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(54) Benævnelse: **Telomerase revers transkriptase-baserede terapier til behandling af tilstande associeret med myokardieinfarkt**

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DESCRIPTION

FIELD OF INVENTION

[0001] This invention falls within the field of molecular biology, biotechnology and medicine. More particularly, it relates to nucleic acid vectors for use in the treatment of myocardial infarction.

BACKGROUND OF THE INVENTION

[0002] Telomeres are specialized structures at the ends of chromosomes, which have a role in protecting the chromosome ends from DNA repair and degrading activities (10; 11). Mammalian telomeres consist of TTAGGG repeats bound by a multi-protein complex known as shelterin (11). A minimum length of TTAGGG repeats and the integrity of the shelterin complex are necessary for telomere protection (10; 11). Telomerase is a cellular reverse transcriptase (TERT, telomerase reverse transcriptase; also known as TP2; TRT; EST2; TCS1; hEST2) capable of compensating telomere attrition through de novo addition of TTAGGG repeats onto the chromosome ends by using an associated R A component as template (Terc, telomerase RNA component) (12). Telomerase is expressed in most adult stem cell compartments, however, this is not sufficient to maintain telomere length as evidenced by the fact that telomere shortening occurs with age in most human and mouse tissues (15; 61; 14).

[0003] Mice carrying homozygous deletion for the TERC gene (the telomerase RNA component) lack any detectable telomerase activity and showed progressive telomere shortening from one generation to the other at a rate comparable to the rate reported in human cells (65). Severe phenotypes typical of late generation TERC-/- mice (e.g. bone marrow aplasia and signs of premature aging) could be rescued by re-introducing a copy of the TERC gene (86). Multiple tissue degeneration arising in later generations in a conditional mouse model defective for TERT (the catalytic telomerase subunit) could be reversed upon telomerase reactivation even in aged mice (40).

[0004] In the context of wild-type mice, introducing an additional copy of the telomerase gene, which is expressed in a wide range of epithelial tissues, led to an increased wound healing capacity of the skin (66). When this allele was introduced in a tumour-resistant genetic background (Sp53/Sp16/SArf) remarkable delay of aging in concert with an increased median lifespan of 40% compared to mice not expressing the telomerase transgene was observed (24).

[0005] A virus (AAV) based telomerase gene therapy was found to be beneficial to extend health span, in the context of normal physiological aging in wild-type mice. In the study examining this benefit, adult and aged mice were subjected to AAV9-mTERT gene therapy to

broadly express the catalytic subunit of mouse telomerase (mTERT). The health span of the TERT treated mice was significantly increased, and aging was decelerated, as indicated by a number of physiological parameters (glucose and insulin tolerance, osteoporosis, neuromuscular coordination, rota-rod, etc). In addition, their mean lifespan, compared to control groups, was increased by 24% and 13% in adult an old mice, respectively. A single intravenous administration of AAV9-TERT in adult mice resulted in an increase in telomere length in peripheral blood cells (26).

[0006] In the case of cardiovascular diseases (CVD), short telomeres have been linked to cardiac dysfunction both in mice and humans (63, 19, 20, 21, 64). In particular, mice with critically short telomeres owing to telomerase deficiency develop cardiomyopathy characterized impaired cell division, enhanced cardiomyocyte death and cellular hypertrophy, which are concomitant with ventricular dilation, thinning of the wall and cardiac dysfunction (19). Interestingly, analogous to the loss of the full regeneration capacity of the heart, expression of the telomerase essential genes Terc and Tert is lost within the first week of postnatal life (22, 23).

[0007] Heart failure is among the most common causes of mortality and morbidity worldwide, and its prevalence continues to increase. In spite of new therapies, cardiac remodeling and subsequent heart failure remain critical issues after myocardial infarction (1), highlighting the urgency of developing new therapeutic strategies.

SUMMARY

[0008] The present invention provides a nucleic acid vector comprising a coding sequence for telomerase reverse transcriptase (TERT) for use in treating myocardial infarction, wherein the vector in an adeno-associated virus-based non-integrative vector and wherein

1. a) TERT is encoded by
    1. i) a nucleic acid sequence comprising the sequence of SEQ ID NO:1 or SEQ ID NO:3, or
    2. ii) a nucleic acid which encodes a polypeptide that has 50% or more TERT activity compared to TERT encoded by SEQ ID NO:1 or SEQ ID NO:3, and which has at least 75% identity to SEQ ID NO:1 or SEQ ID NO:3, or
    3. iii) a nucleic acid sequence which is a fragment of SEQ ID NO:1 or SEQ ID NO:3 that has TERT activity and which has at least 75% identity to the sequence of SEQ ID NO:1 or SEQ ID NO:3 over the entire length of the fragment;

or

b) TERT comprises

1. i) an amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, or
2. ii) a polypeptide which displays 50% or more TERT activity compared to TERT of SEQ ID NO:2 or SEQ ID NO:4 and which has at least 75% identity to SEQ ID NO:2 or SEQ ID

NO:4, or

3. iii) a polypeptide which is a fragment of SEQ ID NO:2 or SEQ ID NO:4 that has TERT activity and which has at least 75% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:4 over the entire length of the fragment.

[0009] In one embodiment, the TERT is encoded by a nucleic acid sequence comprising a sequence that is at least 75% identical to the sequence of SEQ ID NO: 1 or SEQ ID NO: 3. In one embodiment, the TERT is encoded by a nucleic acid sequence comprising the sequence of SEQ ID NO: 1 or SEQ ID NO: 3. In one embodiment, the TERT is encoded by a nucleic acid sequence consisting of the sequence of SEQ ID NO: 1 or SEQ ID NO: 3 In one embodiment, the TERT comprises an amino acid sequence that is at least 75% identical to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4. In one embodiment, the TERT comprises an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4. In one embodiment, the TERT consists of the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4. In one embodiment, the nucleic acid sequence encoding TERT is operably linked to a regulatory sequence that drives the expression of the coding sequence. The vector is a non-integrative vector, an adeno-associated virus-based non-integrative vector. In one embodiment, the vector is an adeno-associated virus-based vector derived from a serotype 9 adeno-associated virus (AAV9). In one embodiment, the capsid of the adeno-associated virus-based vector is made of capsid proteins of the serotype 9 adeno-associated virus (AAV9), and the nucleic acid sequence contained in the capsid is flanked at both ends by internal terminal repeats corresponding to serotype 2 adenoassociated viruses. In one embodiment, the nucleic acid contained in the capsid comprises a fragment which encodes the amino acid sequence coding for TERT. In one embodiment, the vector comprises a regulatory sequence which is a constitutive promoter. In one embodiment, the regulatory sequence is the cytomegalovirus (CMV) promoter.

[0010] In one embodiment, the vector is for use being administered directly to the cardiac tissue.

[0011] In certain embodiments, the patient treated has previously suffered a myocardial infarction.

[0012] Additionally to the invention, the present application also describes compositions and approaches useful for the treatment and prevention of conditions associated with myocardial infarction. In certain cases, the condition associated with myocardial infarction is selected from the group consisting of a myocardial infarction, tissue damage resulting from myocardial infarction, fibrosis of the myocardium resulting from myocardial infarction, and reduced cardiac function resulting from myocardial infarction.

[0013] The present application also describes an approach for promoting myocardial tissue regeneration in a patient in need thereof comprising a nucleic acid vector comprising a coding

sequence for telomerase reverse transcriptase (TERT) for use for that purpose

[0014] The present application also describes an approach for reducing fibrosis of the myocardium in a patient in need thereof comprising a nucleic acid vector comprising a coding sequence for telomerase reverse transcriptase (TERT) for use for that purpose

[0015] The present application also describes an approach for improving cardiac function in a patient in need thereof comprising a nucleic acid vector comprising a coding sequence for telomerase reverse transcriptase (TERT) for use for that purpose

[0016] The present application also describes an approach for preventing myocardial infarction in a patient in need thereof comprising a nucleic acid vector comprising a coding sequence for telomerase reverse transcriptase (TERT) for use for that purpose

[0017] The present application also describes an approach for promoting cardiomyocyte proliferation in a patient in need thereof comprising a nucleic acid vector comprising a coding sequence for telomerase reverse transcriptase (TERT) for use for that purpose

BRIEF DESCRIPTION OF THE FIGURES

[0018]

Figure 1: Ratio of viral genomes (AAV9-Tert and AAV9-eGFP) per diploid genome of analysed heart tissue sample. Deviation is shown in brackets.

Figure 2: Telomerase expression reduces lethal heart failure after M1 and rescues cardiac function parameters. A) Kaplan-Meier survival curves after MI indicating 17% improved survival after Tert treatment and MI. Statistical analysis was calculated with log-rank test. No sham operated animal died. B-F) Echocardiography at 1 and 3 weeks in mice injected with AAV9-Tert, AAV9-empty or no virus (all MI); or sham mice (no virus, no MI) reveals functional improvement in the Tert group. B) LV endsystolic area, C) LV end-diastolic area, D) ejection fraction, E) longitudinal wall thickness, F) transversal wall thickness in the indicated number of mice was determined (E and F only at 1 week post MI).

Figure 3: FDG-PET scan reveals reduced infarct severity in AAV9-Tert treated mice and coincides with smaller fibrotic scar sizes and less interstitial fibrosis after MI. A) Percentage of AAV9-empty or AAV9-Tert mice that died during or shortly after PET scan. B) Infarct severity in AAV9-Tert and AAV9-empty cohorts was assessed in in vivo FDG-PET scans. A higher number of hearts with signal loss was found in the AAV9-empty group. C) Representative PET scan images of a fully functional heart (left) and failing hearts after MI (right). D) Average scar length relative to the LV endocardial circumference of indicated groups. E) Interstitial fibrotic area relative to total area measured in the infarct remote myocardium. Number of animals and statistical analysis (student's t-test) are depicted.

Figure 4: Telomerase expression rescues serum parameters associated with heart failure. Serum urea concentration as a measure of renal and heart functionality was determined in the indicated groups.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

[0019] The present application, additionally to the invention, describes compositions and approaches useful for the treatment and prevention of conditions associated with myocardial infarction.

[0020] A "condition associated with myocardial infarction" includes myocardial infarction events, tissue damage resulting from myocardial infarction (including loss of cardiomyocytes), fibrosis of the myocardium resulting from myocardial infarction, reduced cardiac function resulting from myocardial infarction,

[0021] As shown in the Examples, treatment of adult mouse heart with the telomerase gene therapy vector described in the application has beneficial effects following M1 by aiding heart regeneration capacity, decreasing fibrosis, and significantly increasing survival time. Treatment with the telomerase gene therapy vector increase telomerase activation in the heart which improves cardiac functional and morphological parameters and significantly reduces heart failure mortality after MI. The treatment rescues gene expression changes associated to the failing heart, including changes in metabolism, and expression of fetal genes.

[0022] Accordingly, the invention provides a nucleic acid vector comprising a coding sequence for telomerase reverse transcriptase (TERT) for use in treating myocardial infarction, wherein the vector is an adenoassociated virus-based non-integrative vector, that is, an agent which increases the telomere length of the patient. In one embodiment, the agent prevents degradation of the chromosomal ends. In one embodiment, the agent increases the activity of telomerase reverse transcriptase (TERT).

[0023] In certain approaches, the TERT sequence used in the gene therapy vector is derived from the same species as the subject. For example, gene therapy in humans would be carried out using the human TERT sequence. Gene therapy in mice would be carried out using the mouse TERT sequence, as described in the examples. In one embodiment, the TERT is encoded by the nucleic acid sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 3 (human TERT variants 1 and 2), or is an active fragment or functional equivalent of SEQ ID NO: 1 or SEQ ID NO: 3. The polypeptide sequence encoded by SEQ ID NO: 1 is set forth in SEQ ID NO: 2. The polypeptide encoded by SEQ ID NO: 3 is set forth in SEQ ID NO: 4. As used herein, "functional equivalent" refers to a nucleic acid molecule that encodes a polypeptide that has TERT activity or a polypeptide that has TERT activity. The functional equivalent may displays 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, 100% or more activity compared to TERT

encoded by SEQ ID NO: 1 or SEQ ID NO: 3. Functional equivalents may be artificial or naturally-occurring. For example, naturally- occurring variants of the TERT sequence in a population fall within the scope of functional equivalent. TERT sequences derived from other species also fall within the scope of the term "functional equivalent", in particular the murine TERT sequence given in SEQ ID NO: 5. In a particular embodiment, the functional equivalent is a nucleic acid with a nucleotide sequence having at least 75%, 80%>, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% identity to SEQ ID NO: 1 or SEQ ID NO: 3. In a further embodiment, the functional equivalent is a polypeptide with an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% identity to SEQ ID NO: 2 or SEQ ID NO: 4. In the case of functional equivalents, sequence identity should be calculated along the entire length of the nucleic acid. Functional equivalents may contain one or more, e.g. 2, 3, 4, 5, 10, 15, 20, 30 or more, nucleotide insertions, deletions and/or substitutions when compared to SEQ ID NO: 1 or SEQ ID NO: 3. The term "functional equivalent" also encompasses nucleic acid sequences that encode a TERT polypeptide with at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% sequence identity to the sequence as set forth in SEQ ID NO:2 or SEQ ID NO: 4, but that show little homology to the nucleic acid sequence given in SEQ ID NO: 1 or SEQ ID NO: 3 because of the degeneracy of the genetic code.

[0024] As used herein, the term "active fragment" refers to a nucleic acid molecule that encodes a polypeptide that has TERT activity or polypeptide that has TERT activity, but which is a fragment of the nucleic acid as set forth in SEQ ID NO: 1 or SEQ ID NO: 3 or the amino acid sequence as set forth in SEQ ID NO: 2 or SEQ ID NO: 4. An active fragment may be of any size provided that TERT activity is retained. A fragment will have at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 100% identity to SEQ ID NO: 1-4 along the length of the alignment between the shorter fragment and SEQ ID NO: 1-4.

[0025] Fusion proteins including these fragments can be comprised in the nucleic acid vectors needed to carry out the invention. For example, an additional 5, 10, 20, 30, 40, 50 or even 100 amino acid residues from the polypeptide sequence, or from a homologous sequence, may be included at either or both the C terminal and/or N terminus without prejudicing the ability of the polypeptide fragment to fold correctly and exhibit biological activity.

[0026] Sequence identity may be calculated by any one of the various methods in the art, including for example BLAST (Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). "Basic local alignment search tool". J Mol Biol 215 (3): 403-410) and FASTA (Lipman, DJ; Pearson, WR (1985). "Rapid and sensitive protein similarity searches". Science 227 (4693): 1435-41; http ://fasta.bioch. Virginia, edu/ fasta_www2/ fasta_list2. shtml) and variations on these alignment programs.

[0027] In one approach, the nucleic acid vector for use according to the invention is a gene therapy vector. Gene therapy methods and vectors are well known in the art and generally comprise delivering a nucleic acid encoding a therapeutically active protein to a subject. In the art, the nucleic acid may be delivered in a number of ways including delivering naked DNA

such as plasmid or mini-circles, the use of liposomes or cationic polymers or other engineered nano-particles containing the nucleic acid, or viral vectors that encapsidate the nucleic acid.

[0028] In the art, the gene therapy can be achieved using stable transformation of organisms with an inducible expression system. Suitable inducible expression systems are known in the art and include the CRE-LOX recombinase based system which is suitable for use in mice and tetracycline-regulated which can be used in the treatment of human subjects.

[0029] The gene therapy vector for use according to the invention is a viral vector: an adeno-associated virus-based non-integrative vector. Viral gene therapy vectors are well known in the art. Vectors of the art include integrative and non-integrative vectors such as those based on retroviruses, adenoviruses (AdV), adeno-associated viruses (AAV), lentiviruses, pox viruses, alphaviruses, and herpes viruses.

[0030] Using non-integrative viral vectors, such as AAV, seems to be particularly advantageous. First, this is because non-integrative vectors do not cause any permanent genetic modification. Second, the vectors target to adult tissues, avoiding having the subjects under the effect of constitutive telomerase expression from early stages of development. Additionally, non-integrative vectors effectively incorporate a safety mechanism to avoid over-proliferation of TERT expressing cells. Cells will lose the vector (and, as a consequence, the telomerase expression) if they start proliferating quickly.

[0031] Particular examples of suitable non-integrative vectors of the art include those based on adenoviruses (AdV) in particular gutless adenoviruses, adeno-associated viruses (AAV), integrase deficient lentiviruses, pox viruses, alphaviruses, and herpes viruses. The non-integrative vector used in the invention is an adeno-associated virus-based non-integrative vector, similar to natural adeno-associated virus particles. AAV preferentially targets post-mitotic tissues, which are considered more resistant to cancer than the highly proliferative ones. Examples of adeno-associated virus-based non integrative vectors include vectors based on any AAV serotype, i.e. AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 and pseudotyped AAV. Tissue specificity is determined by the capsid serotype. Pseudotyping of AAV vectors and capsid engineering to alter their tropism range will likely be important to their use in therapy.

[0032] Vectors derived from adeno-associated viruses (AAVs) have emerged as one of the vectors of choice for many gene transfer applications because of their many desirable properties, including capability to transduce a broad range of tissues at high efficiency, poor immunogenicity and an excellent safety profile (67; 68), toxicity being absent in many preclinical models (69; 70; 71; 72; 73). AAV vectors transduce post-mitotic cells and can sustain long-term gene expression (up to several years) both in small and large animal models of disease (69; 70; 71; 72; 73). Safety and efficacy of AAV gene transfer has been extensively studied in humans with encouraging results in the liver, muscle, CNS, and retina (74, 75, 76; 77; 78).

[0033] AAV2 is the best characterized serotype for gene transfer studies both in humans and experimental models. AAV2 presents natural tropism towards skeletal muscles, neurons, vascular smooth muscle cells and hepatocytes. AAV2 is therefore a good choice of vector to target these tissues, in particular when using the vectors for use according to the invention to treat a condition associated with one of these tissues. For example, treatment of neuromuscular degeneration may be targeted to skeletal muscle and/or neurons in this way.

[0034] Newly isolated serotypes, such as AAV7, AAV8, and AAV9 have been successfully adopted in preclinical studies (28). Although limited immunologic responses have been detected in human subjects treated with AAV2 or AAV1 against the AAV capsid (74; 79; 80; 81), long term expression of the therapeutic gene is possible depending on the target tissue and the route of administration (80; 82). In addition, the use of non-human serotypes, like AAV8 and AAV9, might be useful to overcome these immunological responses in subjects, and clinical trials have just commenced (ClinicalTrials.gov Identifier: NCT00979238). Altogether, these encouraging data suggest that AAV vectors are useful tools to treat human diseases with a high safety and efficient profile.

[0035] The choice of adeno-associated viruses of wide tropism, such as those derived from serotype 9 adenoassociated virus (AAV9) is particularly advantageous when treating conditions associated with short telomere length. AAV9 viruses have shown efficient transduction in a broad range of tissues, with high tropism for liver, heart and skeletal muscle (83) and thus the beneficial effects of gene therapy can be achieved in more tissues. In addition, AAV9 vectors have the unique ability to cross the blood-brain-barrier and target the brain upon intravenous injection in adult mice and cats (84; 85).

[0036] One aspect of the invention provides a system in which the capsid (which is the part of the virus which determines the virus tropism) of the adeno-associated virus- based vector is made of capsid proteins of the serotype 9 adeno-associated virus (AAV9). In one embodiment of the viral vectors for use in the invention, the polynucleotide sequence packed in the capsid is flanked by internal terminal repeats (ITRs) of an adeno-associated virus of serotype 2 which has been extensively characterised in the art, and presents a coding sequence located between the ITRs. As set out above, the nucleic acid preferably codes for a functional TERT polypeptide. In one embodiment, the regulatory sequence operatively linked to the TERT coding sequence is the cytomegalovirus promoter (CMV), although other suitable regulatory sequences will be known to those of skill in the art.

[0037] When treating conditions associated with short telomere length, it is advantageous to target the treatment to the effected tissues. The choice of AAV serotype for the capsid protein of the gene therapy vector may be thus based on the desired site of gene therapy. If the target tissue is skeletal muscle, for example, in treating loss of neuromuscular coordination, AAV1- and AAV6-based viral vectors can be used. Both of these serotypes are more efficient at transfecting muscle than other AAV serotypes. AAV3 is useful for transfecting haematopoietic cells. A thorough review of AAV-based vectors for gene therapy can be found in Shi et al, (2008) "AAV-based targeting gene therapy" Am. J. Immunol. 4:51- 65.

[0038] Alternatively, other viral vectors can be used for the approaches described herein. Any vector compatible with use in gene therapy can be used for the approaches described herein. Heilbronn & Weger (2010) Handb Exp Pharmacol. 197: 143-70 provides a review of viral vectors that are useful in gene therapy. In accordance with all the previous discussion, vectors comprising a coding sequence for telomerase reverse transcriptase (TERT) suitable for use in gene therapy are an important point for putting the invention into practice. Suitable gene therapy vectors include any kind of particle that comprises a polynucleotide fragment encoding the telomerase reverse transcriptase (TERT) protein, operably linked to a regulatory element such as a promoter, which allows the expression of a functional TERT protein demonstrating telomerase reverse transcriptase activity in the targeted cells. Preferably, TERT is encoded by the nucleic acid sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 3, or is an active fragment or functional equivalent of TERT. The term gene therapy vector, as used in the present application, includes within its scope naked DNA molecules such as plasmids or mini-circles, i.e. circular DNA molecules which do not contain bacterial DNA sequences, provided that the TERT coding sequence and its linked regulatory element are inserted in the plasmid, as well as to more complicated systems, such as particles with the structure of virions (viral particles), comprising at least a capsid and at least a polynucleotide sequence, with a size that allows the polynucleotide sequence to be packed within the capsid in a manner similar to that of the native genome of the virus of origin of the capsid. The polynucleotide sequence must include a region where the TERT coding sequence and its linked regulatory element are inserted such that the telomerase reverse transcriptase protein can be expressed from that polynucleotide sequence once the viral particle has infected a cell.

[0039] The gene therapy vector for being used in the invention is a non- integrative vector: an adeno-associated virus-based non-integrative vector. For the purposes of the invention, the choice of non-integrative vectors seems to be particularly advantageous, because they do not cause any permanent genetic modification. Also, as stated before, such vectors incorporate a safety mechanism to avoid over-proliferation of TERT expressing cells that will lose the vector if the cells start proliferating quickly. Adeno-associated virus-based vectors derived from a serotype 9 adeno-associated virus (AAV9) are preferred because the beneficial effects can be achieved in more tissues (see above). In one particularly preferred embodiment, the regulatory sequence operatively linked to the TERT coding sequence is the cytomegalovirus promoter (CMV). The nucleic acid sequence encoding TERT is operably linked to a regulatory sequence that drives the expression of the coding sequence. As used herein, the term "regulatory element" means a nucleic acid sequence that serves as a promoter, i.e., regulates expression of a nucleic acid sequence operably linked to the promoter. Such "regulatory elements" or "promoters" can control the expression of linked nucleic acid sequences either constitutively or inducible.

[0040] The regulatory sequence may be a constitutive promoter. An example of a regulatory sequence which is a constitutive promoter is the cytomegalovirus (CMV) promoter.

[0041] The expression of TERT following gene therapy, as shown in Examples of the present

application, persists for a time of several months to several years. In mice, TERT expression was detectable after 5 months. In monkey, gene expression following gene therapy with an AAV- based vector has been detected up to 6 years after treatment and up to 8 years in dogs (Rivera et al Blood 2005, and 69). Frequent repetition of administration of the vectors for use according to the invention is therefore not necessary. In one approach compatible with the invention, the vector is for use in treating a subject once. In an alternative compatible approach, the vector is for use in treating a subject initially, and in treating the subject then again once TERT expression levels decrease by about 50% of those attained immediately following treatment. Treatment may be repeated with the same or alternative vector to maintain the reduction in age-related disorders if necessary, for example annually, or once every 5 years or once a decade. When administering a second or subsequent dose, it may be necessary to use a different gene therapy vector, for example when using an AAV-based vector the second and subsequent administrations may be a vector with a capsid derived from a different serotype than that used for the first administration. It is possible that a subject may develop neutralising antibodies to the first gene therapy vector, making it ineffective if administered a second or subsequent time (Amado et al (2010) Science Translational Medicine 2(21):21ral6).

[0042] In particular embodiments, the gene therapy vector is for use being administered to a patient after that patient has suffered a myocardial infarction. It is preferable to administer the vector as soon as possible after the myocardial infarction, for example within twelve hours, twenty-four hours, thirty-six hours, forty-eight hours, sixty hours, seventy-two hours, or eighty-four hours after the myocardial infarction event.

[0043] In the treatment of a condition associated with myocardial infarction, it is preferable to use the AAV9 vector. While this vector has a wide tropism, it preferentially transduces cardiac tissue, thereby allowing use of a low dose of the vector resulting in minimal transduction of non-cardiac tissue.

[0044] In one embodiment of the invention, the vector is for use being administered directly into the cardiac tissue, further reducing the potential of cross-reactivity of other tissues. Direct administration to the cardiac tissue is also advantageous as it allows for a lower dose of vector to be effective in the treatment. In one embodiment of the invention, the vector is for use being administered directly to the cardiac tissue in a dose which is effective to transduce cardiac tissue with minimal transduction to liver, brain, or other non-cardiac tissue.

[0045] As demonstrated in the Examples, treatment of adult mouse heart with the gene therapy vector for use according to the invention improves cardiac functional and morphological parameters and significantly reduces heart failure mortality after a myocardial infarction event. Specific beneficial effects of the gene therapy treatment following MI included promotion of cardiac tissue regeneration capacity, decreased fibrosis, and significantly increased survival time of the treated mice.

[0046] Importantly, the treatment rescued gene expression changes associated with a failing

heart, including changes in metabolism, and expression of fetal genes. Moreover, sustained TGFβ-signaling and matrix remodeling was found in the treated heart as was induction of cardio-protective pathways such as the EGF pathway. Gene expression changes induced by the treatment are enriched in the regenerative gene expression signature described in neonatal mice, which have a full regenerative potential.

[0047] Accordingly, the approaches described herein have the effect of treating and/or preventing conditions associated with myocardial infarction. Described herein is the use of a nucleic acid vector as described above, for use in the treatment or prevention in a patient of a condition associated with myocardial infarction, including but not limited to myocardial infarction events, tissue damage resulting from myocardial infarction (including loss of cardiomyocytes), fibrosis of the myocardium resulting from myocardial infarction, reduced cardiac function resulting from myocardial infarction.

[0048] As it is disclosed herein, the gene therapy results in an increased lifespan of the treated patient, based on the prevention or delay of a myocardial infarction. As it is disclosed herein, the gene therapy prevents or reduces fibrosis of the cardiac tissue. As it is disclosed herein, the gene therapy improves cardiac function. As it is disclosed herein, the gene therapy promotes cardiac tissue regeneration. As it is disclosed herein, the gene therapy promotes cardiomyocyte proliferation. In particular embodiments, the patient administered the gene therapy has suffered a previous myocardial infarction event.

[0049] The effectiveness of treatment of the conditions associated with myocardial infarction can be measured by various methods known in the art. As it is disclosed herein, the effectiveness of the treatment can be measured by an increase in lifespan of a treated patient suffering from a condition associated with myocardial infarction as compared to the expected lifespan of an untreated patient suffering from the same condition. The lifespan may be extended by 5%, 10%, 15%, 20%, 30%, 40%, 50%, or more, with reference to the expected lifespan for a patient suffering from the same condition.

[0050] The gene therapy treatment is useful in preventing or delaying the occurrence of a myocardial event in a patient who has already suffered a previous myocardial event. As it is disclosed herein, the effectiveness of the treatment can be measured by a delayed onset of a subsequent myocardial infarction after a prior myocardial infarction. The delay in the onset of a subsequent myocardial infarction of a treated patient may be extended by 5%, 10%, 15%, 20%, 30%, 40%, 50%, or more, with reference to the expected timing of subsequent myocardial infarction for an untreated patient.

[0051] As disclosed herein, the effectiveness of the treatment can be measured by a reduction in fibrosis of the myocardium resulting from myocardial infarction. The reduction in fibrosis of the myocardium resulting from myocardial infarction may be reduced by 5%, 10%, 15%, 20%, 30%, 40%, 50%, or more, with reference to the fibrosis in the myocardium prior to the treatment. Fibrosis can be measured using various techniques known in the art. Fibrosis can be measured by a pathological analysis, such as determination of fibrotic areas (scar) using

Masson's trichrome staining.

[0052] As it is disclosed herein, the effectiveness of the treatment can be measured by an increase in cardiac function following myocardial infarction. The cardiac function following myocardial infarction may be increased by 5%, 10%, 15%, 20%, 30%, 40%, 50%, or more, with reference to the level of cardiac function prior to treatment. Cardiac function can be measured using various techniques known in the art. As it is described herein, cardiac function can be measured using echocardiography. As it is described herein, cardiac function can be measured using Micro-PET Imaging. As it is described herein, cardiac function can be also measured based on kidney function, wherein a reduction in kidney function in a patient indicates a reduction in cardiac function in the patient. As it is described herein, cardiac function can be also measured based on the urea level in urine, wherein a reduction in urea level in the urine of a patient indicates a reduction in cardiac function in the patient.

[0053] The efficacy of the treatment can also be measured by directly determining telomere length in sample taken from the patient. Telomere length can be measured, for example, by using standard hybridization techniques, such as fluorescence in situ hybridization (FISH), Quantitative Fluorescent in situ hybridization (Q-FISH), or High Throughput Quantitative Fluorescent in situ hybridization (HT Q-FISH). (66). Telomere length can also be measured as described in Canela et al. (56).

[0054] In a particular approach, samples are taken from the patient undergoing treatment throughout the course of the treatment so that both absolute telomere length and the rate of telomere lengthening or shortening over the course of treatment can be determined. Samples may be taken every day during the course of treatment, or at longer intervals. In one approach, samples are taken once a week, once every two weeks, once every three weeks, once every 4 weeks, once every five weeks, once every six weeks or longer.

[0055] Comparison of telomere length can be measured by comparing the proportion of short telomeres in a sample taken from a patient. In one approach, the proportion of short telomeres is the fraction of telomeres presenting an intensity below the mean intensity of the sample as measured by a in situ hybridization technique, such as FISH or Q-FISH. In one approach, the proportion of short telomeres is the fraction of telomeres presenting an intensity 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40% or more below the mean intensity of the sample. In one particular approach, the proportion of short telomeres is the fraction of telomeres presenting an intensity 50% or more below the mean intensity of the sample.

[0056] In another approach, the proportion of short telomeres is the fraction of telomeres below a certain length, e.g. 8 kb, 7 kb, 6 kb, 5 kb, or shorter. In one approach, the proportion of short telomeres is the fraction of telomeres 8 kb or shorter . In another approach, the proportion of short telomeres is the fraction of telomeres 7 kb or shorter. In another approach, the proportion of short telomeres is the fraction of telomeres 6 kb or shorter. In another approach, the proportion of short telomeres is the fraction of telomeres 5 kb or shorter. In another approach, the proportion of short telomeres is the fraction of telomeres 4 kb or

shorter. In another approach, the proportion of short telomeres is the fraction of telomeres 3 kb or shorter.

[0057] In one approach, the effectiveness of the treatment is measured by a decrease in the proportion of short telomeres in sample taken from a treated patient suffering from a condition associated with myocardial infarction as compared to a control sample. In one approach, the proportion of short telomeres in a sample taken from a treated patient is decreased by 10%, 20%, 30%, 40%, 50%, 60%, 70%, or greater as compared to a control sample. In one approach, the control sample is a sample taken from the same patient prior to the treatment, or taken at an earlier stage of the treatment. In another embodiment, the control sample is a sample taken from a patient suffering from the same condition and not provided the treatment.

[0058] In a further approach, the invention is applied to the subject by administering a pharmaceutical composition comprising an effective amount of any one of the gene therapy vectors compatible with the invention described above.

[0059] A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use in vitro, in vivo or ex vivo.

[0060] "Composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active. An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

[0061] They will usually include components in addition to the active component (such as the gene therapy vector) e.g. they typically include one or more pharmaceutical carrier(s) and/or excipient(s). A thorough discussion of such components is available in Gennaro (2000) Remington: The Science and Practice of Pharmacy. 20th edition, ISBN: 0683306472.

[0062] Compositions will generally be administered to a subject in aqueous form. Prior to administration, however, the composition may have been in a non-aqueous form. For instance, although some viral vectors are manufactured in aqueous form, then filled and distributed and administered also in aqueous form, other viral vectors are lyophilised during manufacture and are reconstituted into an aqueous form at the time of use. Thus a composition compatible with the invention may be dried, such as a lyophilised formulation. The composition may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the composition should be substantially free from (i.e. less than 5µg/ml) mercurial material e.g. thiomersal-free.

[0063] To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml e.g. about 10+2mg/ml NaCl. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride,

etc.

[0064] Compositions will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg.

[0065] Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer (particularly with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the 5-20mM range.

[0066] The composition may include material for a single administration, or may include material for multiple administrations (i.e. a 'multidose' kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose compositions, the compositions may be contained in a container having an aseptic adaptor for removal of material.

[0067] Compositions compatible with the invention for use in humans are typically administered in a dosage volume of about 0.5ml, although a half dose (i.e. about 0.25ml) may be administered to children.

[0068] The present application describes a nucleic acid sequence encoding a TERT for use in therapy. The present application also describes a nucleic acid vector comprising a coding sequence for telomerase reverse transcriptase (TERT), for use in therapy and a gene therapy vector comprising a coding sequence for telomerase reverse transcriptase (TERT), for use in a therapy. In particular, the therapy may be treating or preventing a condition associated with myocardial infarction. The TERT nucleic acid sequence may be the sequence as recited in SEQ ID NO: 1 or SEQ ID NO: 3 or a fragment or functional equivalent thereof. The TERT protein may have a sequence as recited in SEQ ID NO: 2 or SEQ ID NO: 4, or a fragment or functional equivalent thereof.

[0069] The term "patient" refers to a mammal. In certain approaches compatible with the invention, the patient is a rodent, primate, ungulate, cat, dog, or other domestic pet or domesticated mammal. In certain compatible approaches, the mammal is a mouse, rat, rabbit, pig, horse, sheep, cow, domestic cat or dog, or a human. Preferably, the patient is a human.

Examples

Example 1 - Mouse model of myocardial infarction

[0070] A clinical mouse model of myocardial infarction **(MI)** after coronary artery ligation, which

resembles the situation found in the human heart after acute heart failure was developed. The adeno-associated viruses of serotype 9- MV9, which preferentially transduces the heart (28, 83, 55) was used as the gene therapy vector to deliver wild-type TERT expression specifically to the heart.

Mice

[0071] Male mice of a FVB/N background were produced and housed at the specific pathogen-free barrier area of the CNIO, Madrid. All animal procedures were approved by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CElyBA) and conducted in accordance to the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA).

Viral production Measurement of virus transduction efficiency

[0072] Viral vectors were generated as described by Matsushita (48), and purified as previously described (49). The titres of viral genomes particles were determined by quantitative real time PCR. To assess viral tropism a control group of mice was injected with different concentrations of a virus carrying eGFP or Tert under the control of CMV promoter following the methodology described before. At 4 weeks post-injection, mice were sacrificed and subjected to either pathological analysis or eGFP and Tert expression assessments. Quantification of eGFP immunostainings was determined by counting the number of peroxidase stained cells over the total number of hematoxylin-stained cells (liver and brain), or the peroxidase stained area relative to total section area (heart). Telomerase expression in different tissues was analysed by western blot analysis, quantitative RT-PCR and TRAP (telomerase repeat amplification protocol) assay following the previously described protocol (50). Viral genome copy number was assessed as previously detailed (51) with specific AAV9 primers, from heart DNA samples 4 weeks post injection.

Myocardial infarction

[0073] After weaning, five mice were housed per cage and fed ad libitum of a non-purified diet (n° 2018, Harlan). MI was induced in male mice by permanent left anterior descending coronary artery (LAD) ligation under isofluorane anaesthesia. The analgesic bupenorphine was administered via intraperitoneal injections before the procedure. A sham operation was performed in control and injected mice. Sham operation consists of the same surgery procedure except from ligation of the LAD. The date of euthanasia was used to estimate mouse survival. For an assessment of post MI survival, mice were followed and inspected daily up to 6 weeks after LAD-ligation.

Echocardiography and micro-PET

[0074] Transthoracic echocardiography was performed with a high-resolution Vevo770 in mice that were sedated with 1 to 2% isoflurane and placed on a heating pad as described (52). Micro-PET Imaging was performed as previously described (53).

Histology

[0075] Hearts were fixed in phosphate-buffered 4%formaldehyde and embedded in paraffin and sectioned (2 mm in thickness) from basal, midventricular, and apical regions using a heart slicer (Zivic instruments, HSMS001-1). Hearts slide sections (5 mm) were stained with Masson's trichrome. Infarct size and fibrotic length were calculated as the average ratio of scar length to total LV circumference of midventricular sections. Interstitial fibrotic area was performed on Masson's trichrome stained transverse heart sections in a semi-quantitative way using the ImageJ software. Three images per heart from noninfarcted area were analyzed.

[0076] Immunohistochemistry and immunofluorescence was performed on deparaffinised tissues and processed with the indicated antibodies: Anti- eGFP (Abeam, ab290); anti-Vimentin (D21H3; Cell Signaling, 5741); anti-heavy chain cardiac Myosin (Abeam, abl5); anti-Troponin T (Thermo Scientific, Ab-1); anti- KI67 (Abeam, ab16667); anti-active caspase 3 (Abeam, ab13847).

Quantitative real-time PCR and Western blots

[0077] Total RNA from tissues was extracted with Qiagen's RNeasy mini kit according to the manufacturer's instructions. Before processing, RNA samples were DNase I treated. Quantitative real- time PCR was performed using an ABI PRISM 7700 (Applied Biosystems), using the following primers:

Actin- FW:GGCACCACACCTTCTACAATG; (SEQ ID NO: 7)

Actin-RV:GTGGTGGTGAAGCTGTAG; (SEQ ID NO: 8)

TERT-FW:GGATTGCCACTGGCTCCG; (SEQ ID NO: 9)

TERT-RV:TGCCTGACCTCCTCTTGTGAC; (SEQ ID NO: 10)

ANP-FW: GTGGCTTGTGGGAAAATAGTTGA; (SEQ ID NO: 11)

ANP-RV: CTGGCTTGATGATCTGCCTTTAC; (SEQ ID NO: 12)

β-MHC-FW:CCAATGAGTACCGCGTGAA; (SEQ ID NO: 13)

β-MHC- RV:ACAGTCATGCCGGGATGAT; (SEQ ID NO: 14)

BMPRB1-FW: CCCTCGGCCCAAGATCCTA; (SEQ ID NO: 15)

BMPRB1-RV:CCACAGGCATTCCAGAGTCATC; (SEQ ID NO: 16)

FGFR3- FW:GGAGGACGTGGCTGAAGAC; (SEQ ID NO: 17)

FGFR3-RV:GGAGCTTGATGCCCCCAAT; (SEQ ID NO: 18)

IRF7-FW:GAGACTGGCTATTGGGGGAG; (SEQ ID NO: 19)

IRF7-RV:GACCGAAATGCTTCCAGGG; (SEQ ID NO: 20)

ITGB6-FW:CAACTATCGGCCAACTCATTGA; (SEQ ID NO: 21)

ITGB6-RV:GCAGTTCTTCATAAGCGGAGAT. (SEQ ID NO: 22)

Statistical analyses (student's t-test) were performed on delta-delta Ct values. Western blots were made with whole cell extracts from the indicated tissues with the following antibodies: Anti-h/TERT 1 (Calbiochem), anti-GAPDH (Sigma). Quantification was done using the Scion Image Software.

Telomere analysis

[0078] Q-FISH determination on paraffin-embedded tissue sections and HT-qFISH (in peripheral blood) was done as previously described (54, 55, 56).High throughput Q-FISH (HT-QFISH) TL analysis on blood samples HT-QFISH was performed as described (56). Briefly, peripheral blood was extracted from the facial vein before euthanizing animals, red blood cells lysed, leukocytes plated, fixed and subjected to Tel-Q-FISH. Fluorescence intensities were converted into Kb using L5178-R and L5178-S cells as calibration standards, which have stable TLs of 79.7 kb and 10.2 kb, respectively (62). Samples were analysed in duplicate, or triplicate in the case of calibration standards in the unbiased automated OPERA imaging system.

Serum metabolite analysis

[0079] Blood samples were collected in tubes without any anticoagulant, 6 weeks after MI. Samples were maintained on ice for 20 minutes and the serum supernatant was frozen and kept at 80°C until they were analyzed to minimize freeze-thaw degradation (57). Serum urea concentration in mice (groups and numbers of mice depicted) was determined in an ABX Pentra400 serum analyzer (Horiba Medical).

[0080] Additionally, high- throughput analysis of serum levels was measured with RodentMap

v3.0 (Myriad RBM).

Gene Expression analysis

[0081] Total RNA from frozen heart samples was extracted with Qiagen RNeasy kit, RNA integrity analysed in a Agilent Bioanalyzer (samples with RNA integrity index < 7.8 were discarded) and analysed on Agilent's Mouse Genome DNA microarray following the manufacturer instructions and (58). Briefly, differentially expressed genes were obtained by applying linear models using the R limma package (59) (Bioconductor project, http://www.bioconductor.org).

[0082] To account for testing of multiple hypotheses, the estimated significance level (p value) was adjusted using the Benjamini & Hochberg False Discovery Rate (FOR) correction. Those genes with FOR < 0.15 were selected as differentially expressed between Tert, empty virus injected hearts and sham operated controls.

[0083] Gene set enrichment analysis (GSEA) was applied using annotations from Reactome and KEGG. Genes were ranked based on limma moderated t statistic. After Kolmogorov-Smirnoff testing, those gene sets showing FOR < 0.1 (60) were considered enriched between classes under comparison.

Statistical analysis

[0084] A Log Rank test was used to calculate the statistical differences in the survival curves of the different mice cohorts. An unpaired t-student test was used to calculate statistical significance of mRNA and protein expression levels, TRAP, heart functional parameters, scar size and interstitial fibrosis. Mann-Whitney-U test was used for serum parameters. Pathological assessment either through heart observation after Masson's trichrome staining or FDG-PET scans were calculated with the x2 test.

Example 2 - Downregulation of mouse Tert expression in the heart during the first week after birth

[0085] We first set out to determine whether mouse Tert expression is downregulated in heart during the first week of life, similarly to that previously described for mouse telomerase RNA component in different mouse tissues (22), Terc, as well as for Tert expression in the rat (23). To this end, we isolated neonatal hearts at days 1, 3, 7 and 10 and determined mouse Tert expression by qRT-PCR. We observed a significant downregulation of mouse Tert mRNA levels from day 7 after birth.

Example 3 - Targeting AAV9-Tert specifically to the heart

[0086] AAV9 is known to preferentially target the heart and the liver when administered systemically, although the efficiency of targeting hepatocytes is around 10-fold lower than that of targeting cardiomyocytes (29). We used this differential transduction efficiency to find a minimal dose of AAV9 vectors that would specifically target the heart, with minimal transduction of the liver, and other tissues. Under our experimental conditions, a dose 5×10^{11} ug/mouse of a AAV9 reporter vector (AAV9-CMV-eGFP) could transduce more than 60% of heart cells, as shown by eGFP immunohistochemistry (IHC), but less than 1.2% of liver and brain 21 cells. To estimate the transduction efficiency of the telomerase vector 22 (AAV9-CMV-Tert; hereafter AAV9-Tert), we compared the viral genome copy number per diploid genome in hearts transduced with either the eGFP reporter vector or the AAV9-Tert vector (Fig. 1). We found similar numbers of viral genome copies per cell, indicating similar transduction efficiency for AAV9-eGFP and AAV9-Tert in the heart. Using co-immunostaining with eGFP and markers of either cardiomyocytes (beta-myosin heavy chain, β-MHC) or fibroblasts (vimentin), we found that within the myocardium, AAV9 preferentially targets cardiomyocytes (>60% eGFP positive cardiomyocytes) while we found no evidence for infection of fibroblasts. Specific targeting of Tert to the heart was also confirmed by qRT-PCR showing a -400 fold induction of Tert mRNA amounts in the heart of treated mice compared to non-treated mice, while Tert mRNA upregulation was 40-fold lower in the liver of treated mice. Tert mRNA expression levels in heart remained high during at least 2 months as indicated by >200 fold increased expression in AAV9-Tert treated mice compared to those treated with the empty vector. Finally, increased Tert mRNA expression was paralleled by increased TERT protein expression and increased telomerase activity in the AAV9-Tert treated hearts compared to non-treated mice and AAV9-empty treated controls. Together, these results indicate that we achieved specific Tert expression in the adult heart.

Example 4 - Telomerase targeting to the heart does not alter heart morphology

[0087] Constitutive Tert transgenic expression in the heart from embryonic development onwards results in heart hypertrophy (25). Thus, we first set to determine whether AAV9-Tert treatment during adulthood had any undesired effects on heart morphology. AAV9-Tert treatment in the absence of myocardial infarction (MI) did not change the normal heart structure nor did produce any signs of cardiac hypertrophy as assessed 9-10 weeks after virus administration. Further supporting a normal structure of the heart, we found normal expression of beta-myosin heavy chain (β-MHC) in AAV9-Tert treated hearts. These results indicate that Tert over-expression by means of AAV9 gene therapy does not have any of the adverse effects previously reported for constitutive Tert transgenic expression in the heart.

Example 5 - Telomerase gene therapy reduces mortality after MI

[0088] To assess the therapeutic potential of telomerase activation during adulthood in prevention of heart failure after MI, we used a bona fide mouse pre-clinical model of myocardial infarction after coronary artery ligation, previously shown to recapitulate heart failure induced by MI30. Cardiac specific AAV9-10 mediated transgene expression reaches its maximum after around 2 weeks and remains stable thereafter (31). Even though expression emerges already very shortly after intravenous injection we decided to induce MI 2-3 weeks after virus administration to assure Tert levels are maximal. Furthermore, since we were interested in regeneration and remodelling processes after acute infarction, which in humans predominantly occurs in the adult life, we used adult (1-year old) mice. All mice under experimentation were males to avoid gender bias.

[0089] Interestingly, a single treatment with AAV9-Tert three weeks prior to artery ligation, was sufficient to rescue mouse survival post infarction, with more than 74% of the AAV9-Tert treated mice surviving infarct compared to only 57% of those treated with the empty vector (Fig. 2A). Thus, telomerase activation in adult mice significantly reduces mortality by heart failure after MI in a bona fide mouse model for the human condition.

Example 6 - Telomerase gene therapy improves cardiac function after MI

[0090] To investigate how telomerase expression results in lower mortality rates upon MI, cardiac function was assessed by 2-dimensional echocardiography at 1 and 3 weeks after LAD ligation. Cardiac dimensions such as left ventricle systolic and diastolic area were strongly increased after MI compared to the sham operated (no MI) mice (Fig. 2B,C), which was also accompanied by decreased cardiac ejection fraction (Fig. 2D). Importantly, both the cardiac dimensions and the ejection fraction were significantly rescued in mice that received the Tert gene therapy but not in those that received the empty vector (Fig. 2B-D).

[0091] The rescue in cardiac function parameters upon Tert treatment was also evidenced by a similar longitudinal and transversal heart wall thickness in both the Tert treated and the sham operated mice as compared to a marked decrease in these parameters in mice treated with the AAV9-empy vector (Fig. 2E,F).

[0092] Next, we performed FDG-PET scan analysis, a diagnostic tool commonly used to assess infarct severity in vivo, as it measures metabolic activity in the heart via determining glucose uptake by cardiomyocytes. In particular, heart infarct results in decreased metabolic activity at the damaged regions (32). First, we noticed that AAV9-Tert treated mice present a better survival to the PET procedure per se, which involves anaesthesia and may result in death of mice subjected to MI (Fig. 3A). Of note, survival curves shown in Fig. 2A do not include mice used for PET. As expected, all mice showed reduced PET signals indicative of MI, however, we observed less severe infarcts (characterised by >50% loss of the PET signal) in the AAV9-Tert treated group compared to the AAV9-empty treated group (Fig. 3B-C). These

results indicate that AAV9-Tert treated hearts show a better preservation of metabolically active regions compared to the AAV9-empty group upon MI.

[0093] Six weeks after MI, we performed full pathological analysis including determination of heart scar length using Masson's trichrome staining. We found that AAV9-Tert treated mice presented smaller infarcts compared to AAV-empty or no virus controls, as determined by the ratio of scar length to endocardial circumference (Fig 3D). In addition, AAV9-Tert mice developed less interstitial fibrosis in the noninfarcted region of the left ventricle compared to the AAV9-empty group (Fig 3E). Together, these findings indicate that a single AAV9-Tert treatment resulted in increased survival of the myocardium and smaller infarct sizes, thus preventing heart functional decline after MI.

Example 7- Telomerase treatment rescues short telomeres in the heart cardiomyocytes

[0094] Critical telomere shortening causes heart dysfunction in mice. In particular, telomerase deficient mice bearing critically short telomeres present heart phenotypes resembling those of heart dysfunction in humans, including heart hypertrophy (19). As telomerase main function is to elongate critically short telomeres, here we set out to address the effects of Tert treatment on heart telomere length (TL) by performing quantitative telomere Q-FISH directly on heart sections. We found that AAV9-Tert treated hearts show a net-increase in TL in the infarct remote area (mainly formed by cardiomyocytes) compared to the empty vector group. Thus, AAV9-Tert is a potent tool to decrease short telomeres in the myocardium, and subsequently, the associated risk of heart failure in a short period of time (TL was measured 9-10 weeks after virus administration). Since short telomeres in peripheral blood lymphocytes are also proposed to have prognostic value indicating increased risk factor for CVDs, we also measured TL of peripheral blood mononuclear (PBMC) cells. We detected a modest increase in telomere length in blood cells from AAV9-Tert injected mice compared to the controls, confirming our previous observations (26). No differences in PBMC telomere length were observed between sham operated mice and those that underwent MI and received AAV9-empty or no virus, suggesting that acute MI and subsequent events such as inflammation and remodelling do not negatively impact on PBMC's TL. These findings suggest that telomerase gene therapy may have additional beneficial effects by lowering the abundance of short telomeres in the circulation, which in turn is a known risk factor for CVD.

Example 8 - Telomerase treatment results in increased number of proliferating cardiomyocytes

[0095] To study the impact of Tert treatment in apoptosis and proliferation in the heart after MI, we quantified the percentage of cells showing active caspase-3 and Ki67 positive staining, respectively, 6 weeks post MI. We found very few apoptotic cells 6 weeks post MI regardless of the treatment, in agreement with the fact that apoptosis is an early response following ischemia

(33). Upon MI, we observed increased proliferation (Ki67 positive cells) in the infarct remote area and in the infarct area both in the AAV9-Tert and the AAV9-empty groups compared to the sham operated mice. However, this increase was significantly attenuated in the AAV9-Tert group compared to AAV9-empty, in line with decreased fibrotic scar formation in these mice. Interestingly, co-immunostaining using Ki67 and the cardiomyocyte marker Troponin T, showed the presence of Ki67 positive cardiomyocytes in the infarct vicinity, which were significantly increased in the Tert treated group compared to empty virus group, suggesting increased heart healing and regeneration.

Example 9 - Telomerase impacts on metabolic and signalling networks related to cardiac dysfunction

[0096] To further understand the role of Tert in protection from acute heart failure after MI, we first studied changes in serum metabolites in the different mouse cohorts. Owing to a drastically lowered ejection fraction (Fig. 2D), MI can reduce the blood flow to the kidney and eventually induce kidney dysfunction with reduced glomerular filtration rates leading to increased urea blood levels. We found that 6 weeks after MI, serum urea levels were significantly increased in mice treated with no virus or with the empty vector, and that this was significantly rescued in the AAV9-Tert cohort (Fig. 4). A better renal function upon MI in AAV9-Tert treated mice supports our findings of improved cardiac function in these mice (Fig. 2).

[0097] Next, we performed a comprehensive serum multi-analyte profiling 6 weeks post MI encompassing various biological pathways. We found that MI resulted in significant decrease in the serum levels of factors involved in tissue remodelling such as metalloproteinases (MMP-9) and their inhibitors (TIMP-1) (34), in inflammation such as MDC (macrophage derived chemokine) or interleukin 18 (IL-18), and in the epidermal growth factor (EGF). Importantly, all of them were partially rescued in the AAV9-Tert group. Of interest, increased EGFR has been previously described as cardio protective (35).

[0098] Next, we determined the expression of the atrial natriuretic peptide (ANP), a foetal heart protein which is repressed after birth together with other foetal heart genes, but greatly increases during late stages of heart failure (2). Interestingly, ANP levels were decreased following AAV9-Tert treatment in old WT hearts (non infarcted >2 years old mice), suggesting that Tert treatment represses the pathological expression of foetal genes.

[0099] To gain further insights into the gene expression changes mediated by Tert in the heart, we performed microarray DNA analysis 6 weeks post MI, which reflect later stages of tissue repair process, including cardiac remodelling and scar formation (33). We used RNA from left ventricular heart tissue from the different groups (sham (no MI); MI + AAV9-Tert; MI + AAV9-empty). Gene set enrichment analysis (GSEA) revealed upregulation of pathways associated with inflammation, proliferation and DNA replication in the infarcted mice compared to the sham group (no MI), which were significantly rescued in AAV9-Tert treated mice compared to AAV9-empty controls. Increased inflammation is attributed to the clearance of apoptotic and

necrotic cardiomyocytes upon MI, while increased proliferation is associated to repopulation of the injury zone with fibroblasts (36). Thus, the fact that Tert attenuates the expression of genes involved in inflammation and proliferation supports our functional findings that AAV9- Tert treatment is cardio protective. Moreover, we found a significant enrichment in several signatures related to remodelling of the extracellular matrix and fibroblasts dynamics (e.g. ECM, TGFβ and FGFR) in the Tert treated group compared to the empty vector controls. We confirmed some of the most differentially expressed genes within these gene sets (Fgfr3 and Bmprlb, respectively) by qRT-22 PCR. In addition, Timp1, Thbs1 and Thbs4 (thrombospondin 1 & 4) were also differentially expressed in Tert-treated mice. Increased Timp1 expression is in agreement with higher TIMP1 protein levels in the serum from Tert treated mice. These findings are of interest given that overexpression of Timp1 has been shown to mitigate adverse myocardial remodelling and to improve cardiac function, while Thbs1 and Thbs4 are important regulators of cardiac adaptation post MI by regulating fibrosis and remodelling of the myocardium (37-39)

[0100] Neonatal mice have full regenerative potential during the first week of life, which is characterised by a well-defined gene expression signature. Thus, we next set out to address whether gene expression changes associated to Tert treatment in adult hearts were enriched in the regenerative gene expression signature described in neonatal mice. Strikingly, by comparing our expression data to genes overexpressed or underrepresented at postnatal day 1 (relative to postnatal day 10) (7), a stage at which the heart possesses full regenerative potential after MI, we found a significant enrichment in the AAV9-Tert group compared to AAV9-empty. We confirmed some of the most differentially expressed genes within these gene sets (Irf7 and Itgb6) by qRT-PCR. In summary, Tert treatment can modulate transcriptional programs that favour survival and regeneration after MI and that resemble the gene expression signature of the neonatal heart.

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[0101]

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Patentkrav

1. Nukleinsyrevektor omfattende en kodningssekvens til telomerase revers transkriptase (TERT) til anvendelse i behandling af myokardieinfarkt, hvor vektoren er en ikke-integrativ vektor baseret på en adeno-associeret virus, og hvor
a) TERT er kodet af
i) en nukleinsyresekvens, der omfatter sekvensen med SEQ ID NO:1 eller SEQ ID NO:3, eller
ii) en nukleinsyre, der koder for et polypeptid, som har 50 % eller mere TERT-aktivitet sammenlignet med TERT kodet af SEQ ID NO:1 eller SEQ ID NO:3, og som har mindst 75 % identitet med SEQ ID NO:1 eller SEQ ID NO:3, eller
iii) en nukleinsyresekvens, der er et fragment af SEQ ID NO:1 eller SEQ ID NO:3, som har TERT-aktivitet, og som har mindst 75 % identitet med sekvensen med SEQ ID NO:1 eller SEQ ID NO:3 over hele fragmentets længde;
eller
b) TERT omfatter
i) en aminosyresekvens med SEQ ID NO:2 eller SEQ ID NO:4, eller
ii) et polypeptid, der viser 50 % eller mere TERT-aktivitet sammenlignet med TERT med SEQ ID NO:2 eller SEQ ID NO:4, og som har mindst 75 % identitet med SEQ ID NO:2 eller SEQ ID NO:4, eller
iii) et polypeptid, der er et fragment af SEQ ID NO:2 eller SEQ ID NO:4, som har TERT-aktivitet, og som har mindst 75 % identitet med sekvensen med SEQ ID NO:2 eller SEQ ID NO:4 over hele fragmentets længde.

2. Nukleinsyrevektor til anvendelse ifølge krav 1, hvor vektoren er en vektor baseret på en adeno-associeret virus afledt på en AAV-serotype udvalgt blandt AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 og pseudo-typificeret AAV.

3. Nukleinsyrevektor til anvendelse ifølge krav 2, hvor vektoren er en vektor baseret på en adeno-associeret virus afledt af en serotype 9-adeno-associeret virus (AAV9).

4. Nukleinsyrevektor til anvendelse ifølge et af kravene 1-3, hvor TERT kodes af en nukleinsyresekvens, der omfatter sekvensen med SEQ ID NO: 1 eller SEQ ID NO: 3.

5. Nukleinsyrevektor til anvendelse ifølge et af kravene 1-4, hvor TERT omfatter en aminosyresekvens med SEQ ID NO:2 eller SEQ ID NO: 4.

6. Nukleinsyrevektor til anvendelse ifølge et af kravene 1-5, hvor nukleinsyresekvensen, der koder for TERT, er funktionsmæssigt forbundet med en regulatorisk sekvens, der driver ekspressionen af kodningssekvensen.

7. Nukleinsyrevektor til anvendelse ifølge et af kravene 2 til 6, hvor capsidet af vektoren baseret på en adeno-associeret virus er fremstillet af capsidproteiner af den serotype 9-adeno-associerede virus (AAV9), og nukleinsyresekvensen, der er indeholdt i capsidet, er flankeret ved begge ender af interne terminal repeat-sekvenser svarende til serotype 2-adeno-associerede vira.

8. Nukleinsyrevektor til anvendelse ifølge krav 7, hvor nukleinsyren, der er indeholdt i capsidet, omfatter et fragment, som koder for aminosyresekvensen, der koder for TERT.

9. Nukleinsyrevektor til anvendelse ifølge et af kravene 1-8, hvor vektoren omfatter en regulatorisk sekvens, som er en konstitutiv promoter.

10. Nukleinsyrevektor til anvendelse ifølge krav 9, hvor den regulatoriske sekvens er cytomegalovirus (CMV)-promoteren.

11. Nukleinsyrevektor til anvendelse ifølge et af kravene 1-10, hvor vektoren indgives direkte i hjertevævet.

12. Nukleinsyrevektor til anvendelse ifølge et af kravene 1-11, hvor patienten tidligere har haft et myokardieinfarkt-tilfælde.

13. Farmaceutisk sammensætning omfattende en virksom mængde af en nukleinsyrevektor til anvendelse ifølge et af kravene 1-12.

DRAWINGS

Figure 1

Heart infected with:	vector genomes/ diploid genome
AAV9-*Tert* (n=4)	0,92 (+/- 0,03)
AAV9-eGFP (n=5)	0,88 (+/- 0,04)

Figure 2

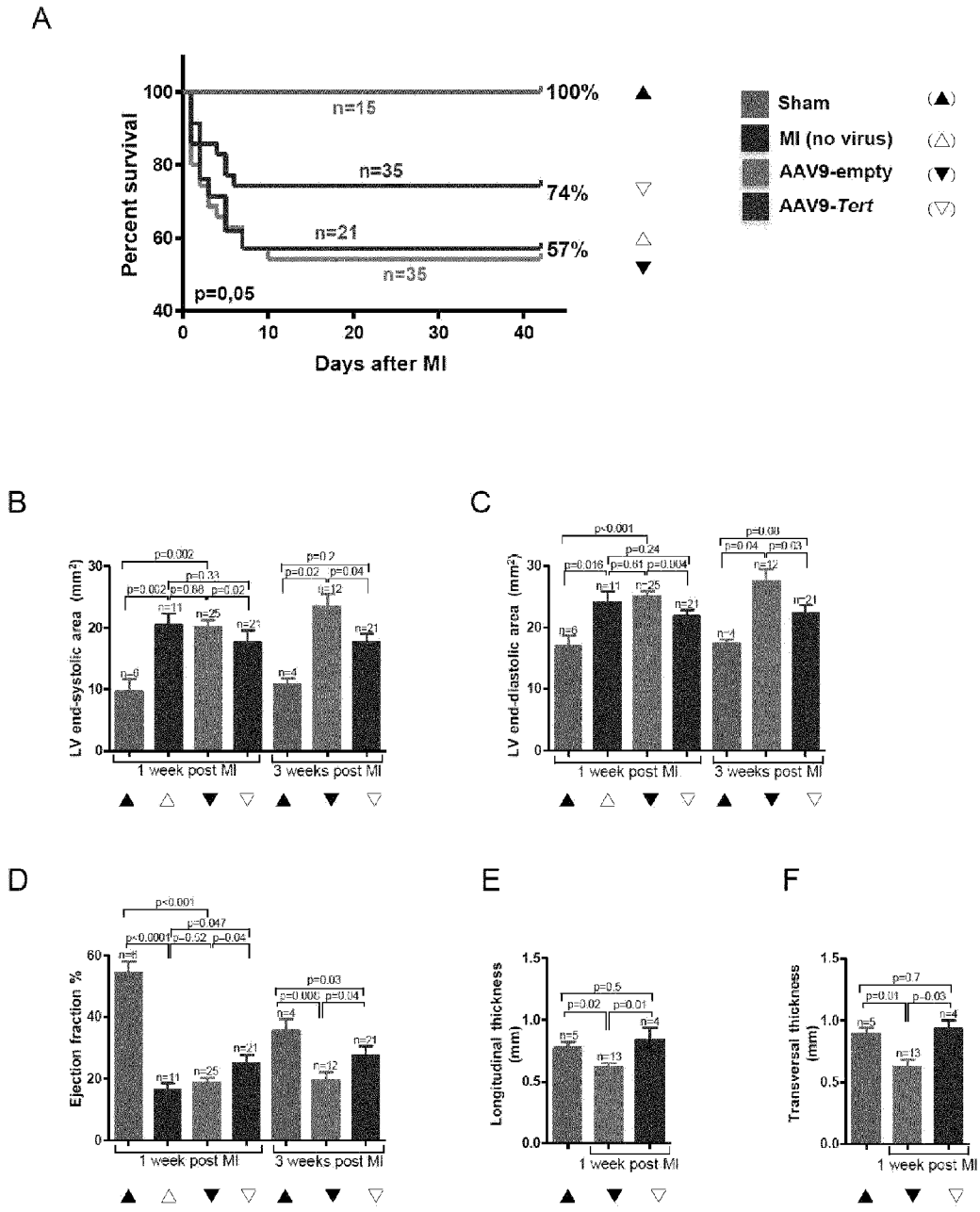

A
Percent survival
Days after MI
n=15
n=35
n=21
n=35
100%
74%
57%
p=0,05
Sham
MI (no virus)
AAV9-empty
AAV9-Tert
B
LV end-systolic area (mm²)
1 week post MI
3 weeks post MI
C
LV end-diastolic area (mm²)
1 week post MI
3 weeks post MI
D
Ejection fraction %
1 week post MI
3 weeks post MI
E
Longitudinal thickness (mm)
1 week post MI
F
Transversal thickness (mm)
1 week post MI

Figure 3

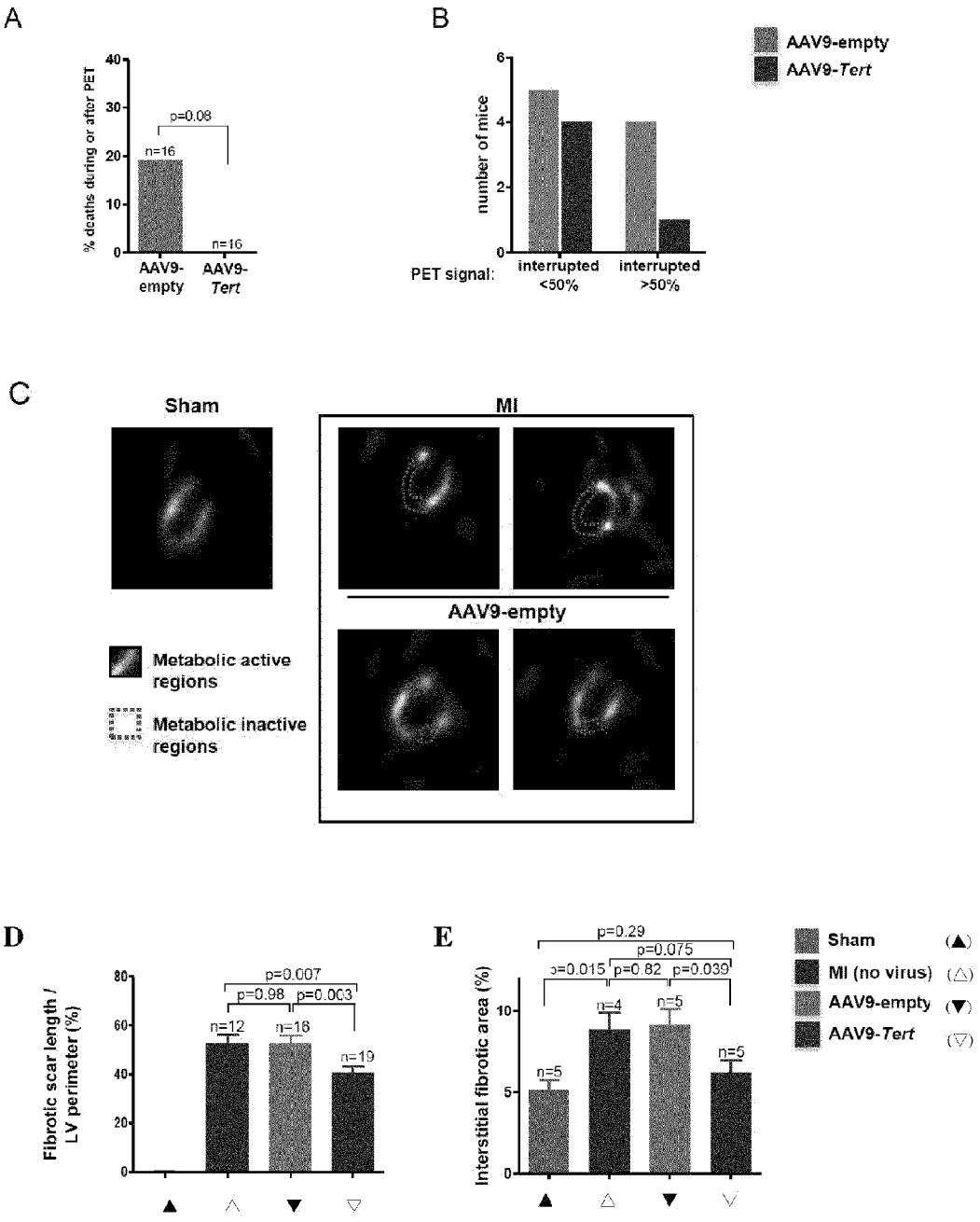
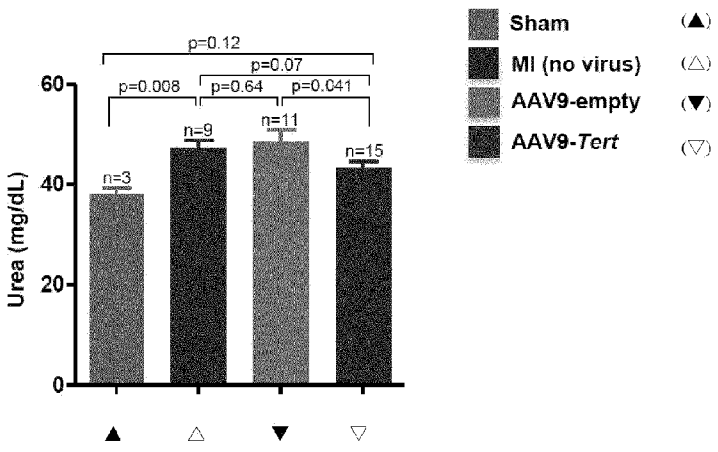

A
% deaths during or after PET
p=0.08
n=16
n=16
AAV9-empty
AAV9-Tert
B
AAV9-empty
AAV9-Tert
number of mice
PET signal:
interrupted <50%
interrupted >50%
C
Sham
MI
AAV9-empty
Metabolic active regions
Metabolic inactive regions
D
Fibrotic scar length / LV perimeter (%)
p=0.007
p=0.98
p=0.003
n=12
n=16
n=19
E
p=0.29
p=0.075
p=0.015
p=0.82
p=0.039
n=5
n=4
n=5
n=5
Interstitial fibrotic area (%)
Sham (▲)
MI (no virus) (△)
AAV9-empty (▼)
AAV9-Tert (▽)

Figure 4


p=0.12
p=0.07
p=0.008
p=0.64
p=0.041
n=3
n=9
n=11
n=15
Urea (mg/dL)
60
40
20
0
▲
△
▼
▽
Sham (▲)
MI (no virus) (△)
AAV9-empty (▼)
AAV9-Tert (▽)

SEKVENSLISTE

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