed gene typically results in decreased levels of gene product encoded by said gene in said cell. Said gene product is preferably RNA produced by said gene, for instance a microRNA.
In another preferred embodiment said compound comprises a protein capable of inhibiting and/or antagonizing the function of said aberrantly expressed gene. In a preferred embodiment said compound comprises noggin or a functional part, derivative and/or analogue thereof. Noggin is capable of inhibiting and/or antagonizing the function of BMP-4[5]. A BMP-4 antagonist inhibits the function of BMP-4. Other BMP-4 inhibitors/antagonists of the invention are: chordin, ventroptin, twisted gastrulation, gremlin or other members of the DAN family of BMP4 antagonists, PRDC, sclerostin, CTGF and follistatin. Thus in a preferred embodiment the invention provides use of a BMP-4 antagonist (for antagonizing expression of BMP4 in a cell), for the manufacture of a medicament for alleviating a symptom of a genetic muscular dystrophy in an individual. Preferably said antagonist comprises noggin, chordin, ventroptin, twisted gastrulation, gremlin or other members of the

DAN family of BMP4 antagonists, PRDC, sclerostin, CTGF and/or follistatin or a functional part, derivative and/or analogue of said protein. Said antagonist may be provided as protein or as a nucleic acid comprising an expression cassette for expression of said antagonist in a cell. In this latter embodiment a compound of the invention preferably comprises said expression cassette for expression of said antagonist in a cell. Further provided is a method for stimulating differentiation of a myoblast cell comprising providing and/or contacting said myoblast cell with a BMP-4 antagonist. Further provided is a method for stimulating differentiation of a myoblast cell comprising providing said myoblast cell with a nucleic acid comprising an expression cassette for expression of said antagonist in said cell. Providing a neighbouring cell with such an expression cassette is considered to be providing and/or contacting the adjacent myoblast cell with said BMP-4 antagonist, i.e. the protein. In a preferred embodiment said neighbouring cell is a muscle cell.

There are many ways to increase expression of (an aberrantly expressed) gene in a cell. Non-limiting examples are the introduction of an expression construct comprising a coding sequence for said gene, expressing a transcription factor that activates or stimulates expression of the endogenously present gene and the expression of analogues, derivatives and/or parts of said gene in said cell. It is also possible to directly transfect protein or RNA encoded by said gene in said cell. These methods are herein collectively referred to as gene therapy. In one embodiment the invention therefore provides a use or a method, wherein modifying said expression comprises gene-therapy. Said gene therapy can be executed in an ex-vivo method or in an in-vivo method. Increasing expression of a gene product in a cell preferably results in increased levels of gene product in said cell. The starting level may be undetectable.

A muscle disorder is a disease that usually has a significant impact on the life of an individual. Any measure that can be taken to alleviate

consequences of a muscle disorder could therefore mean a relief for an individual with said muscle disorder. As differentiated muscle cells are replenished and/or regenerated through myoblast precursor cells, the present invention is particularly suited for the treatment of muscle disorders. The invention provides a use or a method according to the invention, wherein at least a part of the symptoms exhibited by said individual suffering from said genetic disease is due to abnormal differentiation of the muscle precursor cells in addition to the malfunctioning of differentiated muscle cells in said individual when compared to a healthy individual. A muscle disorder can either have a genetic cause or a non-genetic cause. A preferred example of a disease with a genetic cause is a genetic muscular dystrophy.

Genetic muscular dystrophies constitute a group of genetic disorders characterized by progressive muscle wasting and weakness. Many of these disorders are caused by defects in genes for muscle proteins. The different forms of genetic muscular dystrophies often differ in the involved protein(s). Most of the affected genes in these disorders code for proteins that seem to play a role in supporting the structure of muscle fibers, alternatively some proteins may be concerned with biochemical processes that occur in muscle fibers. The invention provides a use or a method according to the invention, wherein said genetic disease comprises a genetic muscular dystrophy. Muscular dystrophies are generally inherited although there are cases in which no family history of the disease exists. The clinical appearance of the different forms of genetic muscular dystrophies varies amongst other things in muscles first and most often affected, the rate at which symptoms progress and age of onset.

There are multiple diagnostic methods available for the diagnosis of the genetic muscular dystrophies and the differentiation between them. Most often a combination of a few methods is used to make a diagnosis. A diagnosis

usually starts with an evaluation of the medical history of a patient and a physical examination. Examples of available diagnostic tests are a blood enzyme test (for example for creatine kinase, CK), assessment of muscle histology, DNA test, magnetic resonance (MR), electromyogram (EMG) and nerve conduction velocity study (NCV). The invention discloses a use or a method according to the invention, wherein said genetic muscular dystrophy is one of the following diseases: Becker Muscular Dystrophy (BMD), Congenital Muscular Dystrophy (CMD), Distal Muscular Dystrophy (DD), Duchenne Muscular Dystrophy (DMD), Emery-Dreifuss Muscular Dystrophy (EDMD), Facioscapulohumeral Muscular Dystrophy (FSH), Limb-Girdle Muscular Dystrophy (LGMD), Myotonic Dystrophy (MMD), Oculopharyngeal Muscular Dystrophy (OPMD). A short description of the causes of aforementioned genetic muscular dystrophies is given below.

Becker Muscular Dystrophy (BMD), insufficient production of functional dystrophin, a protein that helps keep muscle cells intact.

Congenital Muscular Dystrophy (CMD), genetic mutations affecting some of the proteins necessary for muscles and sometimes for the eyes and/ or brain.

Distal Muscular Dystrophy (DD), a mutation in any of at least seven genes that affect proteins necessary to the function of muscles.

Duchenne Muscular Dystrophy (DMD), absence of dystrophin, a protein that helps keep muscle cells intact.

Emery-Dreifuss Muscular Dystrophy (EDMD), mutations in the genes that produce emerin, lamin A or lamin C, proteins in the membrane that surrounds the nucleus of each muscle cell.

Facioscapulohumeral Muscular Dystrophy (FSH), a missing piece of DNA on chromosome 4.

Limb-Girdle Muscular Dystrophy (LGMD), a mutation in any of at least 15 different genes that affect proteins necessary for muscle function.

Myotonic Dystrophy (MMD), a repeated section of DNA on either chromosome 19 or chromosome 3.

Oculopharyngeal Muscular Dystrophy (OPMD), a faulty gene for poly(A)-binding protein 1 (PABPN1), which is suspected to aggregation of RNA and proteins in the nuclei of muscle cells.

In both Duchenne and Becker muscular dystrophy the muscle protein dystrophin is affected. In Duchenne dystrophin is absent, whereas in Becker some dystrophin is present but its production is most often not sufficient and/or the dystrophin present is abnormally formed. Both diseases are associated with recessive X-linked inheritance. DMD results from a frameshift mutation in the DMD gene [6;7]. The frameshift in the DMD gene results in the production of a truncated non-functional Dystrophin protein[8]. BMD occurs as a consequence of multiple mutations in the DMD gene. As in Becker some dystrophin is present in contrast to Duchenne where dystrophin is absent, Becker has less severe symptoms then Duchenne. The onset of DMD is earlier than BMD. DMD usually manifests itself in early childhood, BMD in the teens or in early adulthood. The progression of Becker is slower and less predictable than Duchenne. Patients with BMD can survive into mid to late adulthood. Patients with Duchenne rarely survive beyond their thirties.

Dystrophin plays an important structural role in the muscle fiber, connecting the extracellular matrix and the cytoskeleton. The N-terminal region binds actin, whereas the C-terminal end is part of the dystrophin glycoprotein complex (DGC), which spans the sarcolemma[9]. In the absence of dystrophin, mechanical stress leads to sarcolemmal ruptures, causing an uncontrolled influx of calcium into the muscle fiber interior, thereby triggering calcium-activated proteases and fiber necrosis[10].

A precursor of a differentiated cell has specific characteristics which makes it differentiate into a cell with specific functions. These specific characteristics comprise the presence and activation state of a category of

genes that is involved in the control of the differentiation of a cell. In the present invention it has been found that particularly controlling genes add to the symptoms of the genetic defect when aberrantly expressed in the precursor cell. In a preferred embodiment said aberrantly expressed gene comprises Bone Morphogenetic protein 4 (BMP4). Bone Morphogenetic Proteins (BMPs) are regulatory factors that are members of the transforming growth factor-beta superfamily of proteins. They are synthesized as large precursor molecules which are cleaved by proteolytic enzymes. The active form can consist of a dimer of two identical proteins or a heterodimer of two related bone morphogenetic proteins. Bone Morphogenetic Proteins are implicated in a large variety of cellular processes[12]. BMPs are involved in the differentiation of certain cell types, including myogenic cells. In one preferred embodiment the invention provides a use or a method according to the invention, wherein said transforming growth factor-beta is a Bone Morphogenetic Protein (BMP) or a functional part, derivative and/or analogue thereof, preferably BMP4 or a functional part, derivative and/or analogue thereof.

In another aspect the invention provides a use or a method according to the invention, wherein said aberrantly expressed gene is a controlling factor in the differentiation of said precursor cell into said differentiated cell. A controlling factor is a factor which controls alone, or with other controlling factors, at least one step in the differentiation of a precursor cell into a differentiated cell. Control of said at least one step in the differentiation can be stimulatory or inhibitory. Said controlling factor realizes this control through being part of a signal-transduction cascade. Said controlling factor preferably is located at the beginning of said signal-transduction cascade, receiving signals that come from, for instance, outside the cell in which said cascade is located. In another preferred embodiment of the invention said controlling factor is located at the end of said signal-transduction cascade, effecting

signals that are transduced by said cascade. A controlling factor is for example a growth factor or a transcription factor.

A growth factor is a small protein that attaches to specific receptors on the surface of cells and promote proliferation, growth, differentiation and / or maturation of these cells. Examples of growth factors are : granulocyte-colony stimulating factor (G-CSF); granulocyte-macrophage colony stimulating factor (GM-CSF); platelet-derived growth factor (PDGF); erythropoietin (EPO); thrombopoietin (TPO); myostatin (GDF-8) and/or fibroblast growth factor 2 (FGF-2). In a preferred embodiment the invention provides a use or a method according to the invention, wherein said controlling factor comprises a growth factor or a functional part, derivative and/or analogue thereof, preferably fibroblast growth factor (FGF2) or insulin-like growth factor binding protein (IGFBP3), BMP4, or a functional part, derivative and/or analogue thereof. Fibroblast Growth Factor 2 (FGF2) is a single-chain polypeptide growth factor that plays a significant role in the process of wound healing and is a potent inducer of angiogenesis. Several different forms of the human protein exist ranging from 18-24 kDa in size due to the use of alternative start sites within the fgf-2 gene. It has a 55 percent amino acid residue identity to fibroblast growth factor 1 and has potent heparin-binding activity. The growth factor is an extremely potent inducer of DNA synthesis in a variety of cell types from mesoderm and neuroectoderm lineages.

Insulin-Like Growth Factor II is a multiplication-stimulating factor. It is a well-characterized neutral peptide believed to be secreted by the liver and to circulate in the blood. It has growth-regulating, insulin-like and mitogenic activities. Insulin-Like Growth Factor Binding Protein 3 (IGFBP3) is one of the six homologous soluble proteins that bind insulin-like growth factors (somatomedins) and modulate their mitogenic and metabolic actions at the cellular level.

Transforming growth factor (TGF) is one of many characterized growth factors that exist in nature. Transforming Growth Factors are hormonally active polypeptides that can induce the transformed phenotype when added to normal, non-transformed cells. Their transforming activities are due to the simultaneous action of two otherwise unrelated factors, transforming growth factor alpha and transforming growth factor beta. In a preferred embodiment the invention provides a use or a method according to the invention, wherein said growth factor belongs to the transforming growth factor-ß (TGFß) superfamily or is a functional part, derivative and/or analogue thereof. TGFß is a factor synthesized in a wide variety of tissues. It acts synergistically with TGF-alpha in inducing phenotypic transformation and can also act as a negative autocrine growth factor. TGF-beta has a role in embryonal development, cellular differentiation, hormone secretion, and immune function. TGF-beta is found mostly as homodimer forms of separate gene products TGF-beta1, TGF-beta2 or TGF-beta3. Heterodimers composed of TGF-beta1 and 2 (TGF-beta1.2) or of TGF-beta2 and 3 (TGF-beta2.3) have been isolated. The TGF-beta proteins are synthesized as precursor proteins. Transforming growth factor alpha is a factor that has been isolated in a variety of tissues including epithelium, and maternal decidua. It is closely related to epidermal growth factor and binds to the EGF receptor. TGF-alpha acts synergistically with TGF-beta in inducing phenotypic transformation, but its physiological role is unknown.

Myostatin (also known as Growth and Differentiation Factor 8) is a growth factor that limits muscle tissue growth, i.e. higher concentrations of myostatin in the body cause the individual to have lesser developed muscles. The myostatin protein is produced in muscle cells, circulates in the blood and acts on muscle tissue, apparently by slowing down the development of muscle stem cells[11]. Myostatin is a member of the TGF-beta superfamily of proteins.

In a preferred embodiment the invention provides a use and a method according to the invention, wherein said controlling factor comprises myostatin.

Mechanisms that regulate cell differentiation also involve transcription factors. In one embodiment the invention provides a use or a method according to the invention, wherein said controlling factor comprises a transcription factor. A transcription factor is a protein needed to initiate the transcription of a gene. Transcription factors can be tissue-specific, which means that those factors only have a function in the transcription of one or a few specific gene(s). Alternatively the transcription factors can be general, being involved in the start of transcription of many different genes.

In a particularly preferred embodiment, a method or use of the invention is combined with another treatment for the genetic defect. As a first example, in genetic defects that are the result of an absent or dysfunctional gene product in a differentiated cell, a method or use of the invention is preferably combined with a method for enhancing expression of the absent or dysfunctional gene product in the differentiated cell. Thus the invention further provides a use or a method according to the invention, further comprising providing said individual with a medicament for providing said differentiated cell with at least part of the normal function of said mutated gene. As a second example, in genetic defects that are the result of an absent or dysfunctional gene product in a differentiated cell, a method or use of the invention is preferably combined with a method that uses transplantation of precursor cells for said differentiated cells to the patient in order to generate new and functional differentiated cells. The precursor cells may be derived from healthy donors, but in that case the risk of rejection is high[13]. Therefore, precursor cells derived from the patient are preferred. Said

precursor cells are genetically changed ex vivo to express a functional copy of the gene affected by the mutation. The invention provides a method to correct other defects in the precursor cells secondary to the genetic mutation, and enhances the differentiation capacity of said precursor cells in vivo.

In a further embodiment the invention provides a use or method according to the invention, wherein said aberrantly expressed gene is identified by comparing an expression profile of said precursor cell of an individual suffering from a genetic defect with a corresponding precursor cell of a healthy individual. A gene is said to be aberrantly expressed when it is at least 2-fold differentially expressed compared to said corresponding precursor cell in said healthy individual.

In an example of the invention, a large-scale gene expression time course study using primary human myoblast cultures is performed, wherein myogenesis in DMD cells and the first reaction of the differentiating cell to the absence of dystrophin are monitored. The results show a clear phasing of the different stages in myogenesis. Already at the myoblast stage differences appear and although differentiation seems to initiate at the same time in healthy and DMD cultures, it is shown that the DMD cells differentiate less efficiently. Studies have been performed previously which used gene expression profiling to find pathways involved in the disease mechanism[14-16]. These studies however, did not analyze molecular differences at the precursor cell (myoblast) level but were all focussed on the differentiated cell (muscle cell) itself.

As a preferred example of the invention significant differences in gene expression between healthy and DMD cell cultures have been observed. This observation would not have been expected as the full-length dystrophin is not yet expressed in myoblasts and Dp71 myoblast expression should not be

hampered by the mutations as they are located upstream of the translation initiation site[17]. One of the differentially expressed genes, Fibroblast growth factor 2 (FGF2) is significantly lower expressed in DMD myoblasts. In vitro and in vivo studies demonstrated an important role for FGF2 in the recruitment of satellite cells into proliferation. The addition of recombinant FGF2 enhanced the number of proliferating myoblasts by twofold and did not suppress the initiation of differentiation[18-20]. In addition, Doukas et al. demonstrated that targeted transgene delivery of FGF2 and FGF6 genes led to an enhancement of skeletal muscle repair, showing the importance of the FGF genes in regeneration[21]. These observations indicate that the lower DMD myoblast FGF2 expression observed in an example of the invention, can explain the decreased myoblast proliferation in DMD cultures previously reported[22-24].

Although proliferation capacity is probably reduced and differentiation inhibited in DMD cell cultures, the results of an example of the invention indicate that the timing of the different processes is similar. Genes involved in proliferation are simultaneously downregulated after fusion induction in both healthy and DMD cell cultures (MCM6, CCNB2, CDC28, CKS2 and RPA3, Figure 5, group 'cell growth and maintenance'). During the actual fusion process of myoblasts into myotubes however, gene expression differences appear between healthy and DMD cell cultures, again pointing at an impaired fusion potential of DMD cells. In an example of the invention genes have been found to be aberrantly expressed that are involved in fusion of healthy myoblasts but that probably do not participate in DMD myoblast fusion. Of these, Membrane metallo-endopeptidase (MME) and Adlican (DKFZp564I1922) are thought to be involved in cell adhesion and cell-cell signalling, which are important for cell fusion[25]. In the gene expression study, Laminin alpha 2 (LAMA2) is continuously lower expressed in DMD cell cultures, making them less adhesion competent. This explains the absence of

the Laminin alpha 2 dependent adhesion force which has been reported previously by Angoli et al. [26].

Surprisingly, Mitochondrial tumor suppressor 1 (MTUS1) and Endothelin receptor type A (EDNRA), both presumed to be involved in signalling, are only upregulated in the DMD cells upon initiation of differentiation. These genes are possibly part of alternative signalling pathways due to the absence of Dystrophin.

In a time-course study, differentiation and fusion of primary human myoblasts into myotubes takes approximately 4 days. After this, almost no expression changes are visible and genes are stably expressed[27]. A striking phenomenon is the upregulation of the sarcomeric gene expression after initiation of differentiation in both healthy and DMD cell cultures and a subsequent significant decline, starting at day 6, detectable in DMD myotubes only. The absence of dystrophin causes sarcomeric instability, resulting in a secondary response which initiates downregulation of structural genes. Other functional classes of proteins that are simultaneously up or downregulated during myoblast differentiation do not show a difference in the later timepoints, indicating that this negative feedback is a unique characteristic of the sarcomeric proteins in DMD cultures.

Two present promising gene therapies are based on the re-establisment of dystrophin in the DGC complex by either AAV-mediated introduction of a micro-DMD gene or by skipping an exon to restore the reading frame of the gene[28-36]. The results of an example of the invention indicate that the effectiveness of these therapies might not fulfill the present expectations. Thus far, studies focussed on the dystrophin protein and its localization, but did not look specifically at the regeneration capacity of the muscle after therapy. The presence of dystrophin only corrects the sarcomeric instability and might just

alleviate (temporarily) but not cure the patients because regeneration signalling pathways are not restored. From this viewpoint, it would be crucial to start therapy early in life, before these changes have taken place. Alternatively, additional (pharmaceutical) intervention to regain the normal muscle regeneration capacity should be pursued. A use or a method according to the invention could be applied in combination with a promising gene therapy. In one embodiment the invention provides a use or a method according to the invention, wherein said genetic muscular dystrophy is Becker Muscular Dystrophy (BMD) or Duchenne Muscular Dystrophy (DMD). In a preferred embodiment the invention provides a use or a method according to the invention, comprising skipping an exon of a dystrophin gene.
An example of the invention shows molecular differences between healthy and DMD myoblasts during myogenesis. Decreased FGF2 levels and elevated expression of BMP4 in DMD myoblasts reduce proliferation capacity and make them less differentiation competent. In addition, lower expression of sarcomeric proteins in DMD myotubes is observed. This combination of reduced proliferation, impaired fusion and impaired maintenance of the DMD myotubes leads to inefficient muscle regeneration and contributes to the severe phenotype of DMD patients.

Transplantation of ex vivo expanded myoblasts to DMD patients is an alternative and promising therapy[37]. There are at least two options. Treatment with myoblasts from healthy subjects or autologous cell transplantation. In the first case, the rejection of the cells by the host due to the induction of an immune response is a major concern[13]. Immunocompatible donors, preferably family members[37;38], or immunosuppressants[39;40] are employed to control this immune response. Autologous cell transplantation has the advantage of a reduced chance of rejection by the immune system. Before autologous transplantation, the gene defect has to be corrected by introduction of a functional transgene[41] or an

expression cassette that produces exon skipping antisense sequences[4]. In an example of the invention, a microDMD gene is introduced using an recombinant AAV. In one embodiment the invention provides a use or a method according to the invention of an antisense oligonucleotide or siRNA against BMP4 to reduce the expression of BMP4 in autologous myoblasts, and to enhance the regenerative potential of said autologous myoblasts in a patient.

The invention further provides the use of a compound for modifying expression of a gene, for the manufacture of a medicament for alleviating a symptom of a genetic disease in an individual, wherein said disease is the result of a genetic defect in a differentiated cell of said individual, wherein said gene is aberrantly expressed in a precursor of said differentiated cell and wherein said aberrant expression is not directly related to the mutation underlying said genetic defect. In a preferred embodiment said mutation underlying said genetic defect is associated with a gene and is a result of no or defective protein synthesis from said gene (mutated gene). In a further preferred embodiment modifying said expression comprises lowering expression when said aberrantly expressed gene is over-expressed in said precursor cell and elevating expression when said aberrantly expressed gene is under-expressed in said precursor cell. In a preferred embodiment said aberrantly expressed gene is a controlling factor in the differentiation of said precursor cell into said differentiated cell. Preferably said controlling factor comprises a growth factor or a functional part, derivative and/or analogue thereof. Preferably said growth factor belongs to the transforming growth factor-ß (TGFß) superfamily or is a functional part, derivative and/or analogue thereof. Preferably said growth factor belonging to the transforming growth factor-beta superfamily is Bone Morphogenetic Protein 4 (BMP4) or a functional part, derivative and/or analogue thereof. Preferably said controlling

factor comprises a (transcription) factor that influences the expression of said aberrantly expressed gene.

The invention further provides a method for stimulating differentiation of myoblast cells comprising providing said myoblast cells with a compound for inhibiting expression of a growth factor in said myoblast cells. Preferably said inhibition comprises inhibiting BMP-4 mRNA expression in said myoblast cells. Preferably said method further comprises contacting said myoblast cells with mature muscle cells. Preferably said mature muscle cells are derived from a subject that suffers from a genetic muscular dystrophy. Preferably said contacting is done by transplanting said myoblast cells into said individual. Preferably said transplanted myoblast cells are from a matched donor. Preferably, the myoblast cells are autologous myoblast cells. Preferably inhibiting BMP-4 mRNA expression in said myoblast cells comprises providing said myoblast cells or precursors thereof with a BMP-4 oligonucleotide that is complementary to said BMP-4 gene (anti-sense). Preferably said myoblast cells or precursors thereof are in vitro provided with said BMP-4 anti-sense oligonucleotide. The invention further provides a collection myoblast cells or precursors thereof comprising a BMP-4 anti-sense oligonucleotide.

Further provided is a use or a method of the invention wherein said precursor is a myoblast or a precursor thereof. Preferably said expression is inhibited by means of a virally transduced DNA sequence. Preferably said genetic disease comprises a genetic muscular dystrophy. Preferably said genetic muscular dystrophy comprises one of the following diseases: Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Congenital Muscular Dystrophy (CMD), Distal Muscular Dystrophy (DD), Emery-Dreifuss Muscular Dystrophy (EDMD), Facioscapulohumeral Muscular Dystrophy (FSH), Limb-Girdle Muscular Dystrophy (LGMD), Myotonic Dystrophy (MMD), Oculopharyngeal Muscular Dystrophy (OPMD). Preferably said compound comprises a protein inhibitor. Preferably wherein said protein inhibitor comprises a growth factor inhibitor or antagonist, preferably a BMP-

4 inhibitor or antagonist, preferably wherein said inhibitor or antagonist comprises noggin, chordin, ventroptin, twisted gastrulation, gremlin or other members of the DAN family of BMP4 antagonists, PRDC, sclerostin, CTGF and/or follistatin or a functional part, derivative and/or analogue or a functional part, derivative and/or analogue thereof.
Preferably a method or use of the invention further comprises use of a second compound for the preparation of a medicament of for treatment of said individual wherein said second compound provides said differentiated cell with at least part of the normal function of said mutated gene. Preferably said genetic muscular dystrophy is Duchenne Muscular Dystrophy (DMD) or Becker Muscular Dystrophy (BMD). Preferably said second compound comprises an oligonucleotide, or functional equivalent thereof, for skipping an exon of a dystrophin gene. Preferably said second compound comprises an oligonucleotide or functional equivalent thereof complementary to an exon of a dystrophin gene.

In a preferred aspect the invention provides the use of a compound for reducing, inhibiting and/or antagonizing expression of Bone Morphogenetic Protein 4 (BMP4) in a cell, for the manufacture of a medicament for alleviating a symptom of a genetic muscular dystrophy in an individual. Preferably said cell is a myoblast cell or a precursor thereof. Preferably said compound comprises an antisense RNA or a functional equivalent thereof.

The invention further provides a method for stimulating differentiation of a myoblast cell comprising providing said myoblast cell with a compound for reducing, inhibiting and/or antagonizing expression of BMP4 in said myoblast cell. Preferably said compound (is capable of reducing and/or inhibiting) decreases and/or inhibits BMP4 mRNA expression in said myoblast cell. In another preferred embodiment said compound (is capable of antagonizing) antagonizes the function of BMP-4. A use and/or method according to the

invention further comprises contacting said myoblast cell with a mature muscle cell. In a preferred embodiment said mature muscle cell is derived from a subject that suffers from said genetic muscular dystrophy. A method of the invention for reducing and/or inhibiting BMP4 mRNA expression in said myoblast cell comprises providing said myoblast cell or a precursor thereof with a BMP4 oligonucleotide that is complementary to said BMP4 gene (anti-sense). Preferably said myoblast cells or precursors thereof are in vitro provided with said BMP-4 anti-sense oligonucleotide.

The invention further provides a collection of myoblast cells or precursors thereof comprising a BMP-4 anti-sense oligonucleotide.

Reducing and/or inhibiting expression of BMP4 is preferably achieved by means of a virally transduced DNA sequence. Preferably said compound is provided to said cell by means of a viral vector. Preferably said genetic muscular dystrophy comprises one of the following diseases: Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Congenital Muscular Dystrophy (CMD), Distal Muscular Dystrophy (DD), Emery-Dreifuss Muscular Dystrophy (EDMD), Facioscapulohumeral Muscular Dystrophy (FSH), Limb-Girdle Muscular Dystrophy (LGMD), Myotonic Dystrophy (MMD), Oculopharyngeal Muscular Dystrophy (OPMD). Preferably said compound comprises a protein inhibitor. Preferably said compound comprises a BMP4 inhibitor or antagonist. Preferably said inhibitor or antagonist comprises noggin, chordin, ventroptin, twisted gastrulation, gremlin or other members of the DAN family of BMP4 antagonists, PRDC, sclerostin, CTGF and/or follistatin or a functional part, derivative and/or analogue or a functional part, derivative and/or analogue thereof. Preferably a method or use of the invention further comprises use of a second compound for the preparation of a medicament for treatment of said individual wherein said second compound provides a muscle cell of said individual with at least part of the normal function of a gene that is associated with said genetic muscular dystrophy (mutated gene). Preferably said genetic muscular dystrophy is

Duchenne Muscular Dystrophy (DMD) or Becker Muscular Dystrophy (BMD). Preferably said second compound comprises an oligonucleotide, or functional equivalent thereof, for skipping an exon of a dystrophin gene.

In a further aspect the invention provides a method for determining whether a BMP4 anti-sense oligonucleotide or a functional equivalent thereof is capable of inducing skipping of an exon in a BMP4 pre-mRNA containing said exon, said method comprising providing a cell expression said BMP4 pre-mRNA with said oligonucleotide and determining whether said exon is absent from mature mRNA produced from said pre-mRNA. Preferably, the skipping of an exon in the BMP4 mRNA reduces the production level of functional BMP4 protein levels. Exon skipping in BMP4 and DMD has different objectives: reading frame disruption (or exclusion of an essential stretch of amino acids; BMP4) vs reading frame correction (DMD). A oligonucleotide or functional equivalent thereof is said to be effective in inducing skipping of said exon, if more than 5 and preferably more than 10% of the mRNA resulting from splicing of said pre-mRNA in said method does not contain said exon, as measured in vitro by means of an nucleic acid amplification reaction (preferably polymerase chain reaction, PCR) with primers in exons flanking said exon (that is targeted by said anti-sense oligonucleotide or functional equivalent thereof). Preferably said anti-sense oligonucleotide or a functional equivalent thereof is complementary to said exon. In a particularly preferred embodiment said anti-sense oligonucleotide or a functional equivalent thereof is complementary to an exon-internal part of said exon. An exon-internal part of said exon is herein defined as a part that is complementary to said exon and that does not include intronic sequence immediately flanking said exon. Preferably said exon-internal oligonucleotide or functional equivalent thereof does not comprise one or both of the two exon splice acceptor and/or exon splice donor nucleotides flanking said exon. Preferably said anti-sense oligonucleotide or a functional equivalent thereof is complementary to exon 4

of BMP4. Some BMP4 transcripts start in exon-2. Alternative splice variants may also exist. In these case the numbering of the exons is counted from the exon identified by AON hBMP4#2, and this exon is given the number 4 also if the actual number of exons included in the mRNA before exon 4 is lower or higher than 3. The invention further provides oligonucleotide having the sequence of AON hBMP4#2.

The invention further provides the use of an anti-sense oligonucleotide or a functional equivalent thereof that is complementary to an exon of BMP-4, for skipping said exon in a BMP4 pre-mRNA.
An anti-sense oligonucleotide or a functional equivalent thereof is complementary to an exon of BMP-4 for use in the treatment of a genetic muscular dystrophy. Further provided is the use of an anti-sense oligonucleotide or a functional equivalent thereof is complementary to an exon of BMP-4 for the preparation of a medicament for the treatment of a genetic muscular dystrophy. Said anti-sense oligonucleotide or functional equivalent thereof can be provided to said cell by a number of different methods. A preferred method is without any means for enhancing transfer of said oligonucleotide or functional equivalent thereof to said cell. This is particularly preferred in case of in vivo administration of said oligonucleotide or functional equivalent thereof. In another preferred embodiment said anti-sense oligonucleotide is provided to said cell by means of a viral vector. In a preferred embodiment said viral vector comprises an expression cassette for expression of said anti-sense oligonucleotide.

Examples

Example 1

Material and methods

Cell culture

Primary human myoblasts were isolated from skeletal muscle biopsies [42] of three healthy individuals (KM109, KM108 and HPP4) and three DMD patients (DL589.2 [exon 51-55 deletion], DL470.2 [exon 46-50 deletion] and 50685.1[exon 48-50 deletion])[42;43]. The age at time of biopsy varied from 2-14 years. The cultures consist of myoblasts and other cell types that were present in the original biopsy. The proportion of myoblasts was determined for each biopsy by desmin staining and cell counting as described[44]. Healthy and DMD cultures did not differ in the average percentage of myoblasts (57 ± 20%). Cells were grown in proliferation medium in collagen-coated culture flasks. When cells were 80% confluent, differentiation was initiated by replacing the high-serum medium with low-serum medium[27]. All cell cultures used for the experiments had passage numbers between 4 and 10.

cDNA hybridization

cDNA microarrays containing 4417 muscle-related genes and ESTs (spotted in triplicate) from a human sequence-verified 40K I.M.AG.E. cDNA library (Research Genetics) were used and these were PCR-amplified, printed and pre-hybridized as described[27;45]. Total RNA from the six different cell cultures was isolated at days 0, 1, 2, 4, 6, 10 and 14, amplified, labeled and co-hybridized with a common reference as described[27;45]. The quality and quantity of the total RNA and cRNA was checked with the Bioanalyzer Lab-on-a-Chip RNA nano assay (Agilent Technologies).

Data analysis

All slides were scanned with an Agilent scanner (Model 2565BA) and spot intensities were quantified with the GenePix Pro 3.0 program (Axon Instruments). Raw intensity files were imported into Rosetta Resolver® v4.0 (Rosetta Biosoftware) and normalized with the Axon/Genepix error model. Per condition (healthy or DMD), 9 measurements per gene were considered (3 biological replicates, 3 technical replicates) and a stringent data-analysis procedure was performed. Genes with a normalized intensity higher than the average + 2 standard deviations (SDs) of the negative array controls, were analyzed. This had to be consistent in one condition (healthy or DMD) and in at least one timepoint. Error-weighted two-way ANOVA was performed with time, disease state and the interaction between time and disease state as variables. Genes were considered differentially expressed when the P-value for disease state was < 1 x 10-5 (Bonferroni corrected). Genes differentially expressed in time (P < 1 x 10-5) were functionally divided into groups using Gene Ontology Tree Machine[46].

Quantitative RT-PCR

cDNA was prepared from total RNA of all 6 cell cultures by reverse transcription using random hexamers and 0.5 mg total RNA as template. PCR primer pairs were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/). The oligonucleotide primer pairs used for each of the genes in this study correspond to the following nucleotides: glyceralde-hyde-3-phospate dehydrogenase, 510-529 and 625-644 (NM_002046); BMP4 394-413 and 484-503 (NM_001202) and AQP1 1129-1148 and 1227-1246 (NM_198098.1). Quantitative PCRs (Lightcycler, Roche) were performed as described with an annealing temperature of 58°C (for GAPDH and BMP4) or 62°C (for AQP1)[27]. Optimal cDNA dilutions and relative concentrations were determined using a dilution series per gene. Each gene was normalized to the

abundance of glyceraldehyde-3-phospate dehydrogenase mRNA (shows constant expression over time on the arrays).

RT-MLPA

MLPA probes were designed following the criteria described in[47]. The reaction was carried out on 50 ng total RNA as described in[48], except that all oligonucleotides used as half probes were chemically synthesized (Illumina Inc, San Diego), and two fluorophores were used during the PCR reaction[49]. Two ml of labelled PCR product was mixed with 10 ml formamide and 0.05 ml ROX500 size standard and separated on an ABI3700 capillary sequencer (Applied Biosystems). As there was a considerable range in peak heights 1:10 dilutions were loaded where necessary to obtain sufficient non-saturated signals. Data was exported to Excel for further analysis.
For each probe the relative peak height was used as a measure of intensity. Normalization was performed by dividing the relative peak height of each probe by the sum of the peak height of two control probes amplified with the same fluorophore. The control probes were targetted to genes that showed no significant change in expression level in the array analysis (Calnexin and Protein phosphatase 3 regulatory subunit B). Standard error was calculated on basis of 6 measurements (3 cultures measured in duplicate).

Differentiation assay and immunohistochemical analysis

Recombinant Human BMP4 (R&D Systems Inc.) was added to the cells at different concentrations (between 0.3 and 30 ng/ml) upon initiation of differentiation. During medium change, new BMP4 was added (every 4 days). Cells were fixed with 100% methanol (-20°C) at 7 or 11 days after serum deprivation. Immunohistochemical staining was performed as described previously[44;49]. Differentiation index was calculated by dividing the number of myosin positive cells by the number of desmin positive cells (= myogenic cells able to differentiate) x 100%. For the analysis, a generalized linear

regression model was fitted to explain the differences in the differentiation as a function of day (7 or 11), concentration (0, 0.3, 1, 3, 10 and 30 ng/ml) and cell line (healthy and DMD). The computations were run using R 2.0.1[50]. The model included a term representing the interaction between cell line and concentration, which is the term used to identify individual concentration levels for which the cell line displayed statistically significant differentiation proportions. Probability distributions were associated with the (model) error, and both normal and binominal error distribution were calculated.

Results

To explore the differentiating potential of human DMD myoblasts, a large-scale gene expression analysis has been performed. Human primary skeletal muscle cell cultures of 6 different individuals (3 healthy, 3 DMD) were analyzed with a muscle-related cDNA array. RNA was isolated at the myoblast stage (day 0) and at different days of differentiation (day 1, 2, 4, 6, 10 and 14). To find genes differentially expressed, an error-weighted two-way ANOVA was performed with time, disease state and the interaction between time and disease state as variables. Of the 4010 unique genes present on the array, 2423 gave a significant signal on at least one timepoint in either all the healthy samples or all the DMD samples. 94 genes were differentially expressed between healthy and DMD cultures (52 down, 42 up in DMD, p (disease state) $< 1 \cdot 10\text{-}5$). Surprisingly, already in undifferentiated cells ($t = 0$), 7 genes showed more than a 2-fold difference in RNA expression level (Table 1 and Supplemental Table 1a/b). Two of these (AQP1 and BMP4), are continuously higher expressed in DMD over the whole time course. This difference was confirmed with quantitative RT-PCR (Figure 1).

Dahlqvist et al. previously demonstrated in immortalized mouse myoblasts (C2C12) that BMP4 has an inhibitory effect on muscle differentiation[51]. To

determine if this holds true in pimary human myoblast cultures and to find out if healthy and DMD cells respond differently, recombinant BMP4 was added to the cells. Addition of recombinant BMP4 to the fusion medium at t = 0 resulted in a concentration-dependent reduction of cell fusion (less multinucleated, myosin-positive cells) in both healthy and DMD cell cultures (Figure 2, 3 and supplemental Figure 1). DMD cells were significantly more sensitive to BMP4 ($p < 0.05$) since a 3-fold lower concentration causes a similar degree of differentiation inhibition (Table 2 and 3). The results presented correspond to using the normal distribution as error distribution (both normal and binominal error distributions yielded similar conclusions).

Along with genes that were differentially expressed between healthy and DMD over the whole time course, there was a small group of genes whose expression patterns start to diverge after induction of differentiation (p (disease state) < $1 \cdot 10\text{-}5$). Figure 4 shows that these genes can be divided into two groups. Firstly, genes upregulated during the fusion in healthy cell cultures that remain low in DMD cultures (BF, Adlican and MME). Secondly, two genes with a constant low expression level in healthy cell cultures, but upregulated during fusion of DMD cells (MTUS1 and EDNRA). These results were confirmed with RT-MLPA (Supplemental Figure 2). The RT-MLPA is a technique that allows the rapid and simultaneous quantification of up to 40 transcripts in a single reaction. This technique was chosen as it allows multiple samples and transcripts to be tested in a faster and cheaper assay than quantitative RT-PCR.

The two-way ANOVA also reveals genes whose expression changes equally in the timecourse of both healthy and DMD cultures ($n = 68$, p (time) < $1 \cdot 10\text{-}5$). The role of these genes in myogenesis has been discussed in a previous paper[27]. Using Gene Ontology Tree Machine, these genes were functionally annotated[46]. Figure 5 shows the average expression patterns for healthy and

DMD cells (functional groups containing ≥ 6 genes). The sarcomeric proteins (n = 10) showed a significantly decreased expression at later time points in DMD compared to healthy cells (paired T-test, $p < 0.01$). Other functional categories showed similar expression pattern changes in healthy and DMD cultures.

Example 2

Downregulation of BMP4 expression in Duchenne myoblasts by exon skipping AONs

Human myoblats, isolated from a skeletal muscle biopsy of a Duchenne patient (DL589.2) were proliferated in 6-well dishes to a confluency of 50% (for details see example 1). Twenty four hours before transfection, cells were put on low-serum differentiation medium. Cells were transfected with the AONs (2'-O-methyl phosphorothioate) mentioned below, at a concentration of 100, 200, or 500 nM, or left untreated:

AON sequences:
hBMP4-#1: 5' gca ugg cuc gcg ccu ccu agc ag 3'
hBMP4-#2: 5' cca gug cug ugg auc ugc ucu u 3'
FAM-AON: 5'-FAM-cuu cca cau ccg guu guu u 3'
The first two oligonucleotides are designed against exon internal sequences in exon 4 of human BMP4, transcript variant 1 (NM_001202). The last oligonucleotide is a control oligonucleotide which should not affect BMP4 expression, and contains a 5' FAM-label to allow fluorescent detection of oligonucleotide uptake.

AONs were transfected with the Exgen500 transfection reagent (MBI Fermentas, 2 µl per µg AON). At 3 hours of transfection, the transfection complex was washed away and fresh differentiation medium was added to the cells. At 6 hours after transfection, nuclei of the FAM-AON transfected myoblasts were brightly fluorescent, indicative of high nuclear uptake of the AONs.

At 24 hours after transfection, cells were lysed in RNABee solution (Isotex Diagnostics) and RNA was isolated using the manufacturer-supplied protocol. RNA was transcribed into cDNA by incubating 200 ng of total RNA with 40 ng of random hexamer primers in 13 µl of DEPC-water for 10 minutes at 70°C. After cooling on ice, 4 µl of 5x first strand buffer, 2 µl of 10 mM dNTPs, and 1 µl of Revertaid RnaseH- reverse transcriptase (MBI Fermentas) was added and the reaction mixture was incubated at 42 °C for 2 hours. The reverse transcriptase was inactivated by incubation at 70°C for 15 minutes. The resulting cDNA was diluted 5 times.

To evaluate AON-mediated exon skipping in the BMP4, the following PCRs were performed:
PCR1: amplification of exon 3 of the BMP4 gene, to assess overall BMP4 mRNA levels. Primer sequences used: BMP4exon3for: 5' TGAGCCTTTCCAGCAAGTTTGTT 3'; BMP4exon3rev: 5' ATCAGCATTCGGTTACCAGG 3'
PCR2: evaluation of the skipping of exon 4 by amplification with primers in exon 3 and exon 5: BMP4exon3for: 5' TGAGCCTTTCCAGCAAGTTTGTT 3'; BMP4exon5rev: 5' GGGATGCTGCTGAGGTTAAA 3'
PCR3: evaluation of cDNA synthesis with primers in GAPDH gene: GAPDHfor: 5' GATCATCAGCAATGCCTCCT 3'; GAPDHrev: 5' CCATCCACAGTCTTCTGGGT 3'
PCRs were performed with 40 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 56°C and 30 seconds extension at 72°C.
PCR fragments were visualized on an ethidium-bromide-stained agarose gel.

Results

Results are displayed in Figure 1. All three PCRs generated fragments of the expected size. AON hBMP4#2 induced skipping of exon 4 at the highest concentration tested (500 nM) (Figure 1; Panel B), visible as a shorter

fragment of 142 bp, in which exon 3 is directly spliced to exon 5. Transfection with a lower concentration of AON hBMP4#2 (200 nM) resulted in a complete absence of the BMP4 transcript. This was confirmed with the exon-internal PCR of exon 3 of the BMP4 gene (Panel A). In contrast, the control gene GAPDH is still stably expressed (Panel C), indicative of a selective loss of the BMP4 transcript. Since skipping of exon 4 disrupts the open reading frame in the BMP4 transcript, we think that exon skipping with this concentration of AON has been so effective that the transcripts is lost due to highly efficient nonsense mediated decay activity. At both concentrations, AON hBMP4#2 reduced the level of the intact BMP4 transcript and thus corrects BMP4 mRNA levels towards the normal levels in myoblast cultures from healthy subjects. AON hBMP4#1 and the control FAM-AON did not induce exon skipping. Occasionally, activation of a cryptic splice site in exon 4 was observed.

Example 3

Antisense against BMP4 and DMD exon skipping antisense sequences in mdx x utrophin -/- mice; evaluation of muscle strength, survival, protein and RNA levels

Dystrophin and utrophin-deficient mice (mdx.utrn$^{-/-}$ mice) were generated by crossing of male mdx$^{+/0}$.utrn$^{-/-}$.hDMD$^{+/-}$ mice female mdx$^{+/+}$.utrn$^{-/-}$.hDMD$^{+/-}$ ('t Hoen et al, manuscript in preparation). Mice were housed under standard laboratory conditions. Animals were fed regular chow and had access to drinking water *ad libitum*.

20-mer 2'-*O*-methyl phosphorothioate ribonucleic acids were were synthesized by Eurogentec. The antisense sequences were targeted against

1. the exon 23 - intron 23 boundary of the murine *Dmd* gene, sequence AON-1: GGCCAAACCUCGGCUUACCU. The sequence of this AON is identical to the AON used by Mann *et al.* and Lu *et al.* in their

experiments in *mdx* mice and results in efficient skipping of the mutated exon 23 of the *Dmd* gene, resulting in production of functional dystrophin protein[31;52].

2. BMP4. Designed sequences AON-2 (AGACUGGAGCCGGUAA) and AON-3 (UGGCUCGGCUGGCGGG) in exon 2 of mRNA sequence NM_007554.

Before injections, AONs were diluted in in 0.9% (w/v) NaCl to a final concentration of 50 mg/ml. Mice were divided randomly in three groups. The first group was injected intravenously, starting at the age of 21 days, for 5 consecutive days with 5 mg of AON-1. The second and third groups received, in addition to AON-1, 5 x 5 mg of AON-2 or AON-3, respectively.
Before the first injection, and 1, 2 and 3 weeks after the first injection, a blood sample was taken for determination of creatine kinase activity (a well-established bioessay for muscle damage). At these same time points, animals were forced to run on a Rotarad device and the period of time before the mice fell off was recorded (a bioessay for muscle strength). At three weeks after injection, Evans blue was administered intraperitoneally (10 mg / kg body weight) to stain damaged muscles. Mice were sacrificed by cercival dislocation, and the gastrocnemius, quadriceps, tibialis anterior, heart and diafragm muscle were isolated and snap frozen in liquid nitrogen before further processing.

Levels of corrected dystrophin mRNA transcript were measured as described before[53]. Dystrophin protein levels were determined by Western blotting as described[53]. Standard hematoxylin/eosin staining was performed on 10 μm cryosections of the muscles to assess muscle histology. The average fibre cross-sectional area was determined. Evans blue positive areas in 10 sections per muscle were determined as a measure for muscle damage. Immunostaining with the NCL-DYS2 and CD4 antibody was performed on the sections to

analyze for the presence dystrophin and the presence of immune infiltrates[53].

Example 4

Genetic correction of gene defect in mdx myoblast cultures and coinjection of these myoblasts to mdx mice with the BMP4 inhibitor Noggin.

Dystrophin and utrophin-deficient mice (mdx.utrn$^{-/-}$ mice) were generated by crossing of male mdx$^{+/0}$.utrn$^{-/-}$.hDMD$^{+/-}$ mice female mdx$^{+/+}$.utrn$^{-/-}$.hDMD$^{+/-}$ ('t Hoen et al, manuscript in preparation). Mice were housed under standard laboratory conditions. Animals were fed regular chow and had access to drinking water *ad libitum*.

Mouse myoblasts were obtained from the isolated hindlimb muscle of male mdx$^{+/0}$.utrn$^{-/-}$ mice by standard collagenase / dispase treatment and culturing on collagen-coated dishes in standard proliferation medium [22]. Proliferating myoblasts (1x10^6 cells / mouse) were transfected with 500 nM of AON-1: GGCCAAACCUCGGCUUACCU (2'-*O*-methyl phosphorothioate, Eurogentec), complexed with polyethylenimine (Exgen500, MBI Fermentas, 3.5 µl per µg AON). At 2 days after transcfection, cells were pelleted and taken up in 7 µl Hank's balanced salt solution, containing or not Noggin, a well-known BMP4 inhibitor (different amounts: 0, 10, 50 or 100 ng). Cells were injected in the tibialis anterior muscle of female mdx$^{+/+}$.utrn$^{-/-}$ mice with a 50 µm-tip glass pipette. At 3 or 10 days after the injection, mice were sacrificed, and serial cryosections were made. Sections were stained with heamoxylin/eosin and with dystrophin and Y-chromosome-specific antibodies to analyze for cell survival, migration and dystrophin expression.

Brief description of the drawings

Figure 1. Gene expression of *AQP1* (A) and *BMP4* (B) determined with oligonucleotide microarrays (upper panel) or quantitative RT-PCR (lower panel) (Lightcycler, Roche). On the x-axis time is displayed in days. On the y-axis either the log 2 expression ratio (normalized to healthy at t = 0) or the -Δ(Ct) (normalized to healthy at t = 0) are displayed. The Ct value is proportional to the ^{2}log of the initial amount of mRNA and thus comparible to the ^{2}log expression ratio. Vertical bars represent standard deviations of the different cultures (n = 3).

Figure 2. Immunohistochemical staining of healthy and DMD myotubes incubated with different concentrations of BMP4, evaluated on day 7 of differentiation. Cells were stained with DAPI (blue) and antibodies to desmin (red) and myosin (green).

Figure 3. Differentiation index of healthy and DMD myoblasts after addition of different concentrations of recombinant BMP4. Cells were fixed at day 7. Regression model, ** $p < 0.01$, * $p < 0.05$.

Figure 4. Log2 gene expression ratios of genes differentially expressed between healthy and DMD myoblasts after induction of differentiation; *BF*, *MME*, *Adlican*, *MTUS1*, *EDNRA*. Vertical bars show standard deviations of the different lines used (n = 3).

Figure 5. Mean log2 gene expression of genes in different functional categories during healthy and DMD myoblast fusion. Sarcomeric genes show in late DMD myogenesis (day 6, 10 and 14) a decrease in gene expression. * Paired t-test, $p < 0.01$. Genes involved in other functional categories (cell growth and maintenance, protein binding, metabolism up and metabolism down) do not

show a significant difference between healthy and DMD at any time point ($n \geq 6$).

Figure 6. Ethidium bromide-stained agarose gel demonstrating the antisense oligonucleotide-mediated skipping of exon 4 of human BMP4 in myoblasts of a Duchenne patient (experiment described asexample 2). Panel A shows the fragments obtained with PCR 1 (exon 3 of the BMP4 mRNA); panel B shows the fragments obtained with PCR 2 (exon 3 – 5 of the BMP4 mRNA). The product in which exon 4 is skipped has a size of 142 nucleotides. Panel C shows the fragments obtained with PCR 3 (GAPDH mRNA).

Supplemental Figure 1. Differentiation index of healthy and DMD myoblasts after addition of different concentrations of recombinant BMP4. Cells were fixed at day 11. Regression model, * $p < 0.05$.

Supplemental Figure 2. Gene expression levels of genes differentially expressed in time between healthy and DMD myoblast fusion tested with RT-MLPA; *BF*, *MME*, *Adlican*, *MTUS1*. Vertical bars show standard error for the different lines used ($n = 3$).

Table 1: Genes showing differential gene expression (> 2-fold) between healthy and DMD myoblasts

Genbank	Symbol	Sequence Description	Fold change
H24316	AQP1	aquaporin 1 (channel-forming integral protein, 28kDa)	6.48
AA463225	BMP4	bone morphogenetic protein 4	3.08
AA704587	EST	ESTs, Weakly similar to hypothetical protein FLJ20958 [Homo sapiens] [H.sapiens]	2.07
R97066	TAL1	T-cell acute lymphocytic leukemia 1	2.30
R38539	FGF2	fibroblast growth factor 2 (basic)	-2.20
AA598601	IGFBP3	insulin-like growth factor binding protein 3	-2.34
AA664101	ALDH1A 1	aldehyde dehydrogenase 1 family, member A1	-2.05

Table 2: Generalized linear regression model for differentiation index

Coefficients	P-value
Intercept	$4.02 \cdot 10^{-7}$ ***
Cell line (DMD)	0.22844
Concentration BMP4 0.3 ng/ml	0.47855
Concentration BMP4 1 ng/ml	0.30271
Concentration BMP4 3 ng/ml	0.86939
Concentration BMP4 10 ng/ml	0.48777
Concentration BMP4 30 ng/ml	0.00867 **
Days in differentiaton (11 days)	0.09281
Int. cell line - con. BMP4 0.3 ng/ml	0.25261
Int. cell line - con. BMP4 1 ng/ml	0.08274
Int. cell line - con. BMP4 3 ng/ml	0.43757
Int. cell line - con. BMP4 10 ng/ml	0.04648 *
Int. cell line - con. BMP4 30 ng/ml	0.00756 **

Intercept: baseline proportion for the healthy cell culture, with 0.3 ng/ml BMP4 at day 7.
*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Table 3: Generalized linear regression model for differentiation index, ANOVA table

Variables	P-value
Cell line	0.04069 *
Concentration BMP4	4.74e-05 ***
Days in diffentiation	0.09281
Int. cell line - concentration	0.08305

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Supplemental table 2: Generalized linear regression model for differentiation index

Coefficients	P-value
Intercept	$4.02 \cdot 10^{-7}$ ***
Cell line (DMD)	0.22844
Concentration BMP4 0.3 ng/ml	0.47855
Concentration BMP4 1 ng/ml	0.30271
Concentration BMP4 3 ng/ml	0.86939
Concentration BMP4 10 ng/ml	0.48777
Concentration BMP4 30 ng/ml	0.00867 **
Days in differentiaton (11 days)	0.09281
Int. cell line - con. BMP4 0.3 ng/ml	0.25261
Int. cell line - con. BMP4 1 ng/ml	0.08274
Int. cell line - con. BMP4 3 ng/ml	0.43757
Int. cell line - con. BMP4 10 ng/ml	0.04648 *
Int. cell line - con. BMP4 30 ng/ml	0.00756 **

Intercept: baseline proportion for the healthy cell culture, with 0.3 ng/ml BMP4 at day 7.
*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Supplemental table 3: Generalized linear regression model for differentiation index, ANOVA table

Variables	P-value
Cell line	0.04069 *
Concentration BMP4	4.74e-05 ***
Days in diffentiation	0.09281
Int. cell line - concentration	0.08305

*** P < 0.001, ** P < 0.01, * P < 0.05

Supplemental Table 1a: Genes showing differential gene expression between healthy and DMD myoblast fusion (Genes upregulated in DMD)

				Log2 expression difference (DMD-healthy)						
Genbank	**Name**	**Sequence**	**p-value**	**0**	**1**	**2**	**4**	**6**	**10**	**14**
H24316	AQP1 *	aquaporin	1.11E-16	2.70	3.27	2.92	3.12	2.44	2.80	1.99
AI268273	MAP3K5 *	mitogen-ac	1.11E-16	0.97	1.15	0.85	0.67	0.61	0.77	0.65
L06622	EDNRA *	endothelin	1.11E-16	-0.17	0.62	1.53	1.52	1.64	1.36	1.65
AA463225	BMP4 *	bone morp	1.55E-15	1.62	1.08	1.53	1.62	1.17	1.36	0.70
AA496896	MTSG1 *	transcriptio	1.59E-14	0.27	0.97	1.36	2.00	1.22	1.39	1.35
AA448194	SMN1 *	survival of	9.14E-12	0.54	0.47	0.65	0.55	0.49	0.71	0.50
AA773333	DPM2	dolichyl-ph	3.86E-11	0.49	0.78	0.67	0.51	0.58	0.71	0.49
AI359037	STX3A	Syntaxin 3/	1.13E-09	0.49	0.82	0.81	0.43	0.68	1.09	0.76
AA404269	PRICKLE1	Prickle1-lik	1.16E-09	0.53	0.78	0.84	1.14	0.71	0.89	0.46
AA437212	AP1S2	adaptor-rel	1.81E-09	0.32	0.55	0.80	0.62	0.98	0.75	0.69
R44617	MDFI	MyoD fami	1.89E-09	0.11	0.62	0.68	1.44	0.99	1.24	0.61
AA456821	NETO2	neuropilin (	3.86E-09	0.84	0.71	0.60	1.00	0.93	0.86	0.96
AI336948	BACH1	BTB and C	6.46E-09	0.59	0.77	0.71	0.65	0.64	0.79	0.63
H42679	HLA-DMA	major histo	7.65E-09	0.93	1.19	0.92	1.02	0.49	0.60	0.56
AA402874	PLTP	phospholip	9.83E-09	0.17	0.42	0.55	1.47	0.98	1.29	0.59
AA424824	DSTN *	destrin (act	1.62E-08	0.75	0.95	0.68	0.77	0.69	0.52	0.59
AA918646	KIAA0830	KIAA0830	4.74E-08	0.73	0.87	0.95	0.27	0.28	0.46	0.20
NM_00426	FADS2	fatty acid d	1.20E-07	0.30	0.62	1.22	1.22	0.11	0.71	0.17
N35907	KIAA0830	KIAA0830	2.07E-07	0.65	0.85	0.95	0.38	0.02	0.28	0.13
AJ249545	HEY2	hairy/enhar	3.62E-07	0.58	0.82	0.75	1.20	0.91	0.55	0.99
N51002	PPFIA2	Protein tyrc	3.77E-07	0.69	0.75	0.42	0.68	0.59	0.74	0.46
H74106	LDB2	LIM domair	4.64E-07	0.29	0.39	0.82	0.82	0.80	0.59	0.61
AA505056	PAK2	P21 (CDKI	5.53E-07	0.86	0.80	0.79	0.44	0.91	0.86	0.44
R26417	STAT5B	Signal tran	6.05E-07	0.43	0.33	0.47	0.53	0.23	0.29	0.36
AA449289	SMTN	smoothelin	9.74E-07	0.40	0.63	0.71	0.71	0.16	0.75	0.36
AI129115	FLJ10151	CDNA FLJ	1.02E-06	0.48	0.61	0.42	0.54	0.64	0.46	0.38
AA458981	FKBP4	FK506 binc	1.03E-06	0.66	0.43	0.43	0.73	0.34	0.81	0.48
AF091555	CTBP1	C-terminal	1.41E-06	0.51	0.78	0.78	0.62	0.18	0.78	0.42
AA699926	SNTA1 *	syntrophin,	1.53E-06	0.17	0.32	0.37	0.47	0.25	0.49	0.33
AA677824	TEAD3	TEA domai	1.99E-06	0.65	0.80	0.58	0.28	0.27	0.41	0.41
H65596	SAP18	sin3-assoc	2.22E-06	0.52	0.33	0.55	0.28	0.33	0.33	0.23
AI291262	EST	Homo sapi	2.94E-06	0.79	0.51	0.76	0.59	0.93	1.06	0.59
N63172	LOC153222	Retina prot	3.21E-06	0.62	0.43	0.65	0.48	0.26	0.59	0.39
AA282983	C6orf129	Chromosor	3.28E-06	0.32	0.68	0.50	0.40	0.23	0.58	0.28
AA485871	MYO1C	myosin IC	4.11E-06	0.51	0.95	0.39	0.66	0.02	1.14	0.66
R97066	TGM2	transglutan	4.45E-06	1.20	1.15	0.70	0.63	0.22	0.02	0.23
AA464736	FLJ14525	hypothetica	5.40E-06	0.24	0.77	0.63	0.21	0.31	0.71	0.45
N70786	ASH1L	Ash1 (abse	5.98E-06	0.34	0.64	0.45	0.53	0.54	0.40	0.14
AA122049	FAM35A	Family with	6.60E-06	0.15	0.46	0.53	0.33	0.41	0.48	0.42
AF176422	HEY1	hairy/enhar	7.92E-06	0.70	0.63	1.36	0.63	0.53	0.86	1.00
H07920	MAP2K6	mitogen-ac	7.96E-06	0.13	0.21	0.28	0.22	0.38	0.34	0.59
AA704226	TNS	tensin	8.70E-06	0.42	0.74	0.74	0.54	0.48	0.48	0.26
L07872	RBPSUH	recombinin	9.79E-06	0.70	0.51	0.31	0.82	0.75	0.53	0.94

* Confirmed by sequencing

Supplemental Table 1b: Genes showing differential gene expression between healthy and DMD myoblast fusion (Genes downregulated in DMD)

				Log2 expression difference (DMD-healthy)						
Genbank	**Name**	**Sequence**	**p-value**	**0**	**1**	**2**	**4**	**6**	**10**	**14**
R38539	FGF2	fibroblast g	1.26E-12	-1.15	-0.44	-1.07	-1.05	-0.94	-0.71	-0.71
AA598601	IGFBP3	insulin-like	5.12E-12	-1.23	-1.27	-1.30	-1.29	-0.69	-0.90	-0.36
H07926	ACAA2 *	acetyl-Coe	1.16E-10	-0.56	-0.87	-0.69	-0.63	-0.54	-0.61	-0.38
R42685	IMPA2 *	inositol(my	1.50E-10	-0.71	-1.25	-1.12	-0.97	-0.98	-1.21	-0.55
R51218	PIG8	Translokin	3.66E-10	-0.63	-1.10	-1.00	-0.57	-0.23	-1.44	-0.31
AA401441	BF *	B-factor, pr	6.90E-10	-0.15	-0.20	-0.66	-1.33	-1.50	-1.31	-1.41
AA669750	EST	Transcribe	9.65E-10	-0.78	-0.80	-0.62	-1.01	-0.97	-0.57	-0.96
J03580	PTHLH *	parathyroid	1.43E-09	-0.44	-0.91	-0.83	-0.72	-0.61	-0.77	-0.42
R98851	MME *	membrane	2.98E-09	-0.49	-0.69	-0.94	-1.82	-1.48	-1.09	-1.54
H23235	PDGFRA	platelet-de	1.04E-08	0.06	-1.44	-5.80	-1.62	-1.05	-1.56	-0.58
AA664101	ALDH1A1 *	aldehyde d	2.40E-08	-1.05	-1.19	-1.05	-0.57	-0.80	-1.06	-1.22
AA630104	LIPA	lipase A, ly	3.87E-08	-0.84	-0.36	-0.18	-0.59	-0.66	-0.45	-0.49
AA975768	EMP1	epithelial m	4.61E-08	-0.75	-0.37	-0.71	-0.79	-0.63	-0.50	-0.56
R59697	EST	transcribed	7.10E-08	-0.46	-0.42	-0.62	-0.63	-0.38	-0.63	-0.24
AA464691	MXRA5 *	matrix-rem	1.17E-07	-0.66	-0.41	-0.80	-1.35	-1.40	-1.12	-1.33
NM_00033	SGCD	Sarcoglyca	1.63E-07	-0.88	-0.97	-0.89	-0.60	-1.11	-1.06	-0.96
AA460353	MAPKAP1	mitogen-ac	2.02E-07	-0.36	-0.52	-0.39	-0.30	-0.69	-0.43	-0.83
H14810	EST	mRNA; cD	2.12E-07	-0.31	-0.48	-0.60	-0.64	-0.53	-0.79	-0.45
AA190882	C20orf3	chromoson	2.59E-07	-0.51	-0.61	-0.56	-0.29	-0.77	-0.55	-0.57
AA034939	LAMA2 *	laminin, alp	3.97E-07	-0.74	-0.68	-0.79	-0.70	-0.64	-0.42	-0.43
R61674	PTP4A1	protein tyrc	4.12E-07	-0.85	-0.75	-0.37	-0.70	-0.39	-0.36	-0.33
W02630	SDCCAG3	serological	5.94E-07	0.05	-0.24	-5.91	-0.51	-1.25	-0.13	-1.82
H18640	MTX2	metaxin 2	6.09E-07	-0.37	-0.26	-0.37	-0.67	-0.55	-0.69	-0.42
AA192765	NDUFA10	NADH dyh	1.02E-06	-0.67	-0.30	-0.42	-0.15	-0.29	-0.57	-0.25
AA629844	LRRFIP2	leucine rich	1.09E-06	-0.59	-0.40	-0.27	-0.10	-0.46	-0.43	-0.38
AA456400	ADSL	adenylosuc	1.45E-06	-0.52	-0.62	-0.39	-0.27	-0.74	-0.64	-0.60
T62048	C1S *	complemer	1.68E-06	-0.80	-0.67	-0.75	-0.63	-0.94	-0.62	-0.44
R49329	C6orf85	Chromosor	1.78E-06	-0.43	-0.75	-0.52	-0.32	-0.62	-0.65	-0.43
W55872	NFKBIA	nuclear fac	1.84E-06	-0.16	-0.32	-0.59	-0.71	-0.99	-0.63	-0.48
AA598400	SFRS3	splicing fac	2.08E-06	-0.64	-0.70	-1.27	-0.55	-0.69	-0.71	-0.81
R85213	UBE3A	ubiquitin pr	2.34E-06	-0.57	-0.78	-0.63	-0.49	-0.69	-0.65	-0.41
AA485773	GRSF1	G-rich RNA	2.55E-06	-0.64	-0.39	-0.25	-0.28	-0.51	-0.42	-0.52
AA488084	EST	cDNA clon	2.60E-06	-0.42	-0.87	-1.72	-1.52	-2.60	-1.05	-1.16
AA935533	E2F6	E2F transc	2.71E-06	-0.91	-1.26	-0.86	-0.99	-1.45	-1.84	-0.98
AA939088	FLJ10781	hypothetica	2.74E-06	-0.89	-0.23	-0.28	-0.43	-0.22	-1.25	-0.82
AA486919	RPL28	ribosomal p	3.31E-06	-0.50	-0.63	-0.30	-0.42	-0.83	-0.74	-0.21
AA056395	HCBP6	hepatitis C	3.52E-06	-0.35	-0.36	-0.21	-0.28	-0.51	-0.50	-0.58
W56356	PEG10	paternally e	3.59E-06	-0.15	-1.26	-1.92	-0.34	-0.64	-1.71	0.17
T72235	NNMT	nicotinamic	3.77E-06	-0.58	-0.43	-0.57	-0.15	-0.71	-0.50	-0.49
AI349935	MYO10	myosin X	3.99E-06	-0.22	-0.84	-1.03	-1.72	-1.10	-0.57	-0.39
AA444051	S100A10 *	S100 calciu	4.10E-06	-0.67	-0.80	-1.32	-1.31	-1.00	-1.21	-0.84
H99699	RPC8	RNA polym	4.81E-06	-0.54	-0.89	-0.40	-0.48	-0.50	-0.62	-0.75
AA774755	SPG3A	spastic par	4.82E-06	-0.18	-0.40	-0.50	-0.22	-0.75	-0.86	-0.38
AA936738	DFNA5	deafness, a	5.25E-06	-0.59	-0.08	-0.27	-0.30	-0.57	-0.29	-0.23
AA188661	SH3BP5	SH3-doma	5.31E-06	-0.34	-0.57	-0.69	-0.33	-0.65	-0.46	-0.60
H08424	HSKM-B	HSKM-B pr	6.43E-06	-0.53	-0.38	-0.40	-0.55	-0.58	-0.24	-0.03
AA292054	GAS1	growth arre	6.62E-06	-0.21	-0.34	-0.70	-0.87	-0.77	-0.71	-0.60
AA456688	DRG2	developme	6.85E-06	-0.42	-0.31	-0.43	-0.59	-0.50	-0.67	-0.24
AA496576	NFE2L1	nuclear fac	7.05E-06	-0.62	-0.34	-0.36	-0.46	-0.65	-0.33	-0.49
H20908	ERCC3	excision re	7.95E-06	-0.37	-0.36	-0.43	-0.16	-0.56	-0.57	-0.25
AA487210	FLJ10081	hypothetica	8.55E-06	-0.39	-0.40	-0.29	-0.25	-0.35	-0.34	-0.24

W48761	TAPBP	TAP bindin	8.99E-06	-0.55	-0.55	-0.33	-0.44	-0.49	-0.45	-0.29

* Confirmed by sequencing

Claims

1. Use of a compound for reducing, inhibiting and/or antagonizing expression of Bone Morphogenetic Protein 4 (BMP4) in a cell, for the manufacture of a medicament for alleviating a symptom of a genetic muscular dystrophy in an individual.
2. Use according to claim 1, wherein said cell is a myoblast cell or a precursor thereof.
3. Use according to claim 1 or claim 2, wherein said compound comprises an antisense RNA or a functional equivalent thereof.
4. A method for stimulating differentiation of a myoblast cell comprising providing said myoblast cell with a compound for reducing, inhibiting and/or antagonizing expression of BMP4 in said myoblast cell.
5. A method according to claim 4 wherein said compound decreases and/or inhibits BMP4 mRNA expression in said myoblast cell.
6. A method according to claim 4 or claim 5, further comprising contacting said myoblast cell with a mature muscle cell.
7. A method according to any one of claims 4-6, wherein said mature muscle cell is derived from a subject that suffers from said genetic muscular dystrophy.
8. A method according to any one of claims 4-7, wherein reducing and/or inhibiting BMP4 mRNA expression in said myoblast cell comprises providing said myoblast cell or a precursor thereof with a BMP4 oligonucleotide that is complementary to said BMP4 gene (anti-sense).
9. A method according to any one of claims 4-8, wherein said myoblast cells or precursors thereof are in vitro provided with said BMP-4 anti-sense oligonucleotide.
10. A collection of myoblast cells or precursors thereof comprising a BMP-4 anti-sense oligonucleotide.

11. Use according to any one of claims 1-3, or a method according to any one of claim 4-9, wherein expression is decreased, inhibited and/or antagonized by means of a virally transduced DNA sequence.

12. Use according to any one of claims 1-3, or a method according to any one of claims 4-9, wherein said compound is provided to said cell by means of a viral vector.

13. Use or method according to any one of claims 1-12, wherein said genetic muscular dystrophy comprises one of the following diseases: Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Congenital Muscular Dystrophy (CMD), Distal Muscular Dystrophy (DD), Emery-Dreifuss Muscular Dystrophy (EDMD), Facioscapulohumeral Muscular Dystrophy (FSH), Limb-Girdle Muscular Dystrophy (LGMD), Myotonic Dystrophy (MMD), Oculopharyngeal Muscular Dystrophy (OPMD).

14. Use or method according to any one of claims 1-13, wherein said compound comprises a protein inhibitor.

15. Use or method according to any one of claims 1-14, wherein said compound comprises a BMP4 inhibitor or antagonist.

16. Use or method according to claim 14 or claim 15, wherein said inhibitor or antagonist comprises noggin, chordin, ventroptin, twisted gastrulation, gremlin or other members of the DAN family of BMP4 antagonists, PRDC, sclerostin, CTGF and/or follistatin or a functional part, derivative and/or analogue thereof.

17. Use or method according to any one of claims 1 to 16, further comprising use of a second compound for the preparation of a medicament for treatment of said individual wherein said second compound provides a muscle cell of said individual with at least part of the normal function of a gene that is associated with said genetic muscular dystrophy (mutated gene).

18. Use or method according to any one of claims 1-17, wherein said genetic muscular dystrophy is Duchenne Muscular Dystrophy (DMD) or Becker Muscular Dystrophy (BMD).

19. Use according to claim 18, wherein said second compound comprises an oligonucleotide, or functional equivalent thereof, for skipping an exon of a dystrophin gene.

20. A method for determining whether a BMP4 anti-sense oligonucleotide or a functional equivalent thereof is capable of inducing skipping of an exon in a BMP4 pre-mRNA containing said exon, said method comprising providing a cell expression said BMP4 pre-mRNA with said oligonucleotide and determining whether said exon is absent from mature mRNA produced from said pre-mRNA.

21. A method according to claim 20, wherein said anti-sense oligonucleotide or a functional equivalent thereof is complementary to said exon.

22. A method according to claim 20 or claim 21, wherein said anti-sense oligonucleotide or a functional equivalent thereof is complementary to an exon-internal part of said exon.

23. A method according to any one of claims 20-22, wherein said anti-sense oligonucleotide or a functional equivalent thereof is complementary to exon 4 of BMP4.

24. Use of an anti-sense oligonucleotide or a functional equivalent thereof that is complementary to an exon of BMP-4, for skipping said exon in a BMP4 pre-mRNA.

25. An anti-sense oligonucleotide or a functional equivalent thereof is complementary to an exon of BMP-4 for use in the treatment of a genetic muscular dystrophy.

26. Use of an anti-sense oligonucleotide or a functional equivalent thereof is complementary to an exon of BMP-4 for the preparation of a medicament for the treatment of a genetic muscular dystrophy.

27. Use or a method according to any one of claims 20-26, wherein said anti-sense oligonucleotide is provided to said cell by means of a viral vector.

28. Use according to claim 27, wherein said viral vector comprises an expression cassette for expression of said anti-sense oligonucleotide.