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(54) Title: METHODS OF PREVENTING TRANSPLANTATION REJECTION BY CREATING IMMUNOLOGICALLY NEUTRAL STEM CELLS USING GENE SILENCING TECHNOLOGY

(57) Abstract: The present invention provides compositions and methods for modulating the expression of genetic material encoding an immunogenic cell surface molecule. In particular, the present invention relates to nucleic acid derived compounds which hybridize or otherwise interact with nucleic acid molecules encoding major histocompatability (MHC) proteins or with nucleic acid molecules required for or which facilitate expression of MHC-encoding material thereby reducing expression of the genetic material in stem cells or differentiated tissue derived from the stem cells. The subject invention even more particularly provides the uses of gene silencing techniques to modulate the expression of immunogenic cell surface molecules in single cells *in vitro* and *in vivo*, and in tissues derived therefrom.

Methods of preventing transplantation rejection by creating immunologically neutral stem cells using gene silencing technology

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of genetic material encoding an immunogenic cell surface molecule. In particular, the present invention relates to nucleic acid derived compounds which hybridize or otherwise interact with nucleic acid molecules encoding major histocompatability (MHC) proteins or with nucleic acid molecules required for or which facilitate expression of MHC-encoding material thereby reducing expression of the genetic material in stem cells or differentiated tissue derived from the stem cells. The subject invention even more particularly provides the uses of gene silencing techniques to modulate the expression of immunogenic cell surface molecules in single cells *in vitro* and *in vivo*, and in tissues derived therefrom.

DESCRIPTION OF THE PRIOR ART

Bibliographic details of the publications referred to in this specification are also collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the general knowledge in any country.

The promise of stem cell regenerative medicine is rapidly being fulfilled as demonstrated in numerous studies that are in the process of going from experimental to clinical protocols. Aside from the classic treatments for pathologies that necessitate the rebuilding of the hematopoietic system, applications of stem cell therapeutics are offering solutions for neurological, endocrine and cardiac pathologies.

The properties that make the stem cell so important therapeutically, such as the ability to alter its physiological state to suit its environment and to localize towards regions of injury or inflammation are most potent in those derived from young tissues or persons. Thus research has focused on the most potent of stem cells, those with embryonic derivation. This source of stem cells for therapeutic applications as well as research remains ethically controversial and has limited the availability of government funding and support. Presently, most countries have adopted legislation that promotes restrictive guidelines as to the use of stem cell derived from multi-celled embryos or foetuses.

Compounded with the restrictions of obtaining pools of stem cells is the physiological limitation that is imposed on all cells and tissues that are introduced to an organism other than that of its origin – graft *versus* host disease (GVHD). The most vicious rejection of grafts is provoked by differences in the major histocompatibility complex (MHC) genes between donor and recipient.

The primary function of the MHC molecules is to bind and "present" antigenic peptides on the surfaces of cells for recognition (binding) by the antigen-specific T-cell receptors (TCRs) of lymphocytes. Differential structural properties of MHC Class I and Class II molecules account for the respective roles in activating different population of T lympocytes. MHC Class I molecules are expressed on all nucleated somatic cells except neurons, consistent with the protective function of the cytotoxic lymphocytes (CTLs) which continuously survey cell surfaces and kill cells harboring foreign peptides. MHC Class I molecules bind peptide fragments that have been derived from proteolytically degraded proteins endogenously synthesized in the cell (small peptides are transported to the endoplasmic reticulum where they associate with nascent MHC I molecules before being routed through the Golgi apparatus and displayed on the surface for tecognition by T-cells. MHC Class II molecules are restricted to antigen presenting cells (APC) consistent with the functions of helper (Th) lymphocytes which are locally wherever these cells encounter macrophages, dendritic cells or B cells that have internalized and processed antigens produced by pathogenic mechanisms. Thus, while MHC Class I molecules

provide identity of self information to the immune system, MHC Class II molecules provide defence against foreign microbiologicals.

The two major types of MHC protein molecules- Class I and Class II- that span the membrane of almost every cell in an organismare encoded by several genes, all clustered in the same region on chromosome 6. Each gene has an unusual number of alleles (alternate forms of a gene). As a result, it is very rare for two individuals to have the same set of MHC molecules, which are collectively called a tissue type.

The MHC Class I membrane spanning molecule is composed of two proteins: the spanning protein which is approximately 350 amino acids in length, with approximately 70 amino acids at the carboxylic end which comprises of the transmembrane and cytoplasmic portion, with the remaining 270 amino acids divided into 3 globular domains, alpha-1, alpha-2 and alpha-3 (closest to membrane). The second portion of the molecule is a globular protein, beta-2 microglobulin which associates with the alpha-3 prome domain and is necessary for MHC stability.

In humans, the MHC I and II genes are located at separate but nearby loci on the 6^{th} chromosome. The Class I locus contains three smaller loci of genes for 3 Class 1 genes, A, B and C. Every human processes at least one version of the A, B and C Class I molecules. Since each individual inherits one strand of DNA from each parent most people have 2 distinct variants of each of A, B and C for a total of 6 distinct MHC I genes. The Class I gene codes only for the alpha (α) component of the protein of the Class I molecule. The beta-2 component of the Class I molecule is located elsewhere in the genome and is non-variable. The complex is also called the human leukocyte antigen (HLA) system. The prevalence of different HLA types vary widely in different populations.

MHC molecules are important components of the immune response. They allow cells that have been invaded by an infectious organism to be detected by cells of the immune system called T lymphocytes, or T-cells. The MHC molecules do this by presenting fragments of proteins (peptides) belonging to the invader on the cell's surface. The T-cell recognizes

the foreign peptide attached to the MHC molecule and binds to it, stimulating the T-cell to either destroy or cure the infected cell. In uninfected healthy cells the MHC molecule presents peptides from its own cell (self peptides), to which T-cells do not normally react. However, if the immune mechanism malfunctions and T-cells react against self peptides, an autoimmune disease arises.

MHC molecules were initially defined as antigens that stimulate an organism's immunologic response to transplanted organs and tissues. In the 1940s skin graft experiments carried out in mice by the American geneticist George D. Snell showed that graft rejection was an immune reaction mounted by the host organism against foreign tissue. This response was elicited because the host recognized the MHC molecules on cells of the graft tissue as foreign antigens and attacked them. Thus, the main challenge to a successful transplantation is to find a host and a donor with tissue types as similar as possible.

Transplantation studies have focused on the suppression of the host immune response to enable allograft survival. Unfortunately, interference in the immune response results in patients being susceptible to infections and some cancers. Furthermore, classic applications of stem cell therapeutics such as bone marrow transplantation require several months to locate a suitably matched donor based on the human leukocyte antigen (HLA) typing system. Even then due to the many thousands of different HLA types, matching is performed on a best contingency basis subject to available donors at the time. Presently siblings provide the best chance of allograft survival.

In accordance with the present invention, the level of an immunogenic cell surface molecule is selectively reduced to minimize its impact on tissue rejection and thus ameliorate GVHD. In particular, the present invention enables stem cells to be manipulated *in vitro* to alter their immunogenic potential and then used in tissue bioengineering, transplantation and regeneration.

SUMMARY OF THE INVENTION

Throughout the specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not to the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifier numbers is provided in Table 1. A sequence listing is provided after the claims.

The present invention is directed to compounds, especially nucleic acid and nucleic acid-like oligomers or complexes comprising same, which are targeted to an endogenous nucleic acid encoding an immunogenic cell surface molecule or a nucleic acid molecule required for or which facilitates expression of an immunogenic cell surface molecule-encoding material. In a preferred embodiment, the nucleic acid and nucleic acid-like oligomers or complexes comprising same are targeted to a nucleic acid encoding major histocompatability (MHC) molecule in stem cells. Pharmaceutical and other compositions comprising the compounds of the subject invention are also provided. Methods of screening for modulators of MHC gene expression in cells, tissues or animals are also contemplated. Methods of preventing organ or tissue rejection following transplantation are also set forth herein. Such methods comprise contacting a cell, organ or tissue with an effective amount of a compound or composition of the present invention, such that the expression of one or more immunogenic cell surface molecules is down-regulated or abolished, such that transplantation of the cell, tissue or organ into a recipient will not induce an immune response, and thus prevent rejection of the genetically altered cell, tissue or organ. Alternatively, the methods of the present invention contemplate the administration of a compound or composition of the present invention at the time of transplantation.

More particularly, stem cells may be manipulated *in vitro* to generate an immunologically neutral stem cell. The modified stem cells are then capable of proliferation and differentiation into transplantable tissue or administered prior to substantial proliferation and differentiation into a subject in need of tissue transplantation, repair and/or regeneration.

The present invention provides, therefore, anti-sense, sense, siRNA, RNAi, fRNA, miRNA, sisRMA, snmRNA, stRNA, snoRNA, ribozymes and DNAzymes which selectively reduce directly or indirectly, the level of cell surface immunogenic proteins, especially MHC proteins.

A summary of the sequence identifier's used throughout the subject specification is provided in Table 1.

TABLE 1

Summary of Sequence Identifiers

SEQUENCE ID NO:	DESCRIPTION
1	human β-microglobin cDNA
2	β-microglobin antisense siRNA oligonucleotide
3	β-microglobin sense siRNA oligonucleotide
4	β-microglobin antisense siRNA oligonucleotide
5	β-microglobin sense siRNA oligonucleotide
6	top strand β-microglobin oligo template
7	bottom strand β-microglobin oligo template
8	β-microglobin single hairpin oligo

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention employs compounds, preferably nucleotides and similar species for use in modulating the function or effect of nucleic acid molecules encoding an immunogenic cell surface molecule and, in a particular embodiment, an MHC molecule or of nucleic acid molecules required for or which facilitate expression of an MHC molecule (e.g. a promoter region). Preferably, the cell is a stem cell. Preferably, the compounds of the present invention down-regulate expression of MHC genetic material. This is accomplished by providing oligonucleotides which specifically hybridize or otherwise interact with one or more nucleic acid molecules encoding a MHC or a nucleic acid molecule required for or which facilitates an MHC gene expression. As used herein, the terms "nucleic acid" and "nucleic acid molecule encoding an MHC" have been used for convenience to encompass DNA encoding an MHC, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. The hybridization or interaction of a compound of the present invention with a target nucleic acid may encompass antisense or sense targeting. The latter is also referred to herein as sense suppression. Consequently, the present invention provides for antisense or sense inhibition. Such antisense or sense inhibition is typically based upon hydrogen bonding-based hybridization of oligonucleotide strands or segments such that at least one strand or segment is cleaved, degraded, or otherwise rendered inoperable. Alternatively, post-transcriptional gene silencing (PTGS) may be achieved using sense suppression (formally known as co-suppression). In yet another alternative, complexes comprising nucleic acid molecules and proteins (e.g. ribonucleases) such as anti-sense, sense, siRNA, RNAi, fRNA, miRNA, sisRMA, snmRNA, stRNA, snoRNA, ribozymes and DNAzymes may be employed.

The present invention enables manipulation of stem cells *in vivo* and *in vitro*. Manipulation of stem cells in culture (i.e. *in vitro*) to generate cells with an altered, and in particular, reduced immunological potential. In one embodiment, the stem cells are rendered immunogenically neutral by down regulation of expression of MHC-encoding nucleic acid sequences. The immunogenically neutral stem cells are administered to a

subject or allowed to proliferate and/or differentiate *in vitro* prior to transplantation administration to a subject.

Accordingly, one aspect of the present invention contemplates a method for generating an immunogenically altered stem cell, said method comprising introducing to said stem cell a compound which hybridizes or otherwise interacts with a nucleic acid molecule encoding a major histocompatability (MHC) protein or a nucleic acid molecule encoding a protein required for expression of MHC-encoding material thereby reducing the level of immunogenic molecules on the surface of the stem cell.

The target nucleic acid molecules include the MHC coding sequences, a promoter region, a 3' regulatory region or a nucleotide sequence, the expression of which, facilitates or inhibits MHC gene expression (e.g. a regulatory gene, activator gene or reporter gene).

As used herein, reference to an immunogenic cell surface molecule includes any molecule which when on the cell surface is capable of stimulating an immune response in a recipient of a transplant. In a preferred aspect, reference to "MHC", includes reference to the isoforms HLA-A, HLA-B, HLA-C, HLA-DQ, HLA-DP, HLA-DM, HLA-DR, or subtypes thereof.

Reference to a "stem cell" includes progenitor cells, germ cells and early differentiated cells. Stem cells contemplated by the present invention include but are not limited to embryonic stem cells, somatic stem cells, germ stem cells, human embryonic stem cells, human epidermal stem cells, adult neural stem cells, human neurons, human astrocytes, human keratinocyte stem cells, human keratinocyte transient amplifying cells, human melanocyte stem cells, human melanocytes, human foreskin fibroblasts, human duct cells, human pancreatic islets, human pancreatic β-cells, human adult renal stem cells, human embryonic renal epithelial stem cells, human kidney epithelial cells, human hepatic oval cells, human hepatocytes, human bile duct epithelial cells, human embryonic endodermal stem cells, human adult hepatocyte stem cells, human mammary epithelial stem cells, Bone marrow-derived stem cells, human lung fibroblasts, human bronchial epithelial cells,

human alveolar type II pneumocytes, human skeletal muscle stem cells, human cardiomyocytes, bone marrow mesenchymal stem cells, simple squamous epithelial cells, descending aortic endothelial cells, aortic arch endothelial cells, aortic smooth muscle cells, limbal stem cells, corneal epithelial cells, CD34+ hematopoietic stem cells, mesenchymal stem cells, osteoblasts, peripheral blood mononuclear progenitor cells, osteoclasts, stromal cells, human splenic precursor stem cells, human splenocytes, human CD4+ T-cells, human CD8+ T-cells, human NK cells, human monocytes, human macrophages, human dendritic cells, human b-cells, goblet cells, pseudostriated ciliated columnar cells, pseudostratified ciliated epithelium, stratified epithelial cells, ciliated columnar cells, goblet cells, basal cells, cricopharyngeus muscle cells, female primary follicles and male spermatogonium.

Although the present invention is particularly applicable to stem cells, the present invention further extends to immunogentically neutral adult cells or fully or partially differented cells.

The functions of the nucleic acid molecule to be down-regulated include replication, transcription and/or translation. Where the nucleic acid molecule is RNA, the compounds may target translocation of the RNA to a site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more RNA species, and catalytic activity or complex formation involving the RNA which may be engaged in or facilitated by the R NA. One preferred result of such interference with target nucleic acid function is reduction in the level of expression of MHC. Inhibition is the preferred form of modulation of expression and mRNA is the preferred target nucleic acid. In a particularly preferred embodiment, the expression of the immunogenic gene may be regulated to control the level of the molecule on the cell surface, or the timing of when the molecule is on the cell surface.

In the context of the present invention, "hybridization" means the pairing of complementary strands of nucleic acids. In the present invention, the preferred mechanism

of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleotides) of the strands of oligomeric compounds. For example, adenine and thymine are complementary nucleotides which pair through the formation of hydrogen bonds. Hybridization can occur under varying circumstances.

An antisense or sense compound is specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense or sense compound to non-target nucleic acid sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and under conditions in which assays are performed in the case of *in vitro* assays.

In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which a compound of the invention will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances and in the context of this invention, "stringent conditions" under which oligomeric compounds hybridize to a target sequence are determined by the nature and composition of the oligomeric compounds and the assays in which they are being investigated.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides of an oligomeric compound. For example, if a nucleotide at a certain position of an oligonucleotide (an oligomeric compound), is capable of hydrogen bonding with a nucleotide at a certain position of a target nucleic acid, said target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position. The oligonucleotide and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleotides which can hydrogen bond with each other.

Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleotides such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

It is understood in the art that the sequence of an antisense or sense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). It is preferred that the antisense or sense compounds of the present invention comprise at least 70% sequence complementarity to a target region within the target nucleic acid, such as 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% complementarity to the nucleic acid sequence to which they are targeted. For example, an antisense or sense compound in which 18 out of 20 nucleotides of the antisense or sense compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleotides may be clustered or interspersed with complementary nucleotides and need not be contiguous to each other or to complementary nucleotides. As such, an antisense or sense compound which is 18 nucleotides in length having 4 (four) noncomplementary nucleotides which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an antisense or sense compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., *J. Mol. Biol. 215:* 403-410, 1990; Zhang and Madden, *Genome Res. 7:* 649-656, 1997).

According to the present invention, compounds include antisense or sense nucleic acids, antisense or sense oligomeric compounds, antisense or sense oligonucleotides, ribozymes, sense oligonucleotides, full-length sense molecules, external guide sequence (EGS)

oligonucleotides, alternate splicers, primers, probes, and other oligomeric compounds which hybridize to at least a portion of the target nucleic acid. As such, these compounds may be introduced in the form of single-stranded, double-stranded, circular or hairpin oligomeric compounds and may contain structural elements such as internal or terminal bulges or loops. Once introduced to a system, the compounds of the invention may elicit the action of one or more enzymes or structural proteins to effect modification of the target nucleic acid.

As used herein, an "antisense" or "sense" molecule includes an RNA molecule which, by binding to a complementary sequence in either RNA or DNA, inhibits the function and/or completion of synthesis of the latter molecule. Artificial antisense or sense RNAs have been used to inhibit translation of specific mRNA molecules both in living cells (eukaryotic and bacterial) and in cell-free systems.

One such anti-sense molecule is RNAi. The precise mechanisms behind RNAi are not yet fully understood, but what was established early on was that the gene-silencing effect occurs after the creation of RNA from DNA (transcription), because dsRNA created to target the introns (which are spliced out of the mRNA) did not activate RNAi — hence the umbrella term of post-transcriptional gene silencing (PTGS) used to describe RNAi, co-suppression and quelling.

The central process of RNAi is the chopping of dsRNA into smaller pieces of a defined length by the appropriately named enzyme Dicer. Dicer chops dsRNA into two classes of smaller RNAs — microRNAs (miRNAs) and small interfering RNAs (siRNAs) — that are around 21-23 nucleotides in length. Although miRNAs also stop protein production (and, as described in another article, the boundaries between siRNAs and miRNAs is becoming blurred), siRNAs are thought to be the main protagonists in RNAi. Dicer delivers these siRNAs to a group of proteins called the RNA-induced silencing complex (RISC), which uses the antisense strand of the siRNA to bind to and degrade the corresponding mRNA, resulting in gene silencing. RNAi is astonishingly efficient, because the RISC is an enzyme and catalyses multiple rounds of RNAi, perhaps hundreds or thousands *in vivo*. It

also shows an amazing persistence in some organisms, such as worms, which suggests there could also be an amplification response.

An enzyme, RNAse H, is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense or sense compounds which are "DNA-like" elicit RNAse H. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. Similar roles have been postulated for other ribonucleases such as those in the RNase III and ribonuclease L family of enzymes. Therefore, the compositions of the present invention include anti-sense, sense, siRNA, RNAi, fRNA, miRNA, sisRMA, snmRNA, stRNA, snoRNA, ribozymes and DNAzymes.

While the preferred form of antisense or sense compounds are single-stranded oligonucleotides, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, miRNAs, short-interfering RNA molecules (siRNA) and full-length dsRNAs.

Small interfering RNAs (siRNAs) have an integral role in the phenomenon of RNA interference (RNAi). In RNAi, dsRNAs introduced into certain organisms or cells are degraded into approximately 22 nucleotide fragments. These 22 nucleotide siRNA molecules then bind to the complementary portion of their target mRNA and tag it for degradation.

A second class of regulatory small RNAs contemplated by the present invention are referred to as small temporal RNAs. Approximately 22 nucleotide *lin-4* and *let-7* RNAs are example of this group. These RNA molecules have a role in temporal regulation of *C. elegans* development. These are initially processed from an approximate 70 nucleotide ssRNA transcript folded into a stem loop structure. After processing, these stRNAs are thought to prevent translation of their target mRNAs by binding to the targets

complementary 3' untranslated regions (UTRs). Dicer, RNAase enzyme processes both the types of RNAs (Grishok *et al. Science* 287(5462):2494-2497, 2000).

In the context of this invention, the term "oligomeric compound" refers to a polymer or oligomer comprising a plurality of monomeric units. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics, chimeras, analogs and homologs thereof. This term includes oligonucleotides composed of naturally occurring nucleotides, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for a target nucleic acid and increased stability in the presence of nucleases.

While oligonucleotides are a preferred form of the compounds of this invention, the present invention comprehends other families of compounds as well, including but not limited to oligonucleotide analogs and mimetics such as those herein described.

The compounds in accordance with this invention preferably comprise from about 10 to about 2000 nucleotides (i.e. from about 10 to about 2000 linked nucleosides). One of ordinary skill in the art will appreciate that the invention embodies compounds of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680,690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1080, 1090, 1100, 1110, 1120, 1130, 1140, 1150, 1160, 1170, 1180, 1190, 1200, 1210, 1220, 1230, 1240, 1250, 1260, 1270, 1280, 1290,

1300, 1310, 1320, 1330, 1340, 1350, 1360, 1370, 1380, 1390, 1400, 1410, 1420, 1430, 1440, 1450, 1460, 1470, 1480, 1490, 1500, 1510, 1520, 1530, 1540, 1550, 1560, 1570, 1580, 1590, 1600, 1610, 1620, 1630, 1640, 1650, 1660, 1670, 1680,1690, 1700, 1710, 1720, 1730, 1740, 1750, 1760, 1770, 1780, 1790, 1800, 1810, 1820, 1830, 1840, 1850, 1860, 1870, 1880, 1890, 1900, 1910, 1920, 1930, 1940, 1950, 1960, 1970, 1980, 1990 or 2000 nucleotides in length.

Antisense or sense compounds 10-2000 nucleotides in length comprising a stretch of at least ten (10) such as 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 consecutive nucleotides selected from within the illustrative antisense or sense compounds are considered to be suitable antisense or sense compounds as well.

Exemplary preferred antisense or sense compounds include oligonucleotide sequences that comprise at least the 10 consecutive nucleotides from the 5'-terminus of one of the illustrative preferred antisense or sense compounds (the remaining nucleotides being a consecutive stretch of the same oligonucleotide beginning immediately upstream of the 5'-terminus of the antisense or sense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 10 to about 2000 nucleotides). Similarly preferred antisense or sense compounds are represented by oligonucleotide sequences that comprise at least the 10 consecutive nucleotides from the 3'-terminus of one of the illustrative preferred antisense or sense compounds (the remaining nucleotides being a consecutive stretch of the same oligonucleotide beginning immediately downstream of the 3'-terminus of the antisense or sense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 10 to about 2000 nucleotides). One having skill in the art armed with the preferred antisense or sense compounds illustrated herein will be able, without undue experimentation, to identify further preferred antisense or sense compounds.

Candidate compounds are also referred to herein as "lead" compounds. In the present invention, the target nucleic acid encodes an immunogenic cell surface molecule or is a gene required for immunogenic cell surface molecule gene expression. As indicated

above, an immunogenic cell surface molecule can be an MHC molecule which include MHC I or MHC II molecules. In a preferred aspect, the molecules are HLA-A, HLA-B, HLA-C, HLA-DQ, HLA-DR, HLA-DP and/or HLA-DM.

The targeting process usually also includes determination of at least one target region, segment, or site within the target nucleic acid for the antisense or sense interaction to occur such that the desired effect, e.g., to reduce expression, will result. Within the context of the present invention, the term "region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic. Within regions of target nucleic acids are segments. "Segments" are defined as smaller or sub-portions of regions within a target nucleic acid. "Sites," as used in the present invention, are defined as positions within a target nucleic acid.

Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes). It is also known in the art that eukaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA transcribed from a gene encoding an immunogenic cell surface molecule, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively).

The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. Consequently, the "start codon region" (or "translation initiation codon region") and the "stop codon region" (or "translation termination codon region") are all regions which may be targeted effectively with the antisense or sense compounds of the present invention.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Within the context of the present invention, a preferred region is the intragenic region encompassing the translation initiation or termination codon of the open reading frame (ORF) of a gene.

Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene), and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene). The 5' cap site of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA *via* a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap site. It is also preferred to target the 5' cap region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are

spliced together to form a continuous mRNA sequence. Targeting splice sites, i.e., intron-exon junctions or exon-intron junctions, may also be particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred target sites. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". It is also known that introns can be effectively targeted using antisense or sense compounds targeted to, for example, DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and exonic sequence.

Upon excision of one or more exon or intron regions, or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby

producing transcripts that terminate at unique polyA sites. Within the context of the invention, the types of variants described herein are also preferred target nucleic acids.

The locations on the target nucleic acid to which the preferred antisense or sense compounds hybridize are hereinbelow referred to as "preferred target segments." As used herein the term "preferred target segment" is defined as at least a 10-nucleotide portion of a target region to which an active antisense or sense compound is targeted. While not wishing to be bound by theory, it is presently believed that these target segments represent portions of the target nucleic acid which are accessible for hybridization.

While the specific sequences of certain preferred target segments are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional preferred target segments may be identified by one having ordinary skill.

Target segments 10-2000 nucleotides in length comprising a stretch of at least ten (10) consecutive nucleotides selected from within the illustrative preferred target segments are considered to be suitable for targeting as well.

Target segments can include DNA or RNA sequences that comprise at least the 8 consecutive nucleotides from the 5'-terminus of one of the illustrative preferred target segments (the remaining nucleotides being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target segment and continuing until the DNA or RNA contains about 10 to about 2000 nucleotides). Similarly preferred target segments are represented by DNA or RNA sequences that comprise at least the 10 consecutive nucleotides from the 3'-terminus of one of the illustrative preferred target segments (the remaining nucleotides being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target segment and continuing until the DNA or RNA contains about 10 to about 2000 nucleotides). One having skill in the art armed with the preferred target segments illustrated herein will be able, without undue experimentation, to identify further preferred target segments.

Once one or more target regions, segments or sites have been identified, antisense or sense compounds are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect, i.e. to reduce an immungenic cell surface molecule gene expression or levels of an immunogenic cell surface molecule.

In a further embodiment, the "preferred target segments" identified herein may be employed in a screen for additional compounds that modulate the expression of the immunogenic cell surface molecule gene. "Modulators" are those compounds that decrease or increase the expression of a nucleic acid molecule encoding an immunogenic cell surface molecule and which comprise at least a 10-nucleotide portion which is complementary to a preferred target segment. The screening method comprises the steps of contacting a preferred target segment of a nucleic acid molecule encoding an immunogenic cell surface molecule with one or more candidate modulators, and selecting for one or more candidate modulators which decrease or increase the expression of a nucleic acid molecule encoding an immunogenic cell surface molecule. Once it is shown that the candidate modulator or modulators are capable of modulating (e.g. either decreasing or increasing) the expression of a nucleic acid molecule encoding an immunogenic cell surface molecule, the modulator may then be employed in further investigative studies of the function of an immunogenic cell surface molecule, or for use as a research, diagnostic, or therapeutic agent in accordance with the present invention.

The preferred target segments of the present invention may be also be combined with their respective complementary antisense or sense compounds of the present invention to form stabilized double-stranded (duplexed) oligonucleotides.

Such double stranded oligonucleotide moieties have been shown in the art to modulate target expression and regulate translation as well as RNA processsing *via* an antisense or sense mechanism. Moreover, the double-stranded moieties may be subject to chemical modifications (Fire *et al.*, *Nature 391:* 806-811, 1998; Timmons and Fire, *Nature 395:*

854, 1998; Timmons *et al., Gene 263:* 103-112, 2001; Tabara *et al., Science 282:* 430-431, 1998; Montgomery *et al.*, 1998, *supra*; Tuschl *et al., Genes Dev. 13:* 3191-3197, 1999; Elbashir *et al., Nature, 411:* 494-498, 2001; Elbashir *et al., Genes Dev. 15:* 188-200, 2001). For example, such double-stranded moieties have been shown to inhibit the target by the classical hybridization of antisense or sense strand of the duplex to the target, thereby triggering enzymatic degradation of the target (Tijsterman *et al.*, 2002, *supra*).

In a preferred embodiment, the present invention enables stem cells to be prepared *in vitro* for use in bioengineering, tissue generation and as substrate tissue or cells for transplantation, tissue augmentation or tissue repair, the stem cells prepared by contacting the cells with synthetic RNAi or a genetic construct encoding an RNAi or a component of RNAi specific for a nucleic acid sequence within the stem cell encoding an MHC molecule in an amount sufficient to down-regulate expression of the nucleic acid sequence and to reduce the level of immunogenic molecules on the surface of the stem cells.

Such stem cells prepared in this way are immunogenically neutral or have reduced immunogeneicity.

They may be used directly to introduce same into a subject or may be allowed to differentiate or proliferate into a cell mass or tissue mass for subsequent transplantation or grafting or administration to a subject.

The compounds of the present invention can also be applied in the areas of drug discovery and target validation. The present invention comprehends the use of the compounds and preferred target segments identified herein in drug discovery efforts to elucidate the effect of down-regulating or ablating the on the cell surface immunogenic molecules which have been identified as being associated in transplantation rejections. These methods include detecting or modulating cell surface immunogenic cell surface molecules comprising contacting a sample, tissue, cell, or organism with the compounds of the present invention, measuring the nucleic acid or protein level of an immunogenic cell surface molecule and/or a related phenotypic or chemical endpoint at some time after treatment, and

optionally comparing the measured value to a non-treated sample or sample treated with a further compound of the invention. Having determined the nucleotide sequence of the immunogenic cell surface protein a synthetic siRNA can be engineered and introduced into the cell to prevent its expression. In a preferred aspect, the compounds of the present invention down-regulate or ablate cell surface MHC molecules.

The present invention contemplates the use of therapeutic agents to treat subjects undergoing a transplant, especially from non-HLA matched donors. Subjects treated using the compositions and compounds of the present invention include any animal who may benefit from such treatment. These include, without limitation, humans, marmosets, orangutans and gorillas, livestock animals (e.g. cows, sheep, pigs, horses, donkeys), laboratory test animals (e.g. mice, rats, guinea pigs, hamsters, rabbits), companion animals (e.g. cats, dogs) and captured wild animals (e.g. rodents, foxes, deer, kangaroos. A particularly preferred host is a human, primate or livestock animal.

The compounds of the present invention can be utilized for therapeutics, prophylaxis and as research reagents and kits. Furthermore, antisense or sense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes or to distinguish between functions of various members of a biological pathway.

For use in kits and diagnostics, the compounds of the present invention, either alone or in combination with other compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

As one non-limiting example, expression patterns within cells or tissues treated with one or more antisense or sense compounds are compared to control cells or tissues not treated with antisense or sense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signalling pathway, cellular localization, expression level, size, structure or function of the genes

examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett. 480:* 17-24, 2000; Celis *et al.*, *FEBS Lett. 480:* 2-16, 2000), SAGE (serial analysis of gene expression)(Madden *et al.*, *Drug Discov. Today 5:* 415-425, 2000), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.303:* 258-272, 1999), TOGA (total gene expression analysis) (Sutcliffe *et al.*, *Proc. Natl. Acad. Sci. USA 97:* 1976-1981, 2000), protein arrays and proteomics (Celis *et al.* 2000, *supra*; Jungblut *et al.*, *Electrophoresis 20:* 2100-2110, 1999), expressed sequence tag (EST) sequencing (Celis *et al.*, 2000, *supra*; Larsson *et al.*, *J. Biotechnol.80:* 143-157, 2000), subtractive RNA fingerprinting (SuRF) (Fuchs *et al.*, *Anal. Biochem. 286:* 91-98, 2000; Larson *et al.*, *Cytometry 41:* 203-208, 2000), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol. 3:* 316-321, 2000), comparative genomic hybridization (Carulli *et al.*, *J. Cell Biochem. Suppl.31:* 286-296, 1998), FISH (fluorescent *in situ* hybridization) techniques (Going and Gusterson, *Eur. J. Cancer, 35:* 1895-1904, 1999) and mass spectrometry methods (To, *Comb. Chem. High Throughput Screen, 3:* 235-241, 2000).

The compounds of the invention are useful for research because these compounds hybridize to nucleic acids encoding an immunogenic cell surface molecule. For example, oligonucleotides that are shown to hybridize with such efficiency and under such conditions as disclosed herein as to be effective immunogenic cell surface molecule inhibitors or immunogenic cell surface molecule gene expression inhibitors will also be effective primers or probes under conditions favoring gene amplification or detection, respectively. These primers and probes are useful in methods requiring the specific detection of nucleic acid molecules encoding an immunogenic cell surface molecule and in the amplification of said nucleic acid molecules for detection or for use in further studies of immunogenic cell surface molecule or its gene. Hybridization of the antisense or sense oligonucleotides, particularly the primers and probes, of the invention with a nucleic acid encoding an immunogenic cell surface molecule can be detected by means known in the

art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of immunogenic cell surface molecule in a sample may also be prepared.

The specificity and sensitivity of antisense or sense is also harnessed by those of skill in the art for therapeutic uses. Antisense or sense compounds have been employed as therapeutic moieties in the treatment of transplantation rejection in animals, including humans. Antisense or sense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that antisense or sense compounds can be useful therapeutic modalities that can be configured to be useful in treatment regimes for the treatment of cells, tissues and animals, especially humans.

For therapeutics, an animal, preferably a human, requires a transplantation of cells, organs and/or tissue from a donor which is not identically matched. As such, the compounds and compositions of the present invention can be use to down-regulate or ablate immunogenic molecules which do not correlate between a recipient and a donor. In one embodiment, the expression of the immunogenic cell surface molecule gene is treated by administering antisense or sense compounds in accordance with this invention either *in vitro* or *in vivo*. For example, in one non-limiting embodiment, the methods comprise the step of administering to the animal in need of treatment, a therapeutically effective amount of an immunogenic cell surface molecule gene expression inhibitor at the time of transplantation. Preferably, the cell is a stem cell. In a related aspect, the cell, organ or tissue which is to be transplanted is pre-incubated with a composition or compound of the present invention prior to the cell, organ or tissue being transplanted. The immunogenic cell surface molecule gene expression inhibitors of the present invention effectively inhibit the activity of the immunogenic cell surface molecule protein or inhibit the expression of the immunogenic cell surface molecule gene. In one embodiment, the activity or expression of the immunogenic cell surface molecule or its gene in an animal is inhibited by about 10%. Preferably, the activity or expression of the immunogenic cell surface

molecule or its gene in an animal is inhibited by about 30%. More preferably, the activity or expression of the immunogenic cell surface molecule or its gene in an animal is inhibited by 50% or more. In a most preferred embodiment, the activity or expression of the immunogenic cell surface molecule or its gene in an animal is inhibited by 90% or more. Even more preferably, the activity or expression of the immunogenic cell surface molecule or its gene in an animal is ablated.

For example, the reduction of the expression of the immunogenic cell surface molecule gene may be measured in serum, adipose tissue, skin cells, liver or any other body fluid, tissue or organ of the animal. Preferably, the cells contained within said fluids, tissues or organs being analyzed contain a nucleic acid molecule encoding an immunogenic cell surface molecule protein.

In a preferred aspect, however, stem cells are collected from one source, subjected to conditions permitting posttranscriptional gene silencing of selected nucleic acid sequences encoding MHC molecules and then the stem cells administered to a subject in need of tissue replacement, augmentation, repair or transplantation or first allowed to proliferate or differentiate to form a cell population or partially differential cell mass which is then administered or transplanted to a subject.

The compositions and compounds of the present invention can be used to prevent rejection of cell, tissue or organ transplants. The present invention contemplates treatment with the compounds and compositions of the present invention for use in the prevention of any heterologous transplantation procedure. Including, transplantation to replace such diseased or defective tissue as corneas and hearts, paired organs such as kidneys, or large or regenerating organs or tissues such as skin, bowel, lung, liver, or blood components, skin autografts, bone, and cartilage and other connective tissue, bone marrow, heart, hip sockets, tissue and cells of the central nervous system (CNS) including tissues and cells of the brain and the peripheral nervous system (PNS).

The compositions and compounds of the present invention can also be used in the treatment of autoimmune diseases. The immune system protects the body from potentially harmful substances (antigens) such as microorganisms, toxins, cancer cells, and foreign blood or tissues from another person or species. Antigens are destroyed by the immune response, which includes production of antibodies (molecules that attach to the antigen and make it more susceptible to destruction) and sensitized lymphocytes (specialized white blood cells that recognize and destroy particular antigens).

Immune system disorders occur when the immune response is inappropriate, excessive, or lacking. Autoimmune disorders develop when the immune system destroys normal body tissues. This is caused by a hypersensitivity reaction similar to allergies, where the immune system reacts to a substance that it normally would ignore. In allergies, the immune system reacts to an external substance that would normally be harmless. With autoimmune disorders, the immune system reacts to normal "self" body tissues.

Normally, the immune system is capable of differentiating "self" from "non-self" tissue. Some immune system cells (lymphocytes) become sensitized against "self" tissue cells, but these faulty lymphocytes are usually controlled (suppressed) by other lymphocytes. Autoimmune disorders occur when the normal control process is disrupted. They may also occur if normal body tissue is altered so that it is no longer recognized as "self." The mechanisms that cause disrupted control or tissue changes are not known. One theory holds that various microorganisms and drugs may trigger some of these changes, particularly in people with a genetic predisposition to an autoimmune disorder.

Autoimmune disorders result in destruction of one or more types of body tissues, abnormal growth of an organ, or changes in organ function. The disorder may affect only one organ or tissue type or may affect multiple organs and tissues. Organs and tissues commonly affected by autoimmune disorders include blood components such as red blood cells, blood vessels, connective tissues, endocrine glands such as the thyroid or pancreas, muscles, joints, and skin.

Types of autoimmune diseases which can be treated using the compositions and compounds of the present invention include Active Chronic Hepatitis, Addison's Disease, Anti-phospholipid Syndrome, Atopic Allergy, Autoimmune Atrophic Gastritis, Achlorhydra Autoimmune, Celiac Disease, Crohns Disease, Cushings Syndrome, Dermatomyositis, Type I Diabetes, Discoid Lupus, Erythematosis, Goodpasture's Syndrome, Grave's Disease, Hashimoto's Thyroiditis, Idiopathic Adrenal Atrophy, Idiopathic Thrombocytopenia, Insulin-dependent Diabetes, Lambert-Eaton Syndrome, Lupoid Hepatitis, Lymphopenia, Mixed Connective Tissue Disease, Multiple Sclerosis, Pemphigoid, Pemphigus Vulgaris, Pernicious Anema, Phacogenic Uveitis, Polyarteritis Nodosa, Polyglandular Auto. Syndromes, Primary Biliary Cirrhosis, Primary Sclerosing Cholangitis, Psoriasis, Raynauds, Reiter's Syndrome, Relapsing Polychondritis, Rheumatoid Arthritis, Schmidt's Syndrome, Scleroderma – CREST, Sjogren's Syndrome, Sympathetic Ophthalmia, Systemic Lupus Erythematosis, Takayasu's Arteritis, Temporal Arteritis, Thyrotoxicosis, Type B Insulin Resistance, Ulcerative Colitis and Wegener's Granulomatosis.

The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of a compound to a suitable pharmaceutically acceptable diluent, carrier or other delivery mechanism including means of introduction of RNA or DNA by means of vector systems or other genetic engineering approaches known in the art that will allow the insertion of exogenous nucleic acid into a cell. Use of the compounds and methods of the invention may also be useful prophylactically. The compounds or compositions of the present invention may be administered in conjunction with immunosuppresive therapies. These therapies, in conjunction with the compositions and compounds of the present invention, can be administered in lower doses and for longer periods of time, thereby decreasing or preventing the usual side effects associated with immunosuppressive therapies.

Immunosuppressive drugs contemplated by the methods of the present invention include any of a variety of substances used to prevent production of antibodies. They are commonly used to prevent rejection by a recipient's body of an organ transplanted from a

donor. A transplant is rejected when the recipient's immune system acts against it; current methods aim at suppressing the activity of the lymphocytes, the cells that form antibodies. The steroids, such as cortisone, which suppress the antibody-forming lymphocyte cells, have been used to prolong human organ transplants. Steroids may also prevent antigens from entering cells and thereby prevent local allergic inflammation reactions. In another immunosuppressive method, human lymphocytes are injected into horses, stimulating the animals to produce antilymphocyte serum. The serum, administered to humans with transplanted organs, in some way inactivates lymphocyte cells. The procedure will not work effectively for more than a few injections of serum. Another group of immunosuppressive drugs act by interfering with the synthesis of nucleic acids and are especially effective against proliferating cells such as stimulated lymphocytes. Some of these are analogs of purines and pyrimidines, substances that are nucleic acid subunits; the purine analog azothioprine has been used to suppress rejection of transplanted human kidneys. Most substances that inhibit nucleic acid synthesis, such as nitrogen mustard, cyclophosphamide (Cytoxan), chloramphenicol, actinomycin, and colchicine, are not widely used clinically because they are too toxic. Many of the drugs that suppress the function of the immunological system are also used clinically to check growth of cancerous tissue, which is composed of rapidly dividing cells. The drugs currently used to suppress antibody formation also leave an individual susceptible to infection. Immunosuppressive drugs contemplated for use with the compounds and compositions of the present invention include pharmacological agents such as prednisolone, methyl prednisolone, Cyclophosphamide, Azathioprine, Mycophenolate mofetil (MMF), Methotrexate, Cotrimoxazole, Cyclosporin, tacrolimus, everolimus, FTY720 and antibody based agents such as OKT3, Daclizumab, Basiliximab, Rituximab, Alemtuzumab and LEA294.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be

linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleotide complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense or sense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleotide is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent Numbers: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patent Numbers: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage (i.e. the backbone), of the nucleotide units are replaced with novel groups. The nucleotide units are maintained for hybridization with an appropriate target nucleic acid. One such compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA

compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleotides are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent Numbers: 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science 254:* 1497-1500, 1991.

Preferred embodiments of the present invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. Patent Number: 5,489,677, and the amide backbones of the above referenced U.S. Patent Number: 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent Number: 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents

having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta, 78:* 486-504, 1995) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_3)_2$, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-CH=CH_2), 2'-O-allyl (2'-O-CH_2-CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patent Numbers: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920.

A further preferred modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Oligonucleotides may also include nucleotide (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleotides include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine

(T), cytosine (C) and uracil (U). Modified nucleotides include other synthetic and natural nucleotides such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleotides include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleotides may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleotides include those disclosed in United States Patent Number: 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie*, International Edition, *30:* 613, 1991, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleotides are particularly useful for increasing the binding affinity of the compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleotides as well as other modified nucleotides include, but are not limited to, the above noted U.S. Patent Number: 3,687,808, as well as U.S. Patent Numbers: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992, and U.S. Patent Number: 6,287,860, the entire disclosure of which are incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-

oxycholesterol moiety. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patent Numbers: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide.

The present invention also includes antisense or sense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras", in the context of this invention, are antisense or sense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, increased stability and/or increased binding affinity for the target nucleic acid. An

additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNAse H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. The cleavage of RNA:RNA hybrids can, in like fashion, be accomplished through the actions of endoribonucleases, such as RNAseL which cleaves both cellular and viral RNA. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense or sense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patent Numbers: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S. Patent Numbers: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense or sense compounds of the invention encompass any pharmaceutically

acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993 or in WO 94/26764 and U.S. Patent Number: 5,770,713 to Imbach *et al.*

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. For oligonucleotides, preferred examples of pharmaceutically acceptable salts and their uses are further described in U.S. Patent Number: 6,287,860.

The present invention also includes pharmaceutical compositions and formulations which include the antisense or sense compounds of the invention for use in *in vitro* culture or *in vivo*. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration.

Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferably, however, they are formulated to be given to cell cultures.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations of the present invention may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. Emulsions may contain additional

components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as an embodiment of the present invention. Emulsions and their uses are well known in the art and are further described in U.S. Patent Number: 6,287,860, which is incorporated herein in its entirety.

Formulations of the present invention include liposomal formulations. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are positively charged liposomes which are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. Liposomes and their uses are further described in U.S. Patent Number: 6,287,860, which is incorporated herein in its entirety.

The pharmaceutical formulations and compositions of the present invention may also include surfactants. The use of surfactants in drug products, formulations and in emulsions is well known in the art. Surfactants and their uses are further described in U.S. Patent Number: 6,287,860.

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Penetration enhancers and their uses are further described in U.S. Patent Number: 6,287,860.

One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

Preferred formulations for topical administration include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA).

For topical or other administration, oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters, pharmaceutically acceptable salts thereof, and their uses are further described in U.S. Patent Number: 6,287,860.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in

conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts and fatty acids and their uses are further described in U.S. Patent Number: 6,287,860, which is incorporated herein in its entirety. Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents and their uses are further described in U.S. Patent Number: 6,287,860.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Certain embodiments of the invention provide pharmaceutical compositions containing one or more oligomeric compounds and one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to cancer chemotherapeutic drugs such as daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol

(DES). When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. Combinations of antisense or sense compounds and other non-antisense drugs are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense or sense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense or sense compounds targeted to a second nucleic acid target. Alternatively, compositions of the invention may contain two or more antisense or sense compounds targeted to different regions of the same nucleic acid target. Numerous examples of antisense or sense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured

residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. Examples of effective amounts include 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100g/kg body weight.

The present invention is further described by the following non-limiting examples.

EXAMPLE 1

Strategy of MHC I subunit suppression – generic and custom-tailored

The target T-cell surface protein for ameliorating GVHD using RNAi is the MHC Class I molecule, specifically, either the α subunit or the β-2-microglobulin subunit, which is highly conserved. A sequence corresponding to the β-2-microglobulin is described in SEQ ID NO:1. The strategy as to the prevention of the expression of MHC I molecules on the surface of a cell/tissue which is to be grafted can be accomplished by using RNAi specific for the MHC I α and/or β- subunits. One option is to suppress the expression of β-2-microglobulin (whose RNA sequence is presumably conserved across all individuals) and thereby prevent MHC I expression. Alternatively, the unique individual MHC I α subunit RNA sequence which is polymorphic and thus "tailor"-make the sense or antisense RNA can be utilised in the RNAi application.

EXAMPLE 2

RNAi delivery methods

RNAi is introduced into the cells using a variety of constructs that are able to maintain controlled expression of the specific RNAi. Three mechanisms of RNAi are contemplated by the present invention:

1. Systemic RNAi: silencing effects are observed throughout the entire organism;
2. RNAi amplification: a few molecules of dsRNA per cell silence a large excess of target RNA molecules; and
3. Heritable RNAi: progeny is also silenced.

EXAMPLE 3

Manipulation of stem cells in vitro

Stem cells are collected from blood, bone marrow or from the oral cavity and cultured *in vitro*. The cells are contacted with a synthetic RNAi construct specific for MHC Class I subunit. The RNAi construct may also comprise a second construct is provided with a reporter molecule. This serves to determine the rate of transformation or transfection of the constructs. Cells are permitted to maintain themselves or undergo proliferation and/or differentiation and ELISA used to measure the surface levels of the α subunit. Reduced levels indicate PTGS of the MHC Class I α subunit genes.

EXAMPLE 4

Development of Null stems cells in vitro

Stem cells are collected from blood, bone marrow or from the oral cavity and cultured *in vitro*. The cells are contacted with a small interfering RNA (siRNA) sequence specific for β-2-microglobulin.

Several examples of β-2-microglobulin siRNA sequences which are commercially available from Ambion include product ID#14060, ID#14154 and ID#14243.

In addition, the following specific sequences can be used to reduce or prevent the expression of β-2-microglobulin. For example, using nucleotides 1-22 of SEQ ID NO:1 as the target sequence, siRNA can be generated using the Silencer ™ siRNA Construction Kit as per the manufacturer's instructions. Briefly, β-2-microglobulin sense primer 5'-AACGCGACGCCTCCACTTATACCTGTCTC -3' (SEQ ID NO:3) and antisense primer 5'- AATATAAGTGGAGGCGTCGCGCCTGTCTC -3' (SEQ ID NO:2) are used to generate a double stranded siRNA, having a siRNA sense strand 5'-UAUAAGUGGAGGCGUCGCGtt -3' (SEQ ID NO:4) and anti-sense strand 3'-ttAUAUUCACCUCCGCAGCGC -5' (SEQ ID NO:5). This RNA is then mixed with a stem cell population in order to reduce or prevent the expression of MHC Class I on the

stem cell surface. This can be performed using the Silencer™ siRNA Transfection II Kit as per the manufacturer's instructions.

Briefly, stem cells are resuspended to a concentration of 1 x 10^5 cells/ml in normal growth medium. Next, the RNA is prepared so as to be transfected into the stem cells. Either siPORT *NeoFx* or siPORT *Amine* is combined with OPTI-MEM 1 and incubated for 10 minutes. The β-2-microglobulin RNA is diluted in OPTI-MEM 1 medium to a final concentration of 30nM per well. The RNA and transfection reagent are mixed for 10 minutes at room temperature and the mixture transferred into a well (either 96, 24, 12 or 6). The stem cells are mixed with the RNA/transfection reagent at 37°C. mRNA levels for β-2-microglobulin are tested at 48 and 72 hours. Reduced levels indicate PTGS of the MHC Class I β-2-microglobulin subunit.

Stem cells expressing MHC Class I are separated from MHC Class I negative stem cells using an MHC-1-specific immunoaffinity column.

In addition to using siRNA, expression can also be disrupted using hairpin structures. These can be made using the pSilencer™ Adeno 1.0-CMV System as per the manufacturer's instructions. The modified CMV promoter in the kit efficiently expresses hairpin siRNAs, which are effective at gene silencing through the RNAi pathway. An example of a top strand β-2-microglobulin oligo template is given at SEQ ID NO:6 (5'-TCGAGTATAAGTGGAGGCGTCGCGTTCAAGAGACGCGACGCCTCCACTTATAT T A-3') with the bottom strand oligo template at SEQ ID NO:7 (5'-CTAGTAATATAAGTGGAGGCGTCGCGTCTCTTGAACGCGACGCCTCCACTTAT AC-3'). Using the pSilencer™ Adeno 1.0-CMV Systems, these oligos produce a single hairpin oligo such as disclosed in SEQ ID NO:8 (5'-CGGCGAAGCTTTTTCCAAAAAATATAAGTGGAGGCGTCGCGCTACACAAACGC GACGCCTCCACTTATACCGGTGTTTCGTCCTTTCCACAAG -3').

EXAMPLE 5

In vivo suppression of MHC Class I

In addition to use the suppression of MHC Class I on stem cells, RNAi based β-2-microglobulin can also be used in cultured skin grafts, cultured liver cells, pancreatic cells or other organ replacement constructs where such tissues and organs are grown. Such applications can be extended to Xenotransplantation.

EXAMPLE 6

Generation of DNA-derived siRNA molecules

Sustained and propagatable siRNA molecules to target genetic sequences are generated by the method of Paddison *et al., Proc Natl Acad Sci USA 99*:14431448, 2002. The vector is generated using the promoter and vector backbone of *Miyagishi and Taira BioTechnology 19*:497-500, 2002. The target gene encodes human β-2-microglobulin. Stem cells are cultured in vitro and transfected with the genetic constructs. The constructs also encode a reporter gene encoding resistance to a chemical or a GFP. Cells are sorted on the basis of resistance or GFP expression and such cells selected. These cells are then tested for silencing of the target gene. The cells are then administered to a subject or allowed to undergo differentiation and/or proliferation before administration.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes al steps, features, compositions and compounds referred to or indicated in this specification individually or collectively, and any and all combinations of any two or more steps or features.

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CLAIMS:

1. A method for generating an immunologically neutral stem cell *in vitro*, said method comprising collecting stem cells from a source and contacting same with a nucleic acid molecule or complex comprising same which targets a genomic nucleotide sequence or mRNA transcript from the stem cell which encodes an MHC Class I or II molecule wherein said nucleic acid molecule or complex comprising same enables post transcriptional gene silencing of said MHC Class I- or II-encoding genomic nucleotide sequence.

2. The method of Claim 1 wherein the nucleic acid molecule or complex brought into contact with the stem cell is a synthetic RNAi, a construct encoding an RNAi molecule, a complex of targeting RNA and dicer or an anti-sense molecule to the mRNA transcript.

3. The method of Claim 2 wherein the stem cells are contacted with synthetic RNAi.

4. The method of Claim 1 or 2 or 3 wherein the stem cell is selected from embryonic stem cells, somatic stem cells, germ stem cells, human embryonic stem cells, human epidermal stem cells, adult neural stem cells, human neurons, human astrocytes, human keratinocyte stem cells, human keratinocyte transient amplifying cells, human melanocyte stem cells, human melanocytes, human foreskin fibroblasts, human duct cells, human pancreatic islets, human pancreatic β-cells, human adult renal stem cells, human embryonic renal epithelial stem cells, human kidney epithelial cells, human hepatic oval cells, human hepatocytes, human bile duct epithelial cells, human embryonic endodermal stem cells, human adult hepatocyte stem cells, human mammary epithelial stem cells, Bone marrow-derived stem cells, human lung fibroblasts, human bronchial epithelial cells, human alveolar type II pneumocytes, human skeletal muscle stem cells, human cardiomyocytes, bone marrow mesenchymal stem cells, simple squamous epithelial cells, descending aortic endothelial cells, aortic arch endothelial cells, aortic smooth muscle cells, limbal stem cells, corneal epithelial cells, CD34+ hematopoietic stem cells, mesenchymal stem cells, osteoblasts, peripheral blood mononuclear progenitor cells,

osteoclasts, stromal cells, human splenic precursor stem cells, human splenocytes, human CD4+ T-cells, human CD8+ T-cells, human NK cells, human monocytes, human macrophages, human dendritic cells, human b-cells, goblet cells, pseudostriated ciliated columnar cells, pseudostratified ciliated epithelium, stratified epithelial cells, ciliated columnar cells, goblet cells, basal cells, cricopharyngeus muscle cells, female primary follicles and male spermatogonium.

5. The method of Claim 4 wherein the MHC molecule is sleeted from HLA-A, HLA-B, HLA-C, HLA-DQ, HLA-DP, HLA-DM, HLA-DR and a subunit.

6. The method of Claim 5 wherein the MHC molecule is the MHC Class I a subunit.

7. An isolated or cultured stem cell characterized by being immunogenically neutral said stem cell or progency thereof comprising a silenced gene encoding an MHC Class I or II molecule.

8. The isolated or cultured stem cell of Claim 7 selected from the list consisting of embryonic stem cells, somatic stem cells, germ stem cells, human embryonic stem cells, human epidermal stem cells, adult neural stem cells, human neurons, human astrocytes, human keratinocyte stem cells, human keratinocyte transient amplifying cells, human melanocyte stem cells, human melanocytes, human foreskin fibroblasts, human duct cells, human pancreatic islets, human pancreatic β-cells, human adult renal stem cells, human embryonic renal epithelial stem cells, human kidney epithelial cells, human hepatic oval cells, human hepatocytes, human bile duct epithelial cells, human embryonic endodermal stem cells, human adult hepatocyte stem cells, human mammary epithelial stem cells, Bone marrow-derived stem cells, human lung fibroblasts, human bronchial epithelial cells, human alveolar type II pneumocytes, human skeletal muscle stem cells, human cardiomyocytes, bone marrow mesenchymal stem cells, simple squamous epithelial cells, descending aortic endothelial cells, aortic arch endothelial cells, aortic smooth muscle cells, limbal stem cells, corneal epithelial cells, CD34+ hematopoietic stem cells, mesenchymal stem cells, osteoblasts, peripheral blood mononuclear progenitor cells,

osteoclasts, stromal cells, human splenic precursor stem cells, human splenocytes, human CD4+ T-cells, human CD8+ T-cells, human NK cells, human monocytes, human macrophages, human dendritic cells, human b-cells, goblet cells, pseudostriated ciliated columnar cells, pseudostratified ciliated epithelium, stratified epithelial cells, ciliated columnar cells, goblet cells, basal cells, cricopharyngeus muscle cells, female primary follicles and male spermatogonium.

9. The isolated or cultured stem cell of Claim 7 or 8 wherein the MHC molecule is selected from HLA-A, HLA-B, HLA-C, HLA-DQ, HLA-DP, HLA-DM, HLA-DR and MHC Class I a subunit.

10. The isolated or a Human stem cell of Claim 9 wherein the MHC molecule is MHC Class I a subunit.

11. The isolated or cultured stem cell of Claim 7 or 8 or 9 wherein the gene encoding the MHC molecule is silenced by RNAi.

12. Use of synthetic or double stranded RNAi or an anti-sense RNA or DNA to a nucleotide sequence encoding an MHC molecule in the generation of a stem cell which is immunogenically neutral with respect to said MHC molecule.

13. Use of Claim 12 wherein the MHC molecule is selected from HLA-DQ, HLA-DP, HLA-DM, HLA-DR and MHC Class I a subunit.

14. Use of Claim 13 wherein the MHC molecule is MHC Class I a subunit.

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/001795

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 5/10 C12N 5/06 C12N 5/08 C12N 15/12 A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Medline, Caplus, Biosis, Biotechabs, Wpids (stem, hematopoietic, embryonic, progenitor, germ, pluripotent, es, cell? mhc? major histocompatibility, hla? human leukocyte antigen, immunogenic cell surface, mhcI, mhcII, silenc? inhibit? suppress? cosuppress? modulat? Antisense, siRNA, RNAi, fRNA, miRNA, sisRNA, snmRNA, stRNA, snoRNA, ribozym? dnazym? Ptgs, interfere? nonimmunogen? immunologically neutral, modifi? transgen? engineer? graft v host, graft vs host, graft versus host, gvhd, gvhr)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P/X	WO 2004 029219 A (COLD SPRING HARBOR LABORATORY) 8 April 2004. See whole document.	1-14
P/X	WO 2004 048583 A (INSTITUT CLAYTON DE LA RECHERCHE) 10 June 2004. See whole document especially pp19, 57-69, Examples 4, 10.	1-14
X	WO 2003 062394 A (GENETICA INC and COLD SPRING HARBOR LABORATORY) 31 July 2003. See whole document especially pp7-10, 28-29, 58-84.	1-14

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/001795

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2002 061033 A (YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM) 8 August 2002. See whole document especially pp3, 11, Examples 1-6.	1-14
X	WO 1997 025861 A (ORTHO PHARMACEUTICAL CORPORATION) 24 July 1997. See whole document.	1-14
X	Kaufman DS, *et al.* 2000. Transplantation therapies from human embryonic stem cells—circumventing immune rejection. e-biomed. 1:11-15 (available at http://www.liber/pub.com/EBI/defaultstatic.asp.) See page 12	7-10
X	Grusby, M.J. *et al.* 1993. Mice lacking major histocompatibility complex class I and class II molecules. Proceedings of the National Academy of Sciences (USA). 90:3913-3917. See whole document.	7-10
X	Apasov, S. *et al.* 1993. Highly lytic $CD8^+$, $\alpha\beta$ T-cell receptor cytotoxic T cells with major histocompatibility complex (MHC) class I antigen-directed cytotoxicity in β_2-microglobulin, MHC class I-deficient mice. Proceedings of the National Academy of Sciences (USA). 90:2837-2841. See whole document.	7-10
X	Crump, A.L. *et al.* 1993. Thymocyte development in major histocompatibility complex-deficient mice: Evidence for stochastic commitment to the CD4 and CD8 lineages. Proceedings of the National Academy of Sciences (USA). 90:10739-10743. See whole documents.	7-10
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2004/001795

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2004048583	US	2005014166				
WO	2004029219		NONE				
WO	1997025861	AU	16977/97	AU	50331/98	CA	2226695
		CA	2242400	EP	0853122	EP	0900002
		JP	10201396	US	6002066		
WO	2002061033	CA	2430653	EP	1379624	US	2002127715
WO	2002074345		NONE				
WO	2003062394	AU	45793/01	CA	2403397	CA	2473944
		EP	1272630	US	2002162126	US	2003084471
		US	2004018999	US	2004086884	WO	0168836

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END OF ANNEX