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(54) Title: SATELLITE CELL PROLIFERATION IN ADULT SKELETAL MUSCLE

(57) Abstract

Novel methods of retroviral-mediated gene transfer for the *in vivo* incorporation and stable expression of eukaryotic or ʼrokaryotic foreign genes in tissues of living animals is described. More specifically, methods of incorporating foreign genes into ʼitotically active cells are disclosed. The constitutive and stable expression of *E.coli* β-galactosidase gene under the promoter control of the Moloney murine leukemia virus long terminal repeat is employed as a particularly preferred embodiment, by way of example, establishes the model upon which the incorporation of a foreign gene into ba mitotically-active living eukaryotic tissue is based. Use of the described methods in therauptic treatments for genetic deseases, such as those muscular degenerative diseases, is also presented. In muscle tissue, the described processes result in genetically-altered satellite cells which proliferate daughter myoblasts which preferentially fuse to form a single undamaged muscle fiber replacing damaged muscle tissue in a treated animal. The retroviral vector, by way of example, includes a dystrophin gene construct for use in treating muscular dystrophy. The present invention also comprises an experimental model utilizable in the study of the physiological regulation of skeletal muscle gene in intact animals.

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SATELLITE CELL PROLIFERATION
IN ADULT SKELETAL MUSCLE

The present invention relates to the field of molecular genetics and its use in the preparation of therapeutic agents effective _in vivo_ in adult tissue. More particularly, stem cells are genetically modified _in vivo_ and then function to induce regeneration and repair of damaged tissue throughout the life span of an animal. More specifically, the present invention relates to the retroviral-mediated transfer of marker genes into adult tissue in which a mitotically-active state of satellite cells has been induced.

Additionally, the present invention relates to methods of treating genetically-transmitted diseases. The invention also relates to methods and therapeutic treatments for regenerating damaged muscle tissue, as a particular method for muscle regeneration in persons with muscular dystrophy is disclosed.

The insertion of foreign genes or DNA into an organism's genome has become a powerful tool in experimental biology during the past decade. This

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modification of genetic information has been attempted <u>via</u>
the development of a variety of experimental protocols.
Many experiments have transferred genes into mammalian
cells in culture and into newly fertilized mammalian eggs

5   that then develop into an intact animal. Less commonly,
experiments have been performed wherein foreign DNA is
inserted into the genome of cells of young or adult
animals <u>in situ</u>. The insertion of foreign DNA into the
genome of a cell or embryonic tissue has been postulated

10   to be applicable in the treatment of genetic diseases in
higher eukaryotes, particularly in humans. Methods
currently under extensive study in the manipulation of
genes include <u>in vitro</u> DNA transfection techniques, <u>in
vivo</u> whole cell injection (i.e., hematopoetic and myoblast

15   cells), and <u>in vivo</u> as well as <u>in vitro</u> retroviral-
mediated gene transfer.

       The insertion of foreign genes into an organisms
genome thus posses a potentially revolutionary method for

20   treating genetic-defect tractable diseases in man.
However, many of these genetic diseases remain to be more
fully characterized before gene therapy can be used in
their treatment.

25   Some of the more fully characterized examples of
genetic-defect diseases identified in the literature
include, by way of example, the various forms of muscular
dystrophy (see Table 1), diabetes, hemophilia and
albinism. The various forms of muscular dystrophic

30   diseases appear below.

TABLE 1

The Progressive Muscular Dystrophies

Type of Dystrophy	Age of Onset	Pattern of Muscular Involvement	Special Features	CK Level	Inheritance
Duchenne	Infancy or early childhood	Pelvifemoral; later pectoral girdle	Hypertrophy-pseudohyper-trophy; cardiac involvement; mental retardation	High	X-linked recessive
Becker	Childhood, adolescence, or early adult	Pelvifemoral, later pectoral girdle	Cardiac involvement, sight; mentation normal	Moderate	X-linked recessive
Emery-Dreifuss	Childhood, adolescence	Humeroperoneal	Contractures posterior neck and biceps muscles	Moderate	X-linked recessive
Landouzy-Dejerine	Late Childhood, adolescence girdle-late	Facioscapulo-humeral; pelvic normal	Heart normal, mentation	Slight to moderate	Dominant
Scapulohumeral of Seitz	Childhood, or adult	Spinal and humeral, later pelvic girdle girdle-late	Cardiomyopathy	Slight to moderate	Dominant
Limb-girdle (Erb)	Childhood, adolescence, sometimes adult	Pectoral or pelvic or both	Heart usually normal, mentation normal	Slight to moderate	Variable, recessive, or dominant

TABLE 1 CONTINUED

Type of Dystrophy	Age of Onset	Pattern of Muscular Involvement	Special Features	CK Level	Inheritance
von Graefe-Fuchs	Childhood, adolescence	Ocular (sparing pupils); later facial and other muscles (slight)	Kearns-Sayre group have retinitis pigmentosa, heart block, stunting of growth, and ovarian dysgenesis	Slight to moderate	Dominant; Kearns-Sayre recessive
Oculopharyngeal	Middle or late adult	Levators of lids; other ocular-pharyngeal muscles later		Slight to normal	Dominant
Myotonic (Steinert)	Infancy, childhood, adult	Ocular, facial, sternomastoid, forearm, peroneal	Cataracts, testicular atrophy	Slight to normal	Dominant
Congenital	Birth, infancy	Pectoral and pelvic girdles, or diffuse	Mental retardation, arthrogryposis	Slight to moderate	Dominant or recessive

Among the many genetic diseases described, the muscle
degenerative, or muscular dystrophy-causing diseases, have
experienced significant recent technological advances.
For example, the muscular dystrophies have been traced to
5   particular gene defects, the elucidation of which has
experienced particularly significant scientific
breakthroughs in terms of genetic molecular
characterization. The most widely known of the muscular
dystrophies are Duchenne's muscular dystrophy and the less
10   severe Becker muscular dystrophy. Both of these genetic
diseases are characterized by an inability of the muscle
to produce dystrophin, which is a muscle protein. This
defect is an x-linked recessive disease potentially caused
by a defect in the dystrophin gene. The dystrophin gene
15   has most recently been established as having a close
relative gene on human chromosome 6[24].

Prior studies have attempted the use of foreign
normal myoblast injection as a form of gene product
20   replacement. For example, foreign myoblasts containing a
normal dystrophin gene have been injected into dystrophic
tissue to invoke the expression of the dystrophin protein
in the muscle tissue. However, this method presents the
inherent risk of immune rejection, as well as the
25   necessity of injection at multiple, probably closely-
spaced sites[23]. Additionally, injection at multiple sites
is necessary with such a therapy because dystrophin, like
other muscle proteins, tends to remain localized within a
single fiber, close to the nuclei from which it was
30   derived. An additional limitation of currently practiced
myoblast-injection techniques is the low fusion rate of
implanted myoblasts into normal host muscle tissues.

Muscle tissue is characterized by the presence of
35   satellite cells, which are located between the basal
lamina and the sarcolemma of the of skeletal muscle

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fibers. Satellite cells in skeletal muscle are dormant
stem cells which are present outside the muscle fibers in
an adult skeletal muscle. Each of these inactive stem
cells can either proliferate into many satellite cells or
5   mature into an embryonic myoblast upon the appropriate
stimulation.

     By way of example, satellite cells of the muscle have
been shown to become stimulated to replicate by muscle
10   damage (Allbrook, 1981; Carlson et al., 1983). It is also
possible that strenuous exercise may stimulate cells to
become mitotically active and to replicate.[27] Chemical
damage via bupivacaine injection into mammalian skeletal
muscle cells has been shown to result in rapid recovery of
15   the tissue showing maximum proliferative capacity of
satellite cells 36-48 hours following damage [6,14]. As
stem cells, satellite cells function to supply myonuclei
to growing fibers in immature animals as well as to
provide myogenic cells for muscle regeneration and repair
20   throughout the life of the animal.

     Several laboratories have published protocols by
which the hematopoietic cells of mice have had new genes
introduced with retroviral vectors ex vivo, with
25   subsequent reintroduction of the treated cells into the
bone marrow in vivo[22]. In this system, marrow cells are
obtained from a donor and incubated with a monolayer of
vector-generating producer cells. Since marrow cells,
unlike the producer cells, do not attach to the culture
30   disk, they are easily recovered after co-cultivation. The
hematopoietic cells are then introduced into a recipient
animal by intravenous injection. Space in the
hematopoietic system to receive the vector-treated marrow
must be made usually by lethally irradiating the
35   recipient. While this technique has been widely used
experimentally, its use in humans is limited by the

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recurrent risk of host immune rejection, as current
studies on muscle were performed on mdx (immunosuppressed)
animals[23]. Additionally, low proportion of cells
incorporate the normal myoblast dystrophin gene in
5   myoblast transfer procedures. Moreover, multiple
injections of myoblasts are typically required to achieve
proper dispersion of the myoblasts in situ.

        A need thus remains in the art for the development of
10   a therapeutic system which minimizes or eliminates host
immune response, perhaps through the development of an
entirely in vivo vector-induced, host cell gene
incorporation process. However, technical difficulties in
the manipulation of genomes from organisms both in
15   achieving the initial incorporation of the desired gene by
the host cells as well known as achieving expression of
those genes in the host have limited techniques of direct
incorporation of a gene without a "carrier" cell into
damaged tissue in vivo. DNA transfection and retroviral
20   mediated gene incorporation are two methods of such a
"direct gene" incorporation which eliminates the need for
a "carrier" cell.

        The treatment of genetically-related diseases with
25   techniques as DNA transfection has thus far,
unfortunately, not met with great success. In DNA
transfection, DNA (presumably which includes a non-
defective counterpart of the defective gene) is introduced
into cells in culture as part of a coprecipitate with
30   calcium phosphate or dextran sulfate.[9] A successful
result is a viable cell containing one to many copies of
the new gene which continuously expresses the new genetic
information.

35   While several limitations exist in this system, the
most significant limitation is that it is a very

inefficient means of transferring genes into mammalian cells. For example, only one in a thousand cells (more typically, one cell in a million) will incorporate the newly transformed gene. Additionally, not all cultured cell lines are susceptible to this method of gene transfer. For example , while the stem cells located in bone marrow are typically susceptible to this method of gene transfer, they are present only as a small fraction (less than one cell in a thousand) of the total nucleated cells of the tissue. Another limitation is that it is an ex vivo technique.

This method is therefore inefficient as only a small population of cells incorporate the desired gene, failing to accomplish gene delivery to a large fraction of a target cell population. Even where the technical difficulties of gene incorporation are able to be overcome, a second obstacle to the effective employment of this technique in the higher eukaryotes and mammals which has not yet been solved in being able to attain proper expression of successfully incorporated genes by the host.

Retroviral-mediated gene transfer (i.e., the use of retroviruses to deliver genes into cells) is an alternative gene transfer technology which has met with a limited, yet improved, success in host genome incorporation rates. Using this technique, it is now possible to insert a gene into a retroviral vector to obtain a recombinant virus, and then infect target cells with the retrovirus (which includes a particular gene of interest) and achieve the expression of the foreign gene by the host cell's chromosomes. Retroviruses are RNA viruses, that is, the viral genes are encoded in an RNA molecule rather than in a DNA molecule. All RNA tumor viruses are members of the retrovirus family, but not all retroviruses are oncogenic or even pathogenic.

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In retroviral-mediated gene transfer, the viral RNA
is first converted to DNA when an RNA virus penetrates a
cell. If the cell penetrated is a replicating cell (i.e.
mitotically active), the DNA will enter the nucleus and
5   integrate into a chromosome. This integrated DNA becomes
indistinguishable as far as the cell is concerned from any
other cellular gene. It is from this integrated form,
that the viral genes are expressed. In this process,
integration of the viral genome into the cell's chromosome
10   is an essential part of its replication. However, RNA
retroviruses only insert their genome into mitotically
active cells; thus making it highly unlikely that an RNA
retrovirus would infect a mature myonucleus.   Thus, only
immature animal tissues have been used experimentally with
15   retroviral-mediated gene transfer systems with much
improvement of gene-incorporation rates.

Retroviral mediated gene transfer remains a
relatively inefficient gene transfer system for adult
20   tissues owing to low gene incorporation rates of target
cells. It would therefore only be by means of a highly
efficient retroviral vector system that genetic
manipulation of totipotent stem cells would become
practical, as currently practiced methods of retroviral-
25   gene transfer provide only a small fraction of
genetically-altered cell components.

A number of procedures are known to artificially
induce a mitotically-active state in a cell population.
30   For example, exercise,[27] tissue trauma,[6] chemical
injection,[28] and radiation[9,30] to have been shown to make
tissue or cells in culture mitotically active. Many
pharmaceutical agents (especially anesthetics, such as
Marcaine®) have been found to destroy or damage muscle
35   fibers.[28] For example, it has been found that when
bupivacaine (a local anesthetic) is injected into skeletal

muscle, existing muscle fibers (including myonuclei) are
destroyed,[15, 16] curiously, however, the satellite cells in
these tissues are left undamaged.

5   Despite developments in retroviral-mediated gene
transfer and studies regarding the _in vitro_ and _in vivo_
induction of mitotically active states in certain cell
populations, the many unknown aspects of the structure and
function of retroviruses has prevented the combination of
10   these advantages in the development of an efficient gene-
transfer system. Technical difficulties still exist in
the use of retroviral vectors for gene transfer in large
animals (i.e., humans), such as in the efficiency of
infection of pluripotent stem cells and in the long-term
15   stability of expressed genes. Moreover, the use of
retroviral-mediated gene transfer is limited by the size
of the gene constructs which the virus is able to carry.
For example, genes having a 1400 K^6 sequence have been
found to be too large for common vectors. Such results in
20   difficulties in replacing proteins, either for functional
studies or in gene therapy experiments, which involve
genes of this size.

In the development of an efficient retroviral gene
25   transfer system, two parameters must be optimized. First
is the capacity to infect a large proportion of the target
cells, a property dependent at least in part on the
ability to generate a high concentration (or titer) of
recombinant virus. Second is the capacity to have the
30   gene expressed properly in the host.

An intense need exists in the art of molecular
biology to either circumvent the limitations of
retroviral-mediated gene transfer (gene size, gene
35   incorporation ratio in target cells) if retroviral vectors
are to be used in the medical treatment of genetic disease

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in humans. Additionally, the need for an efficient gene
transfer system is desirable for its usefulness to basic
research, as well as being an absolute prerequisite for
application to human therapy.[10]

5   The innovative development of gene transfer
technology to improve target cell incorporation ratio
while achieving successful and acceptable amounts of gene
expression in the host, while eliminating host immune-
10   response problems, would revolutionize currently practiced
methods of treating genetically-induced maladies,
especially those muscle degenerative diseases which have
escaped human intervention over the years.

15   The present invention presents a surprisingly
effective and novel _in vivo_ method of expressing foreign
genes in an adult eukaryotic tissue. This method
accomplishes the incorporation of a foreign gene of
interest using a retroviral-mediated gene transfer system
20   wherein the target tissue is first treated so as to induce
a mitotically-active state in the cells of the target
tissue.

25   The present inventive method also presents an
amazingly accurate and simple technique applicable to the
study of eukaryotic tissue regeneration, especially the
regeneration of muscle tissue. For example, with the
presently disclosed innovative retroviral gene transfer
system, Applicants have been able to reveal for the first
30   time two important aspects of the cell biology of the
muscle. First, that in damaged muscle, a satellite cell
directs its daughter myoblasts almost exclusively to a
single regenerating fiber. Second, Applicants' studies
show that the apparent diffusion domain of a myonucleus
35   overlaps several adjoining myonuclei, confirming the
possibility of internuclear communication of factors that

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may regulate gene expression in tissue, particularly that
in muscle tissue.

    In its broadest embodiment, the present invention
5   comprises a method for the _in vivo_ incorporation of a
foreign gene into an adult tissue comprising infecting a
mitotically-active cell in the living tissue with a
retroviral vector. The retroviral vector of the present
invention most preferably comprises a replication
10 deficient retrovirus and a foreign gene. In one preferred
embodiment of the claimed method, the foreign gene of the
retroviral vector is a prokaryotic foreign gene. This
prokaryotic foreign gene is in one application of the
invention, is the ß-galactosidase gene of _E. coli_.
15

    In still another embodiment of the claimed method,
the foreign gene of the retroviral vector is a eukaryotic
gene. Most preferably, when the foreign gene is a
eukaryotic foreign gene, the gene is a gene encoding
20 dystrophin protein, or dystrophin gene, or a gene encoding
insulin, or an insulin gene. Of these, the eukaryotic
dystrophin gene is most preferred. The dystrophin gene is
in a modified form as a dystrophin gene construct, which
is significantly smaller than the naturally occurring
25 dystrophin gene. Such is provided in order to allow the
containment of the dystrophin gene construct in a single
retroviral vector.

    In that the present methods allow for the
30 incorporation of a single gene in a living tissue,
particularly muscle tissue, the present invention allows
for the follow-up study of the effect of the _in vivo_
specifically-altered genome on tissue function resultant
from the incorporation of a particular foreign gene.
35 Additionally, the study of the control of gene expression
in tissues so genetically altered is also facilitated.

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Foreign gene expression in intact adult animals is achieved with the present invention through the efficient incorporation of the foreign gene into the genome of the target tissue or cells thereof. For this purpose, a retrovirus which if replication deficient is employed, which includes a gene encoding a desired gene product. In that the present invention encompasses a method of gene incorporation, a method of gene therapy for the replacement of a defective or deficient gene is also disclosed. The gene which is to be incorporated by the target cells in this method encodes a product which supplies or is complementary to a particular genetic deficiency present in the animal.

An object of the present invention is to eliminate the problem of immune response attendant many forms of gene transfer. Applicants' method of incorporating a single foreign gene into eukaryotic cells completely _in vivo_, employing only a retroviral vector, effectively eliminates the risk associated with immune rejection inherent to other techniques involving whole cell (e.g., myoblast) injection.

The presently disclosed methods accomplish genetic complementation and repair totally _in vivo_, as compared to other techniques which require _ex vivo_ vector transformation of donor or recipient cells prior to treatment of the diseased or damaged tissue (i.e., donor myoblast retroviral infection and recipient injection of transformed myoblasts). Pursuant thereto, the present invention features the advantage over prior methods of avoiding host immune rejection of vector-transformed cells. While this obstacle does not exist when host autologous cells are used as the vehicle of gene transformation, sufficient host autologous cells may not be available for genetic therapy prior to their

reintroduction into the host. Limitations of the system
attendant the availability of a sufficient population of
the host cells (as the transforming agent) are eliminated
by doing away with the need for extraction and genetic
5   manipulation of the transforming cells. The risk of
potential damage from manipulation of the transforming
agent (i.e. host cells) is also eliminated.

        A preferred embodiment of the presently described method
10   for incorporating a foreign gene into an adult eukaryotic
tissue completely in vivo comprises: inducing a
mitotically-active state in an adult eukaryotic tissue to
produce mitotically active eukaryotic cells; preparing a
retroviral vector comprising a gene of interest; and
15   exposing the mitotically-active cells to the retroviral
vector for a time period sufficient to allow genetic
incorporation of the gene of interest in the genome of the
mitotically-active cells of the eukaryotic tissue. Most
preferably, the retroviral vector is replication
20   deficient, and the gene of interest comprises a gene
encoding an enzyme or protein which is complementary to
the deficiency or supplies the product of a deleted gene
in the host tissue being treated.

25   The gene of interest in one particularly preferred
embodiment of the described method comprises a prokaryotic
foreign gene. In such a preferred embodiment, the
prokaryotic foreign gene is a ß-galactosidase gene. The
gene of interest in still another particularly preferred
30   embodiment of the described method is a eukaryotic foreign
gene. Preferred embodiments of the disclosed methods
which include a eukaryotic foreign gene are further
defined as comprising a eukaryotic gene such as an insulin
gene, a dystrophin gene or a spectrin gene, or
35   phenotypically sufficient fragments or constructs thereof.
Thus, in one particularly preferred embodiment, the gene

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of interest is a dystrophin eukaryotic gene construct
which is a modified dystrophin gene having between 7,000-
10,000 base pairs.

5   In that the present methods may employ any eukaryotic
gene of interest in the retroviral vector, the described
methods also comprise a method of gene therapy. As a
method of gene therapy, a particular defective gene in a
eukaryotic tissue is replaced or substituted in vivo by a
10   complementary non-defective gene in the cells of the
eukaryotic tissue. In such an embodiment, the present
method of gene therapy for the replacement of a defective
gene in a eukaryotic tissue comprises: inducing a
mitotically-active state in the eukaryotic tissue to
15   produce mitotically-active cells; preparing a retroviral
vector comprising a gene complementary to the defective
gene in the eukaryotic tissue; and exposing the
mitotically-active cells to the retroviral vector for a
time period sufficient to allow the incorporation of the
20   gene of interest in the eukaryotic cell, wherein
incorporation of the gene of interest replaces the
defective gene in the cells of the eukaryotic tissue. In
a most preferred embodiment of this method, the gene
complementary to the defective gene is a eukaryotic gene,
25   such as the dystrophin gene for the treatment of animals
with degenerative muscle disease.

          As a method for treating a genetic disease in a
eukaryote, the invention comprises incorporating a
30   complementary gene to a genetic deficiency in a stem cell
of the eukaryote, wherein incorporation of the
complementary gene comprises the steps of: inducing a
mitotically-active state in the cells of the eukaryote to
form a mitotically-active preparation of eukaryotic stem
35   cells; preparing a retroviral vector comprising a
eukaryotic gene complementary to the genetic deficiency in

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the cells of the eukaryote; exposing the mitotically-
active preparation of eukaryotic stem cells to the
retroviral vector; and incubating the retroviral vector
with the mitotically-active preparation of eukaryotic
5   cells a time period sufficient to allow incorporation of
the complementary gene in the eukaryotic stem cell. In
this manner, the deficiency in the eukaryotic genome is
eliminated.

10   Most preferably, the eukaryotic cell described in the
present methods include totipotent eukaryotic cells, such
as a stem cell (i.e., muscle satellite cells,
hematopoietic stem cells (in bone marrow)[13], dark basal
keratinocytes[31]). As used in the present application, the
15   term "totipotent " relates to a cell which is capable of
differentiating into an entire organism or into a part of
an organism, such as a cell which is capable of generating
or differentiating to form a particular tissue (i.e.,
muscle, skin, heart, brain, uterus, testis, blood).
20

The eukaryote described in the present invention
includes all organisms comprising cells which contain a
membrane-bound nucleus. In particularly preferred
embodiments of the described methods, the eukaryotic
25   tissue described in relation to the disclosed methods is
human tissue, rat tissue or mouse tissue.

In the more preferred embodiments of the described
methods, a mitotically-active state in a living eukaryotic
30   cell or tissue comprises inducing cellular-repair
mechanisms in the cell or tissue. Thus, the induction of
a mitotically-active state comprises discomposing the
tissue, exposing the cell to radiation, or administering a
pharmaceutical chemical agent. As used in this
35   application, the term discompose relates to any change in
the normal resting state or non-mitotically active state

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of a eukaryotic cell culture or tissue, particularly that
of a eukaryotic tissue. By way of example, discomposition
of a tissue may be accomplished through vigorous exercise
stimulation[7], agitation, surgical intrusion or other
5   trauma of the tissue or cell culture.

The use of chemicals to induce a mitotically-active
state in the cell (i.e., in cell culture) or tissue
described includes the administration of any of a variety
10   of pharmaceutical agents. By way of example, the
pharmaceutical agents capable of inducing a mitotically-
active state _in vitro_ or _in vivo_ include bupivacaine,
collagenase, dexamethasone, fibroblast growth factor, and
any other reagent capable of inducing a mitotically-active
15   state in a eukaryotic tissue or a eukaryotic cell.

The retroviral vector is a retrovirus (RNA virus)
which is replication deficient or replication incompetent.
That is to say, the retrovirus lacks one or more of the
20   replication genes, gag (group-specific antigen), pol
(polymerase) or env (envelope) protein encoding genes.

The gene therapy of the present invention comprises a
method by which any of a variety of genetic diseases may
25   be eliminated. The invention thus comprises a method of
treating genetic disease. Such is accomplished through
the successful incorporation of a particular complementary
gene or part of a complimentary gene in the genome of
totipotent cells of the diseased organism, thereby
30   facilitating the production of cells in the diseased host
with similarly corrected genomes. By way of example,
genetic diseases which may be treated employing the
described methods include diabetes, albinism, and the
various forms of muscular dystrophy. However, any
35   recessive gene is hypothesized to be treatable employing

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the described methods of gene transfer and gene
replacement.

In a most preferred embodiment, a method for treating
the genetic disease of muscular dystrophy is described.
Muscular dystrophy is a genetic disease characterized by a
defective gene encoding the muscle protein, dystrophin.
In this particular embodiment of the invention, the gene
of interest would be a part of the dystrophin gene (i.e.,
a dystrophin gene construct) which encodes a part, segment
or fragment of the dystrophin protein, this particular
protein segment supplying a sufficient part of the native
dystrophin protein to protect the eukaryotic tissue from
all but only the mildest phenotypic manifestation of
muscle degeneration. This particular dystrophin gene
fragment is described by England et al.[20]

The spectrin gene, which encodes a muscle protein
similar to dystrophin, is also hypothesized to be
effective in the methods described herein for the
treatment of muscular dystrophy. More particularly, a
retroviral vector which includes a spectrin gene or a
fragment of the spectrin gene may be effective in treating
muscular dystrophy through the injection of such a
retroviral vector into a eukaryotic tissue, the eukaryotic
tissue being treated so as to induce a mitotically active
state therein. Various methods for inducing such a
mitotically active state are as otherwise described herein
(radiation, trauma, exercise, chemical injection).

The retroviral vector in one preferred embodiment of
the present invention would include a dystrophin gene
construct which encodes a particular dystrophin protein
fragment which is effective to halt massive or significant
muscle degeneration, as well as to regenerate already
damaged muscle tissue. In a most preferred embodiment of

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the invention for the treatment of muscular dystrophy, the
retroviral vector includes a dystrophin gene construct
comprising between 7,000 - 10,000 base pairs. In an even
more preferred embodiment, the dystrophin gene construct
5   comprises about 9,000 base pairs of the native dystrophic
gene. This dystrophin gene construct encodes a protein
having a relative molecular mass of about 200,000 K.

        As a method of treating muscular dystrophy in a human
10   or other eukaryotic organism, the present invention in a
most preferred embodiment comprises the initial induction
of a mitotically-active state in the tissue of the
eukaryote through the intramuscular administration of
bupivacaine to the skeletal muscle tissue. The retroviral
15   vector of this particular embodiment of the invention
comprises a replication defective murine leukemia virus.
Most preferably, the murine leukemia virus comprises AKR,
Moloney or Friend leukemia virus. However, most
preferably, the retroviral vector comprises the
20   replication defective Moloney murine leukemia virus.

        In still another embodiment of the present invention,
the retroviral vector comprises a prokaryotic gene, such
as a ß-galactosidase gene, wherein the ß-galactosidase
25   gene is under the control of the retroviral 5' long
terminal repeat promoter region. More particularly, the
ß-galactosidase gene is under the control of a
constitutive promoter[3]. A method for monitoring gene
expression in tissue is thereby provided (i.e., focal and
30   diffuse expression). For example, as employed to infect
muscle tissue in eukaryotes, Applicants propose that the
use of such a vector system would track regenerating and
fully recovered muscle fibers, in that regenerating and
fully recovered muscle fibers would express the ß-
35   galactosidase gene product if the infected satellite cells
gave rise to viable myoblasts.

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　　　　The disclosed methods are not to be limited to
treatment of muscular degenerative diseases. For example,
the gene of interest in the present invention may comprise
any gene or part of any gene which complements a
5　　　deficiency in the animal being treated. However, length
of the gene of interest, or the coding region thereof, is
preferably less than 14,000 base pairs so as to facilitate
the inclusion of the particular gene in a single
retrovirus.
10

　　　　Use of the presently described methods to stimulate
stem cell proliferation allows the transformation of the
genome of, in one particular embodiment, skeletal muscle
satellite cells, resulting in the production of
15　　　genetically-corrected myoblasts. These myoblasts then
become the non-defective myonuclei of newly formed and
non-defective muscle fibers. As will be appreciated, the
invention in such an application comprises a method for
regenerating muscle tissue. In a most preferred
20　　　embodiment, this technique comprises a method of treating
degenerated dystrophic muscle tissue. In this preferred
embodiment, the regeneration of dystrophic muscular
tissue, characteristic of such diseases as Duchenne's
muscular dystrophy and Becker muscular dystrophy, is
25　　　specifically envisioned.

　　　　As a therapeutic treatment for muscular dystrophy,
Applicants have found that the technique of stimulating
stem cell proliferation to allow retroviral-mediated gene
30　　　transfer is generally applicable to all eukaryotic
tissues. Potential target cell populations for use with
the present methods are characterized by the presence of
stem cells. By way of example, tissues expected to be
susceptible to the described treatments and methods are
35　　　those tissues which include stem cells. By way of
example, these stem-cell containing tissues include those

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tissues of the uterus, skeletal muscle, liver, kidney, blood, alimentary epithelium, testes, skin and gastrointestinal tissue. The stem cells of muscle tissue are the satellite cells. In the described methods, the

5   stem cells (i.e., satellite cells) in muscle tissue are the cells which are infected with the retroviral vector to facilitate incorporation of the eukaryotic gene of interest in the satellite cell itself. These satellite cells may then function to supply myonuclei to growing

10   fibers in immature animals and to provide myogenic cells for muscle regeneration and repair throughout the life of an animal.

       In that any of the methods described in the present

15   application may be used _in vivo_, the retroviral vector may be further defined as comprising a retroviral vector conjugated to a protein. Such may be accomplished by methods well known to those of skill in the art, and will function to enhance the targeting of the vector to

20   particular populations of target cells.

       The following abbreviations are used throughout the Specification:

25   CFU =   colony forming units
        ug =   microgram
       EGTA =   methylene glycol tetraacetic acid
        ß   =   beta
        um =   micromolar
30   ß-galactosidase = beta-galactosidase (EC 3.2.1.23)
       MoLV =   Moloney murine leukemia virus

       Figure 1 - ß-galactosidase expression in soleus muscle sections. 24-48 hours after muscle damage the

35   bupivacaine-damaged muscle and undamaged contralateral muscle were injected with 0.3 ml of the retrovirus

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carrying the ß-galactosidase gene (2×10^5 CFU/ml). The
animals were then housed in a biohazard facility for one
week or four weeks. 16 um frozen sections were stained
for ß-galactosidase activity using the method of
Dannenberg and Suga[6] to produce a distinct indigo blue
precipitate. (A) Cross-section. Diffuse ß-galactosidase
activity occurs within the circumference of the fibers
with adjacent fibers occasionally exhibiting less intense
staining. (B) Cross-section. More focal staining is
often observed associated with the sarcolemma.
(C) Longitudinal section. Diffuse ß-galactosidase
activity occurs uniformly over many nuclear domains.
(D) Longitudinal section. Focal activity along the length
of a fiber. The magnifications in all panels are
identical; the length bar in (D) represents 10 um.

The inventions of the present disclosure relate
generally to gene transfer systems in eukaryotic cells and
tissues. The practice of the invention provides an
improved transfer system for eukaryotic and prokaryotic
genes surprisingly improved over other practiced gene-
transfer systems. For example, improvements of the
present system include an enhanced gene incorporation rate
in a target cell population, successful target cell
expression of the host-incorporated foreign gene, and
elimination of host-immune rejection response. These
methods of gene transfer are effected through the
induction of a mitotically-active state in a target cell
population prior to exposure to a retroviral vector.
Target cells thus become receptive to retroviral gene
insertion, thus greatly enhancing the rate of gene
incorporation. Use of the direct _in vivo_ injection of a
retroviral vector eliminates the risk of host-immune
response existent in "carrier cell" type gene transfer
systems (i.e., _in vitro_ myoblast modification and
subsequent _in vivo_ injection).

It is desirable that the retrovirus employed be
replication deficient and be a murine leukemia virus. In
most preferred embodiments of the invention, the
retrovirus is an AKR, Moloney or Friend murine leukemia
virus. Moreover, it is particularly desirable that the
retrovirus include a particular gene of interest which is
complementary to or provides a suitable substitute for a
defective or deficient gene in the target cell population
or tissue.

In a most preferred use of the described invention, a
method for the _in vivo_ incorporation of a foreign gene
into an adult tissue is described comprising infecting a
mitotically-active cell in the adult tissue with a
retroviral vector. The retroviral vector in such a
preferred embodiment comprises a eukaryotic foreign gene
and is replication defective.

As a method of gene therapy, the present invention
discloses a technique whereby a defective gene in a
eukaryotic tissue may be replaced. This particular method
comprises inducing a mitotically-active state in the
eukaryotic tissue to produce mitotically-active cells;
preparing a retroviral vector comprising a eukaryotic gene
complementary to the defective gene in the eukaryotic
tissue; and exposing the mitotically-active cells to the
retroviral vector for a time period sufficient to allow
incorporation of the gene of interest in the eukaryotic
cell. By this method, the incorporation of the gene of
interest functions to replace the defective gene in the
eukaryotic tissue.

The present invention presents a revolutionary method
for treating virtually any disease caused by a genetic
deficiency in a eukaryote. Such is accomplished through
the incorporation of a non-defective gene complementary to

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the particular deficiency in the diseased organism. More
particularly, the incorporation of a particular
complementary gene comprises a series of steps, wherein a
mitotically-active state is first induced in the eukaryote
5   cells, most preferably stem cells of the eukaryote. Thus,
the general scheme of the technique, i.e., stimulating
stem cell proliferation followed by retrovirus injection
is postulated to provide a means to facilitate gene
transfer into many different tissues.
10

Applicants' studies demonstrate the described foreign
gene incorporation into mammalian adult skeletal muscle.
Accordingly, from these studies, it is hypothesized
similar gene incorporation may be accomplished in smooth
15   muscle, gastrointestinal, brain, cardiac muscle, uterine,
blood, skin or testicular tissue, especially those tissues
as they exist in the human.

Turning now to a consideration of the methods by
20   which a mitotically-active state may be induced in a
viable adult eukaryotic tissue or a eukaryotic cell
culture, such a receptive state may be induced via any
discomposition which induces cellular-repair mechanisms in
a cell culture or tissue. As will be recalled,
25   retroviruses are unable to insert genetic information into
another cell unless that target cell is made receptive
(i.e., mitotically active). A receptive state of a target
cell may be induced by making the cell mitotically active.
A variety of techniques are available which induce such a
30   mitotically-active state. By way of specific example,
such includes the irradiation of the tissue or cell
culture, the exposure of the cells or tissue to particular
pharmaceutical agents, the physical agitation of a cell
culture, or the surgical intrusion of a tissue, as well as
35   through the vigorous exercise of a tissue.

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Among those pharmaceutical agents expected to be
effective in inducing the described mitotic or active
state in cell culture or _in vivo_, the following list
presents those agents most particularly preferred:
5   collagenase, fibroblast growth factor, bupivacaine,
estrogen and dexamethasone.

The following tissue sectioning and staining protocol
represents a method which may generally be used in the
10   fixation of any type of tissue from any of a variety of
animal species. Several experimental examples follow
thereafter which are designed to illustrate particularly
preferred embodiments of the inventions, both as they
actually exist and as they are proposed to exist in the
15   future (i.e., prophetic exemplary use in treating genetic
disease in humans).

It should be appreciated that many modifications and
changes can be made in the particular stimulatory agents
20   and their doses, the particular retroviral construct
(i.e., the promoter, the gene of interest, and the
particular retrovirus) and the particular conditions under
which the retrovirus introduced to a culture of cells or
into a living adult tissue without departing from the
25   spirit and scope of the invention.

Tissue Sectioning and Staining

Muscle sections were stained by the method of
30   Dannenberg and Suga.[8] Briefly, 16 um sections were
mounted on 0.5% gelatin slides and fixed 5-10 minutes at
4°C with the same fixative as used in the dissection. The
sections were washed briefly with PBS containing 2 mM
$MgCl_2$ at 4°C and once again for 10 minutes with the same
35   solution. The sections were then washed for 10 minutes in
PBS containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and

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0.02% Nonidet P40 at 4°C. The slides were briefly dried
and stained for 18 hours at 37°C in a PBS solution
containing 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2 mM
$MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P40, and 1

5   mg/ml 5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside
(Xgal). Following staining the slides were rinsed three
times for 5 minutes each with PBS at room temperature,
blotted dry, and stained with 5% eosin.

10   Applicants are in the process of replacing the ß-
galactosidase gene with the rat insulin-like growth factor
I coding region, using the following strategy. The entire
ß-galactosidase gene was removed from the gag region of
the retroviral "BAG" construct of Price, et al.,[3] by

15   restriction with Pvu I and BamHI. The insulin-like growth
factor I coding region will be added to the gag site where
ß-galactosidase was removed as follows. Applicants were
supplied with prIGF-Ib-42-a, a 422 bp insert in pUC-19,
which included a partial 5' untranslated region with 2

20   putative ATG translation start sites, the Pre, B, C, A, D
and part of the E domains of the rat insulin like growth
factor 1 coding region, without introns. The processed
mature insulin-like growth factor I peptide did not
contain the E domain. The 422-bp insulin-like growth

25   factor I coding region was flanked by EcoRI restriction
sites.

BL-2 tissue culture facility

30   An 80 ft^2 room was devoted solely to culture of the
Psi2 packing cell line and 3T3 cells, which were used to
prove that the produced retrovirus is replication-
incompetent, that is no recombinant wild-type retrovirus
is produced. A BL-2 rated laminar flow hood (Nuaire 425-

35   400) and a CO_2 H_2O - jacketed incubator (Nuaire) was also
used in the following studies. An Olympus CK2 inverted

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scope with phase contrast was available outside the
culture facility.

Laboratory facilities

5

Two cryostats (IEC minotome) were available for
production of tissue sections. An automatic microtome
knife sharpener was also available for usage. Applicants'
laboratory has an Olympus BH-2 microscope with attached
10   mOS color video camera so that a slide may be viewed
simultaneously through the binocular observation tube and
on a video color monitor. This system is attached to a PC
Vision Plus Frame Grabber Board in an AT-clone computer.
Applicants have a JAVA image-processing program which
15   permits the determination of relative area of indigo blue
on a tissue section.

Applicants' laboratory also contains apparatus for
electrophoresis of agarose, sequencing and polyacrylamide
20   gels, power supplies, microfuges, water baths, tissue
homogenizers, vacuum oven, sterilizer, freezers, gel
dryers, pH meters, stirrers, IEC-5000 centrifuge, DNA
sequencing apparatus, Geiger counters, recorders, pumps
and electrical stimulators. Applicants have access to
25   Departmental low and high centrifuges (Sorvall RC series
to Beckman L5-50), gamma and beta scintillation counters,
and Gilford spectrophotometer.

EXAMPLES

30

The following examples further illustrate certain
features of particularly preferred methods as provided by
the present invention.

EXAMPLE 1 - PREPARATION OF A RETROVIRAL CONSTRUCT

The replication-defective retroviral construct is
that of Price et al[3] Psi2 cells producing the retrovirus
5   containing the ß-galactosidase and neomycin
phosphotransferase genes (American Type Culture Collection
CRL 9560) were grown to confluence in Dulbecos Modified
Eagles Medium containing 10% fetal calf serum. The E.
coli ß-galactosidase gene used was under the promoter
10   control of the retroviral Moloney murine leukemia virus 5'
long-terminal repeat promoter region, and thus is
constitutively expressed. The medium was withdrawn and
replaced with fresh medium. Following three days of
incubation, the retrovirus-containing medium was
15   collected, filtered through 0.45 um filters, brought to 8
ug/ml polybrene, and stored at -80°C. A titer of 2 X 10^5
colony forming units (CFU) per milliliter was obtained, as
determined by infection of NIH 3T3 cells.

20   The retrovirus was tested for wild-type (replication-
competent) viral particles by testing the ability of the
conditioned media from infected NIH 3T3 cultures to
produce a secondary infection in uninfected cells. No
wild-type viral particles were detected, but as a
25   precaution the retrovirus was handled in type IIB culture
facilities.

The retroviral construct was used in the experiments
described in Examples 2 and 3.
30
EXAMPLE 2 - IN VIVO FOREIGN GENE
INCORPORATION AND EXPRESSION IN ADULT FEMALE RAT MUSCLE

The following experiment was designed to determine if
35   a foreign gene could be successfully inserted and achieve
expression in the tissue of a living animal employing a
totally in vivo system. The experiment tests a method for

inserting a vector which includes a foreign gene, in this
case, the _E. coli_ gene coding for ß-galactosidase, into
the existing DNA of skeletal muscle in adult rats. This
system constitutes an effective marker system to follow
5   the incorporation of a gene of interest into a tissue.

To maximize the probability of retroviral gene
insertion, the retrovirus containing _E. coli_ ß-
galactosidase gene was injected into the bupivacaine-
10   damaged soleus muscle of the rat 24-48 hours following
damage.

In order to produce bupivacaine damage, the soleus
muscles of 125 g female rats were exposed by lateral
15   incisions. The left soleus muscle was injected
bilaterally in each quarter with 0.3-0.5 ml 0.5%
bupivacaine HCl, 0.1% methylparaben in isotonic NaCl (0.5%
Marcaine®, Winthrop-Breon Labs) using a 27g needle. There
was considerable leakage of the liquid from the injection
20   sites. The right soleus muscle served as the
contralateral control and was similarly injected with 0.9%
NaCl, 0.9% benzyl alcohol (bacteriostatic NaCl). 24-48
hours following bupivacaine damage, both left and right
soleus muscles were again exposed and 0.3 ml of the
25   retrovirus-containing medium was administered by injection
in the same pattern as the bupivacaine and saline
injections. The animals were then allowed to recover
either 6 days or one month in a biohazard suite.

30   Upon dissection, the soleus muscles were placed in a
fresh solution of ice-cold 2% paraformaldehyde, 100 mM
PIPES, 2 mM $MgCl_2$, 1.25 mM EGTA, pH 6.9, for 15 min. The
tissue was then placed in an ice-cold PBS solution
containing 2 mM $MgCl_2$ and 30% sucrose for 3 hours. The
35   muscles were blotted dry and embedded in OCT medium (Miles

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Laboratories) on dry ice. Embedded muscle was stored at -
20°C.

The contralateral soleus muscle received <u>saline</u>
<u>injections</u> instead of <u>bupivacaine</u>, but otherwise was
treated identically to the bupivacaine-damaged muscle.

In all cases, injection of the retrovirus into the
damaged soleus muscle resulted in the expression of
histochemically detectable ß-galactosidase activity in
satellite cells and muscle fibers within the week
following injection (Figure 1). Furthermore, the
expression was stable for at least one month following the
injection of the retrovirus. Several tens of fibers were
routinely found to express ß-galactosidase activity in
serial sections, though only a few fibers expressed the
enzyme activity densely throughout the entire
circumference of the fiber. In contrast, the
contralateral control soleus muscle (which received saline
instead of bupivacaine injections and was also injected
with retrovirus) exhibited far less expression of ß-
galactosidase activity. (Table 1). Bupivacaine-damaged
muscle that did not receive injections of retrovirus did
not express ß-galactosidase activity.

Diffuse and Focal Foreign Gene Expression In Vivo

Two patterns of ß-galactosidase expression, diffuse
and focal, were evident. Diffuse expression occurred
within the circumference of a single fiber (FIG. 1, A and
C). Often an adjacent fiber has a less dense expression
of ß-galactosidase activity within the fiber
circumference; this apparent exclusivity of lineage was
not a result of undamaged neighboring fibers because the
phenomenon also occurred when the neighboring fibers were
regenerating myotubes. Focal ß-galactosidase activity

occurred immediately adjacent to the sarcolemma (Fig. 1 B
and D) and occasionally in the intracellular matrix
between fibers. Because this focal expression was
maintained even after 1 month of regeneration, the
5   structures associated with this expression might have been
satellite cells or their daughter myonuclei. However,
because the apparent diffusible domain of a myonucleus
overlapped several adjoining myonuclei, Applicants did not
expect the expression of the ß-galactosidase to be limited
10   to within the myonuclear envelope. Nonetheless, at the
present time Applicants do not have an explanation for the
mechanism underlying the specific patterns and variability
of ß-galactosidase expression in the regenerating skeletal
muscle, and further do not wish to be limited to any such
15   theory of gene expression in the presently described
invention.

Applicants outlined a method to incorporate a single
foreign gene into mammalian adult skeletal muscle that
20   does not risk the possibility of immune rejection inherent
in techniques involving the injection of infected
myoblasts into an animal.[22] The technique introduced here
opens the future possibility for the introduction of
muscle-specific genes into muscle in vivo, followed by
25   studies of altered muscle function and the control of
muscle gene expression.

The general scheme of the technique, i.e.,
stimulating stem cell proliferation followed by retrovirus
30   injection, may provide a means to facilitate gene transfer
into tissues other than muscle tissue. By way of example
and not limitation, it is envisioned that the present
retroviral mediated gene transfer system would be
effective in such tissues as brain, intestinal tissue,
35   cardiac tissue, skin, blood, testicular tissue and uterine
tissue.

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Table 2
ß-galactosidase Expression

	bupivacaine-damaged	control
no. of muscles	8	5
no. of samples	17	14
Scores		
0 (no stain)	0	7
1 (light stain)	1	7
2 (dark stain in serial sections)	16	0
Fisher's 1-tail test on proportions		
no. of successful trials	17*	7

*p<0.05

Differential expression of retrovirally-transfected ß-galactosidase activity in bupivacaine-damaged and undamaged soleus muscle. From each muscle at least one sample of ten to thirty 16 um serial cross-sections were processed for the histochemical detection of ß-galactosidase activity. The degree of ß-galactosidase expression in each sample was scored according to the following system: 0, no activity; 1, occasional light diffuse or focal staining that does not necessarily appear in serial sections; 2, dark diffuse staining within the circumference of one or more fibers in serial sections. The Fisher's 1-tail test on proportions was performed (where scores of 1 and 2 were considered successful trials) and indicates a significantly greater ß-galactosidase expression in the bupivacaine-damaged soleus muscle.

EXAMPLE 3 - IN VIVO RETROVIRAL GENE INCORPORATION IN RAT UTERINE TISSUE

The following experiment was designed to examine the effectiveness of the presently described retroviral-mediated gene transfer system in mitotically-active uterine tissue of adult ovariectomized rats. Adult Sprague-Dawly female rats were ovariectomized under light

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ether anesthesia. The animals were then allowed to
recover for a period of 7 days.

Following ovariectomy, the uterus of each animal was
examined and found to have atrophied to the unstimulated
state. The fully recovered ovariectomized rats with
completely atrophied uterine tissue were then injected
with estrogen at a dose of about 40 micrograms per Kg body
weight (40 ug/mg B.W.). Upon estrogenic stimulation, the
uterine tissue began to regenerate, with the attendant
increase in cell proliferation.

Retroviral vectors including the ß-galactosidase gene
under the promoter control of the Moloney murine leukemia
virus (MuLV) 5' long-terminal repeat were then injected
into the uterine cavity and allowed to bath in the
estrogen-treated uterus.

After a period of 18 hours, the treated animals were
sacrificed. Uterine tissue samples were then fixed and
examined microscopically as described in Example 1.

Results from this study indicate that the
mitotically-active proliferating uterine cells of these
adult female rats readily incorporated the ß-galactosidase
gene of the retroviral vector. This study also suggests
the use of other hormones, such as testosterone,
prostaglandins, prolactin, follicle stimulating hormone
(FSH), human chorionic gonadotrophin (hCG), luteonizing
hormone (LH), insulin, and other stimulatory hormones well
known to those of skill in the art would be useful in the
induction of a mitotically-active state in stimulating
other cell types.

EXAMPLE 4 - PROPOSED STUDY FOR
THE IN VIVO REGENERATION OF DYSTROPHIC MUSCLE IN MICE

5   In this experiment, Applicants propose to demonstrate
the integration and expression of a foreign reporter gene,
ß-galactosidase, in the replication-incompetent MoMuLV,
into the DNA of replicating satellite cells of skeletal
muscle in the dystrophic mouse. Applicants have already
demonstrated that the described integration and expression
10   can be achieved in adult normal rat skeletal muscle
tissue, and therefore expect that the method will be
successful in dystrophic mouse skeletal muscle tissue as
well. This hypothesis is supported in the literature from
observations that the regenerating capacity of young adult
15   dystrophic muscles is almost equal to that of
nondystrophic muscles.[19]

         The ultimate application of the present methods in
supplying dystrophic tissue with a foreign gene is to
20   employ the method as a therapeutic corrective measure for
the treatment of animals with genetic diseases, such as
muscular dystrophy. In such an application, the
retroviral vector will comprise a fragment of a dystrophin
gene on a dystrophin gene construct.

25

         In order to facilitate the inclusion of the required
genetic information into a single retroviral vector, the
dystrophin gene should comprise a dystrophin gene
construct comprising a number of base pairs less than the
30   14,000 base pairs (14 kb) of the native dystrophin gene.
Most preferably, this dystrophin gene construct comprises
between about 10,000 - 8,000 base pairs of the native
dystrophin gene. In a most preferred embodiment, the
dystrophin gene construct comprises about 9,000 base pairs
35   of the native dystrophin gene. This particular gene

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construct comprises a protein having a relative molecular density of 200 K.54.

Recent studies have found that a very mild dystrophic phenotype was manifest in persons having only about 54% of the native dystrophin protein[20]. Thus, it is hypothesized that significant deletions may be made in the native dystrophin gene without a loss in essential biological function of the protein. Such a modified dystrophin gene is proposed for use in the dystrophin gene construct, as the "gene of interest", in the proposed retroviral vector of the presently claimed methods for treating human muscular dystrophy.

PHASE I

The following experiment is designed to determine if the integration and expression of the coding region for the rat insulin-like growth factor-I peptide in fibers of dystrophic muscle of mice will cause their hypertrophy.

Experimental design Groups	Duration with retrovirus	
	30 day	180 day
dy/dy, mice without bupivacaine-retrovirus	8*	8
dy/dy, mice with bupivacaine-retrovirus containing the coding region of insulin-like growth factor I	8	8

*Number of mice

Chronology

Day 0:   Injection of bupivacaine into soleus and anterior tibialis muscles.

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Day 2:   Injection of replication-incompetent MoMuLV with
          insulin-like growth factor I into the 2-day
          Marcaine damaged, recovering muscles.
The treated designated muscles will then be removed and
fixed as described previously.

Retrovirus description and handling

        Applicants subcloned the insulin-like growth factor I
coding region in the EcoRI site in the multiple cloning
region of pT7/T3-18 (Bethesda Research Laboratories). The
Sma I site in the multiple cloning region of pT7/T^3-18 and
IGF-I has been converted to a PvuI by Sigma linker #L2392.
Applicants plan to convert the EcoRI site at the 3' end of
insulin like growth factor I to a BamHI site by usage of
EcoRI-XmnI adaptor and BamHI-XmnI adaptor (New England
Biolabs #1105 and 1106). Thus, the insulin-like growth
factor I coding region will then have a 5' PvuI
restriction site and a 3' BamHI restriction. Recall that
ß-galactosidase was removed by a 5' restriction at a PvuI
site and a 3' restriction at a BamHI site, into which the
insulin-like growth factor I will be subcloned.

        The handling of the retrovirus, animal handling, and
muscle sectioning are as described for Example 2.
However, staining procedures as described for Example 2
will differ because there is no ß-galactosidase gene in
this particular replication-incompetent MoMuLV construct.

        The following analyses will be made on serial
sections of muscles:
1.   Identification of fibers expressing insulin-like
      growth factor I will be made by either:
      a.   In situ hybridization for insulin-like growth
            factor I mRNA, and/or

      b.   Immunohistochemistry for neomycin
          phosphotransferase II.

    2.   Sizes of fibers with positive identification of
       either insulin-like growth factor I mRNA, or neomycin

5   phosphotransferase _vs_ size of fibers without
       expression in of the aforementioned.

     In situ hybridization for insulin-like growth factor
I mRNA will be performed, as described by Edwall, _et al._[25]

10   Applicants have obtained similar hybridization for the
sense and antisense probes for IGF-I and are increasing
the stringency to obtain preferential hybridization with
the muscle IGF-I mRNA. For more specific localization of
hybridization products, Applicants will employ ^{3}H-labeled

15   probes.

     Biotinylated anti-neomycin phosphotransferase II will
be obtained from 5' - 3' (#5307-621261). Applicants are
using biotinylated probes for _in situ_ hybridization so

20   there will be no difficulty with this detection system.

     Neomycin phosphotransferase II is under constitutive
expression by SV 40 early promoter region. Applicants
have a JAVA image-processing software system which they

25   use with a PC Vision Plus Frame Grabber Board in an AT-
clone computer. Applicants use this system to determine
the quantities of _in situ_ hybridization,
immunohistochemistry, and size of muscle fibers within
serial sections.

30

PHASE II

     Retroviral vectors will be prepared which include a
dystrophin gene construct under the constitutive promoter

35   control of the MoLV 5' long-terminal repeat. This
dystrophin gene construct will comprise between about

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10,000 and 8,000 base pairs of the native dystrophin gene, and encode a dystrophin protein having a relative molecular mass of 200,000. This particular gene fragment will be prepared as described by England et al.[20] In a

5   most preferred embodiment, the dystrophin gene construct will comprise about 9,000 base pairs.

Dystrophic muscle of mice will then be treated with bupivacaine, as described above. The tissue will then be

10   exposed to the described dystrophin-construct containing retroviral vector. Excision, fixation and analysis of the treated tissue of the dystrophic animals will then be performed as described in the previous examples.

15   **EXAMPLE 5 - PROPOSED _IN VIVO_ USE IN**
       HUMAN GENE THERAPY OF MUSCULAR DEGENERATIVE DISEASE

The gene encoding human dystrophin has been isolated as a "mammoth" gene, postulated to comprise 2.3 megabases.

20   However, recent reports indicate the isolation of a segment of the native gene which has been demonstrated to impart to human subjects only mild dystrophic phenotypes[19]. Applicants propose the use of this "mini gene" together with the presently described and claimed

25   retroviral mediated gene transfer system as applied to satellite muscle cells for the gene therapy of humans with genetic muscular degenerative diseases. These diseases include, by way of example, Duchenne's and Becker muscular dystrophies.

30

Use of the retroviral-mediated gene transfer systems described by the Applicants will effectively eliminate currently-recognized limitations in the art of gene therapy, such as the risk of immune rejection, diffusion,

35   retroviral gene insert limitations and the requirement for

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multiple site injections (as in the case of myoblast
injection to degenerative human muscle sites).

5   While the determination of exact treatment regimens
for such a therapy await human _in vivo_ clinical trials, it
is hypothesized that the same pharmaceutical agents,
radiation treatments, hormones, and other cell or tissue-
discomposing events would elicit a mitotically-active
state in human tissue and cells as they have in
10   Applicants' experimental rats and mice. Current reviews
suggest the process of muscle regeneration in humans shows
few significant differences from the processes in
mammalian experiments, saving those of scale due to the
large size of human muscles, nor is there currently any
15   reason to believe that the control mechanisms of muscle
regeneration differ to any significant degree[5]. Thus, the
successful gene incorporation accomplished with the
techniques provided and described herein in rats are
expected to be mimicked for the successful gene
20   incorporation in humans.

                              * * *

       The instant invention has been disclosed in
25   connection with specific embodiments. However, it will be
apparent to those skilled in the art that variations from
the illustrated embodiments may be undertaken without
departing the spirit and scope of the invention.

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CLAIMS:

1.   A method for the <u>in vivo</u> incorporation of a foreign
gene into an adult eukaryotic tissue comprising infecting
5   a mitotically-active cell in the adult tissue with a
retroviral vector.

2.   The method of claim 1, wherein the retroviral vector
10   is replication defective.

3.   The method of claim 1, wherein the retroviral vector
comprises a prokaryotic foreign gene.
15

4.   The method of claim 3, wherein the prokaryotic
foreign gene comprises a ß-galactosidase gene.

20
5.   The method of claim 1, wherein the retroviral vector
comprises a eukaryotic foreign gene.

25   6.   The method of claim 5, wherein the eukaryotic foreign
gene is an insulin gene or a dystrophin gene.

7.   A method for incorporating a foreign gene into an
30   adult eukaryotic tissue comprising:

inducing a mitotically-active state in an adult
eukaryotic tissue to produce mitotically-active
eukaryotic cells;
35

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preparing a retroviral vector comprising a foreign
gene of interest; and

exposing the mitotically-active cells to the
retroviral vector for a time period sufficient
to allow incorporation of the foreign gene of
interest in the mitotically- active cells of the
eukaryotic tissue.

8.   The method of claim 7, wherein the foreign gene of
interest is a prokaryotic gene.

9.   The method of claim 8, wherein the prokaryotic gene
is a ß-galactosidase gene.

10.   The method of claim 7, wherein the foreign gene of
interest is a eukaryotic gene.

11.   The method of claim 10, wherein the eukaryotic gene
is an insulin gene, a dystrophin gene, or a spectrin gene.

12.   A method of gene therapy for the replacement of a
defective gene in a eukaryotic tissue comprising:

inducing a mitotically-active state in the eukaryotic
tissue to produce mitotically-active cells;

preparing a retroviral vector comprising a eukaryotic
gene complementary to the defective gene in the
eukaryotic tissue; and

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exposing the mitotically-active cells to the
      retroviral vector for a time period sufficient
      to allow incorporation of the gene of interest
      in the eukaryotic cell,

5

wherein incorporation of the gene of interest replaces the
defective gene in the eukaryotic tissue.

10   13. A method for treating a genetic disease in a
eukaryote comprising incorporating a complementary gene to
a genetic deficiency in a stem cell of the eukaryote,
wherein incorporation of the complementary gene comprises
the steps of:

15

inducing a mitotically-active state in the eukaryote
      to form a mitotically-active preparation of
      eukaryotic stem cells;

20   preparing a retroviral vector comprising a
      complementary gene to the genetic deficiency in
      the eukaryote;

exposing the mitotically-active preparation of
25   eukaryotic stem cells to the retroviral vector;
      and

incubating the retroviral vector with the
      mitotically-active preparation of eukaryotic
30   stem cells a time period sufficient for
      incorporation of the complementary gene into the
      eukaryotic stem cell.

35   14. The method of claim 1, 7, or 12, wherein the
eukaryotic tissue is human, mouse, or rat tissue.

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15.   The method of claim 1, 7, or 12, wherein the
eukaryotic tissue comprises skeletal muscle, cardiac
muscle, smooth muscle, brain, gastrointestinal,
5   testicular, blood, skin or uterine tissue.

16.   The method of claim 1, 7, or 12, wherein inducing a
mitotically-active state comprises inducing cellular-
10   repair mechanisms in the cell.

17.   The method of claim 15 wherein, the induction of a
mitotically-active state comprises: discomposing the
15   eukaryotic cell, exposing the eukaryotic cell to
radiation, or administering a pharmaceutical agent
selected from the group consisting of collagenase,
fibroblast growth factor, bupivacaine, estrogen and
dexamethasone.
20

18.   The method of claim 7 or 12, wherein the eukaryotic
tissue is skeletal muscle.

25

19.   The method of claim 13, wherein the genetic disease
comprises muscular dystrophy, diabetes or albinism.

30   20.   The method of claim 13, wherein the genetic disease
is muscular dystrophy.

21.   The method of claim 20, wherein the muscular
35   dystrophy is Duchenne's muscular dystrophy or Becker
muscular dystrophy.

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22. The method of claim 20, wherein the foreign gene comprises a dystrophin gene or a spectrin gene.

23. The method of claim 20, wherein the foreign gene comprises a dystrophin gene construct.

24. The method of claim 23, wherein the dystrophin gene construct is a modified dystrophin gene having between about 7,000 - 10,000 base pairs.

25. The method of claim 24, wherein the dystrophin gene construct comprises about 9,000 base pairs.

26. The method of claim 24, wherein the dystrophin gene construct encodes a protein comprising a relative molecular mass of about 200,000 Kd.

27. The method of claim 20, wherein the induction of a mitotically-active state comprises administering bupivacaine to the skeletal muscle tissue.

28. The method of claim 1, 7 or 12, wherein the retroviral vector comprises a murine leukemia virus.

29. The method of claim 28, wherein the murine leukemia virus comprises AKR, Moloney, or Friend murine leukemia virus.

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30. The method of claim 29, wherein the murine leukemia virus is Moloney murine leukemia virus.

5   31. The method of claim 30 wherein Moloney murine leukemia retroviral vector comprises a ß-galactosidase gene.

10   32. The method of claim 30, wherein the ß-galactosidase gene is under the control of an <u>E. coli</u> long-terminal repeat promoter region.

15   33. The method of claim 1, 7, or 12, wherein the retroviral vector is conjugated to a protein.
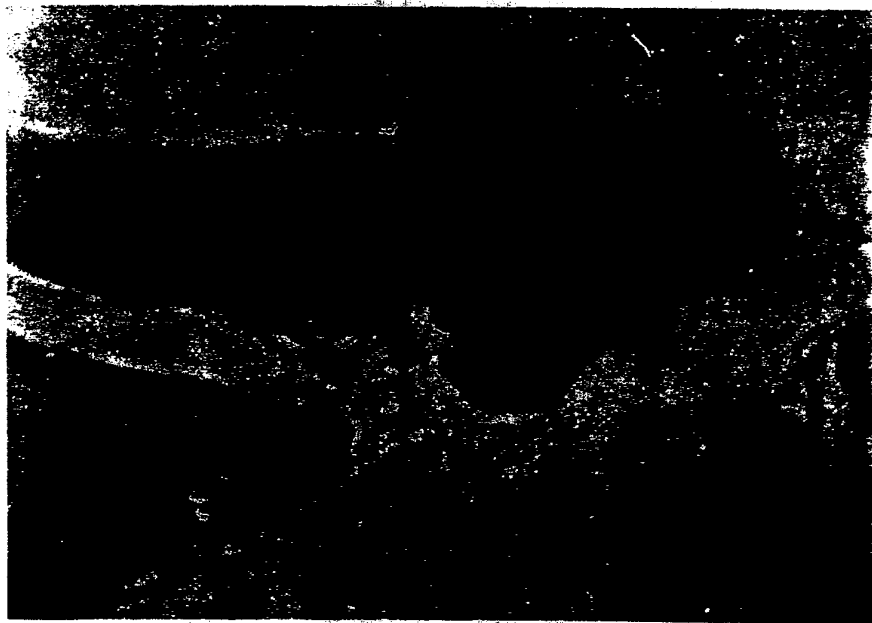
FIG.1A

FIG.1B

FIG.1C

FIG.1D