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(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR CARDIAC REGENERATION

(57) Abstract: The adult mammalian heart regeneration is largely prevented by the limited proliferative capacity of the resident cardiomyocytes (CMs). Here, the inventors identify Ephrin-B1 as a new critical regulator of adult CM proliferation. CM-specific transgenic repression of Ephrin-B1 promotes adult CM cell cycle reentry until division both *in vitro* and *in vivo* upon stimulation only, thus leading to substantial cardiac tissue regeneration through atypical CM proliferation and contractile function improvement to compensate for ageing stress, and apex resection. Cardiac deletion of *efnb1* after myocardial infarction also improves considerably cardiac function and survival in mice. Together, these findings highlight Ephrin-B1 as a promising original target for future therapeutic strategies in cardiac regenerative medicine. Accordingly, the present invention relates to methods and pharmaceutical composition for cardiac regeneration based on use of Ephrin-B1 inhibitors.



METHODS AND PHARMACEUTICAL COMPOSITIONS FOR CARDIAC REGENERATION

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for cardiac regeneration.

BACKGROUND OF THE INVENTION:

In the recent years, reinitiating the proliferative activity of differentiated resident cardiomyocytes (CMs) has emerged as an exciting avenue for cardiac regenerative medicine^{4,5}. Theoretically, this challenging concept provides the opportunity to yield sufficient CMs to replace their profuse loss after myocardial damages. Several evidences support the natural self-renewal potential of mammalian adult CMs but this mechanism remains too weak to efficiently repair the heart¹⁻³. Thus, strategies now focus on boosting this regenerative process pharmacologically^{6, 7, 10} or genetically by exploiting factors involved in the postnatal loss of CM proliferation^{8, 9, 11-13}. However, while most of these studies have successfully extended the neonatal regenerative window, they demonstrated only modest impact on the adult CM proliferation and most target manipulations were associated with concomitant cardiac defects, precluding them as ideal candidates for future regenerative therapies. Thus, the characterization of the molecular mechanisms specifically governing the post-mitotic state of adult CMs remains a crucial issue for future therapeutics in human cardiac regenerative medicine.

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for cardiac regeneration. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The adult mammalian heart regeneration is largely prevented by the limited proliferative capacity of the resident cardiomyocytes (CMs)¹⁻⁵. Recent findings challenged this dogma and raised the possibility to reboot adult CM proliferation to compensate for cardiac tissue injury⁶⁻¹³. However, little is known about the molecular mechanisms governing the specific blockade of proliferation in adult CM. Here, the inventors identify Ephrin-B1 as a new critical regulator of adult CM proliferation. CM-specific transgenic repression of Ephrin-B1 promotes adult CM cell cycle reentry until division both *in vitro* and *in vivo* upon stimulation only, thus leading to substantial cardiac tissue regeneration through atypical CM proliferation and contractile function improvement to compensate for ageing stress, apex resection but also under

neuregulin1 (NRG-1). Cardiac deletion of *efnb1* after myocardial infarction also improves considerably cardiac function and survival in mice. The inventors provide evidence that the post-mitotic state of the adult CM relies on Ephrin-B1 acting as a direct spatial hub for Cdc42-Cip4 and inactive Yap1 at the lateral membrane, thus coordinating CM rod-shape polarity and the canonical Hippo/Yap/proliferation pathway. Absence of Ephrin-B1 results in downregulated Cdc42 expression, loss of the adult CM rod-shape, and release of inactive Yap1 in the cytosol thus available for potential stress-induced Yap1-dependent proliferation. Inhibition of Cdc42 activity phenocopies Ephrin-B1 deletion, leading to NRG-1-induced Yap1-dependent proliferation of adult CMs. Together, these findings reveal a new pivotal role for Ephrin-B1 that tunes the adult CM to a postmitotic state by scaffolding key components of both the Hippo/Yap and cell polarity pathways and highlight Ephrin-B1 as a promising original target for future therapeutic strategies in cardiac regenerative medicine.

Accordingly, the first object of the present invention relates to a method of promoting proliferation of cardiomyocytes in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Ephrin-B1 inhibitor.

A further object of the present invention relates to a method of promoting cardiac regeneration in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Ephrin-B1 inhibitor.

A further object of the present invention relates to a method of treating cardiac injury in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Ephrin-B1 inhibitor.

As used herein, the term “cardiomyocyte” has its general meaning in the art and refers to sarcomere-containing striated muscle cells, naturally found in the mammalian heart, as opposed to skeletal muscle cells. Cardiomyocytes are characterized by the expression of specialized molecules e.g. proteins like myosin heavy chain, myosin light chain, cardiac alpha-actinin.

As used herein, the term “cardiac regeneration” refers to reinitiating the proliferative activity of differentiated resident cardiomyocytes. The term “cardiac regeneration” also refers to improving cardiomyocytes proliferation to replace their profuse loss after cardiac tissue injury.

As used herein, the term “cardiac tissue injury” has its general meaning in the art and refers any kind of reversible or irreversible damage to the myocardium and characterized but the death of cardiomyocytes. The term is synonym to “cardiac damage”. Cardiac tissue injury may be caused by any condition. Typically, cardiac tissue injury is caused by myocardial

ischemia. Ischemia may be a reversible or persisting (i.e. permanent) ischemia. Persisting ischemia is characterized in that the myocardium is inappropriately supplied by blood and, thus, hypoxic, even in a resting subject. In some embodiments, cardiac tissue injury may be caused by any form of chemical or physical agents, such as, drugs, environmental toxicants, or any other substance that contacts a subject and results directly or indirectly, in damage to the cardiac tissue. Also included, is damage that results from successful therapeutic treatment of a subject, such as for example, the treatment which results in induction of cardiac apoptosis (e.g. chemotherapy).

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., pain, disease manifestation, etc.]).

In some embodiments, the subject of the present invention suffers from a cardiac pathology. As used herein, the term "cardiac pathology" refers to any disease or condition affecting the heart, in particular to any disease or condition associated with cardiac tissue injury. The term encompasses, for example, injuries, degenerative diseases and genetic diseases.

5 Examples of cardiac degenerative diseases include, but are not limited to, heart failure, myocardial infarction, cardiac ischemia, myocarditis, arrhythmia, and the like. Examples of cardiac genetic diseases include, but are not limited to, Duchenne muscular dystrophy, Emery Dreiffuss dilated cardiomyopathy, mental retardation caused by genetic abnormality such as fragile X chromosome and other inborn errors of metabolism such as phenylketonuria gene
10 defect, and the like.

In some embodiments, the Ephrin-B1 inhibitor is administered to a subject having one or more signs or symptoms of acute myocardial infarction. In some embodiments, the subject has one or more signs or symptoms of myocardial infarction, such as chest pain described as a pressure sensation, fullness, or squeezing in the mid portion of the thorax; radiation of chest
15 pain into the jaw or teeth, shoulder, arm, and/or back; dyspnoea or shortness of breath; epigastric discomfort with or without nausea and vomiting; and diaphoresis or sweating. In some embodiments, the Ephrin-B1 inhibitor is administered simultaneously or sequentially (i.e. before or after) with a revascularization procedure performed on the subject. In some
20 embodiments, the subject is administered with the Ephrin-B1 inhibitor before, during, and after a revascularization procedure. In some embodiments, the subject is administered with the Ephrin-B1 inhibitor as a bolus dose immediately prior to the revascularization procedure. In some embodiments, the subject is administered with the Ephrin-B1 inhibitor continuously during and after the revascularization procedure. Typically, the revascularization procedure is
25 selected from the group consisting of percutaneous coronary intervention; balloon angioplasty; insertion of a bypass graft; insertion of a stent; directional coronary atherectomy; treatment with one or more thrombolytic agent(s); and removal of an occlusion.

In some embodiments, the Ephrin-B1 inhibitor is particularly suitable for preventing heart failure in a subject who experienced a myocardial infarction.

As used herein, the term "Ephrin-B1" has its general meaning in the art and refers to the
30 Ephrin-B1, a member of ephrin family. The term "Ephrin-B1" also refers to EFNB1, the B subclass of ephrin family which have a transmembrane domain and short cytoplasmic region. Ephrin-B1 is encoded by *efnb1* gene (Gene ID: 1947). The term is also known as EFNB1, CFND; CFNS; EFB1; EFL3; EPLG2; Elk-L; and LERK2. An human amino acid sequence is represented by SEQ ID NO:1.

SEQ ID NO:1

MARPGQRWLG KWLVMVVWA LCRLATPLAK NLEPVSWSSL
 NPKFLSGKGL VIYPKIGDKL DIICPRAEAG RPYEYYKLYL VRPEQAAACS
 TVLDPNVLVT CNRPEQEIRF TIKFQEFSPN YMGLEFKKHH DYYITSTSNG
 5 SLEGLENREG GVCRTTRTMKI IMKVGQDPNA VTPEQLTTSR PSKEADNTVK
 MATQAPGSRG SLGDSDGKHE TVNQUEEKSGP GASGGSSGDP DGFFNSKVAL
 FAAVGAGCVI FLLIIIFLTV LLLKLRKRHR KHTQQRAAAL SLSTLASPKG
 GSGTAGTEPS DIIPLRTTE NNYCPHYEKV SGDYGHPVYI VQEMPPQSPA NIYYKV

As used herein, the term “Ephrin-B1 inhibitor” has its general meaning in the art and
 10 refers to a compound that inhibits the activity or expression of Ephrin-B1. In particular, the
 inhibitor selectively blocks or inactivates Ephrin-B1. The term “Ephrin-B1 inhibitor” also
 refers to a compound that selectively blocks the binding of Ephrin-B1 to its downstream
 effectors. As used herein, the term “selectively blocks or inactivates” refers to a compound that
 15 preferentially binds to and blocks or inactivates Ephrin-B1 with a greater affinity and potency,
 respectively, than its interaction with the other sub-types of the ephrin family. Compounds that
 block or inactivate Ephrin-B1, but that may also block or inactivate other ephrin sub-types, as
 partial or full inhibitors, are contemplated. The term “Ephrin-B1 inhibitor” also relates to a
 compound that selectively blocks Ephrin-B1 nuclear translocation. The term “Ephrin-B1
 20 inhibitor” also relates to a compound that selectively phosphorylates Ephrin-B1 at its tyrosine
 Y328. The term “Ephrin-B1 inhibitor” also refers to a compound that inhibits Ephrin-B1
 expression. Typically, an Ephrin-B1 inhibitor compound is a small organic molecule, a
 polypeptide, an aptamer, an antibody, an intra-antibody, an oligonucleotide or a ribozyme. Tests
 and assays for determining whether a compound is an Ephrin-B1 inhibitor are well known by
 the skilled person in the art such as described in Arvanitis and Davy, 2008; Cho et al., 2014.

25 In some embodiments, the Ephrin-B1 of the present invention is an antibody, in
 particular an antibody having specificity for Ephrin-B1. In some embodiments, the antibody of
 the present invention promotes the internalisation of Ephrin-B1 and thus limits its expression
 at the surface membrane of the cells (i.e. cardiomyocytes). As used herein, the term "antibody"
 is thus used to refer to any antibody-like molecule that has an antigen binding region, and this
 30 term includes antibody fragments that comprise an antigen binding domain such as Fab', Fab,
 F(ab')₂, single domain antibodies (DABs), TandAbs dimer, Fv, scFv (single chain Fv), dsFv,
 ds-scFv, Fd, linear antibodies, minibodies, diabodies, bispecific antibody fragments, bibody,
 tribody (scFv-Fab fusions, bispecific or trispecific, respectively); sc-diabody; kappa(lamda)
 bodies (scFv-CL fusions); BiTE (Bispecific T-cell Engager, scFv-scFv tandems to attract T

cells); DVD-Ig (dual variable domain antibody, bispecific format); SIP (small immunoprotein, a kind of minibody); SMIP ("small modular immunopharmaceutical" scFv-Fc dimer; DART (ds-stabilized diabody "Dual Affinity ReTargeting"); small antibody mimetics comprising one or more CDRs and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art (see Kabat et al., 1991, specifically incorporated herein by reference). Diabodies, in particular, are further described in EP 404, 097 and WO 93/1 1 161; whereas linear antibodies are further described in Zapata et al. (1995). Antibodies can be fragmented using conventional techniques. For example, F(ab')₂ fragments can be generated by treating the antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')₂, scFv, Fv, dsFv, Fd, dAbs, TandAbs, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques or can be chemically synthesized. Techniques for producing antibody fragments are well known and described in the art. For example, each of Beckman et al., 2006; Holliger & Hudson, 2005; Le Gall et al., 2004; Reff & Heard, 2001 ; Reiter et al., 1996; and Young et al., 1995 further describe and enable the production of effective antibody fragments. In some embodiments, the antibody of the present invention is a single chain antibody. As used herein the term "single domain antibody" has its general meaning in the art and refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains. Such single domain antibody are also "nanobody®". For a general description of (single) domain antibodies, reference is also made to the prior art cited above, as well as to EP 0 368 684, Ward et al. (Nature 1989 Oct 12; 341 (6242): 544-6), Holt et al., Trends Biotechnol., 2003, 21(11):484-490; and WO 06/030220, WO 06/003388. In some embodiments, the antibody is a humanized antibody. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby incorporated by reference. In some embodiments, the antibody is a fully human antibody. Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of which are incorporated herein by reference. In some embodiments, the antibody of the present invention does not mediate antibody-dependent cell-

mediated cytotoxicity and thus does not comprise an Fc portion that induces antibody dependent cellular cytotoxicity (ADCC). In some embodiments, the neutralizing antibody does not comprise an Fc domain capable of substantially binding to a FcγRIIIA (CD16) polypeptide. In some embodiments, the neutralizing antibody lacks an Fc domain (e.g. lacks a CH2 and/or CH3 domain) or comprises an Fc domain of IgG2 or IgG4 isotype. In some embodiments, the neutralizing antibody consists of or comprises a Fab, Fab', Fab'-SH, F(ab')₂, Fv, a diabody, single-chain antibody fragment, or a multispecific antibody comprising multiple different antibody fragments. In some embodiments, the neutralizing antibody is not linked to a toxic moiety. In some embodiments, one or more amino acids selected from amino acid residues can be replaced with a different amino acid residue such that the antibody has altered C2q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Patent Nos. 6,194,551 by Idusogie et al.

In some embodiments, the Ephrin-B1 inhibitor is an inhibitor of Ephrin-B1 expression. An “inhibitor of expression” refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. In some embodiments, said inhibitor of gene expression is a siRNA, an endonuclease, an antisense oligonucleotide or a ribozyme.

In some embodiments, the inhibitor of expression is a siRNA. Small inhibitory RNAs (siRNAs) can also function as inhibitors of expression for use in the present invention. Ephrin-B1 gene expression can be reduced by contacting a patient or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that Ephrin-B1 gene expression is specifically inhibited (i.e. RNA interference or RNAi).

In some embodiments, the inhibitor of expression is an endonuclease. The term “endonuclease” refers to enzymes that cleave the phosphodiester bond within a polynucleotide chain. Some, such as Deoxyribonuclease I, cut DNA relatively nonspecifically (without regard to sequence), while many, typically called restriction endonucleases or restriction enzymes, cleave only at very specific nucleotide sequences. The mechanism behind endonuclease-based genome inactivating generally requires a first step of DNA single or double strand break, which can then trigger two distinct cellular mechanisms for DNA repair, which can be exploited for DNA inactivating: the error prone non-homologous end-joining (NHEJ) and the high-fidelity homology-directed repair (HDR). In a particular embodiment, the endonuclease is CRISPR-Cas. As used herein, the term “CRISPR-Cas” has its general meaning in the art and refers to clustered regularly interspaced short palindromic repeats associated which are the segments of prokaryotic DNA containing short repetitions of base sequences. In some embodiment, the endonuclease is CRISPR-cas9, which is from *Streptococcus pyogenes*. The CRISPR/Cas9

system has been described in US 8697359 B1 and US 2014/0068797. In some embodiment, the endonuclease is CRISPR-Cpf1 which is the more recently characterized CRISPR from Provotella and Francisella 1 (Cpf1) in Zetsche et al. ("Cpf1 is a Single RNA-guided Endonuclease of a Class 2 CRISPR-Cas System (2015); Cell; 163, 1-13).

5 In some embodiments, the inhibitor of expression is an antisense oligonucleotide. The term "antisense oligonucleotide" refers to an oligonucleotide sequence that is inverted relative to its normal orientation for transcription and so expresses an RNA transcript that is complementary to a target gene mRNA molecule expressed within the host cell (e.g., it can hybridize to the target gene mRNA molecule through Watson-Crick base pairing). An antisense
10 strand may be constructed in a number of different ways, provided that it is capable of interfering with the expression of a target gene. For example, the antisense strand can be constructed by inverting the coding region (or a portion thereof) of the target gene relative to its normal orientation for transcription to allow the transcription of its complement, (e.g., RNAs encoded by the antisense and sense gene may be complementary). Furthermore, the antisense
15 oligonucleotide strand need not have the same intron or exon pattern as the target gene, and noncoding segments of the target gene may be equally effective in achieving antisense suppression of target gene expression as coding segments. As used herein, the term "oligonucleotide" refers to a nucleic acid sequence, 3'-5' or 5'-3' oriented, which may be single- or double-stranded. The antisense oligonucleotide used in the context of the invention may in
20 particular be DNA or RNA. According to the invention, the antisense oligonucleotide of the present invention targets an mRNA encoding Ephrin-B1, and is capable of reducing the amount of Ephrin-B1 in cells, in particular in cardiomyocytes. As used herein, an oligonucleotide that "targets" an mRNA refers to an oligonucleotide that is capable of specifically binding to said mRNA. That is to say, the antisense oligonucleotide comprises a sequence that is at least
25 partially complementary, preferably perfectly complementary, to a region of the sequence of said mRNA, said complementarity being sufficient to yield specific binding under intra-cellular conditions. As immediately apparent to the skilled in the art, by a sequence that is "perfectly complementary to" a second sequence is meant the reverse complement counterpart of the second sequence, either under the form of a DNA molecule or under the form of a RNA
30 molecule. A sequence is "partially complementary to" a second sequence if there are one or more mismatches. The antisense oligonucleotide of the present invention that target an mRNA encoding Ephrin-B1 may be designed by using the sequence of said mRNA as a basis, e.g. using bioinformatic tools. Methods for determining whether an oligonucleotide is capable of reducing the amount of Ephrin-B1 in cells are known to the skilled in the art. This may for example be

done by analyzing Ephrin-B1 protein expression by Western blot, and by comparing Ephrin-B1 protein expression in the presence and in the absence of the antisense oligonucleotide to be tested. In some embodiments, the antisense oligonucleotide of the present invention has a length of from 12 to 50 nucleotides, e.g. 12 to 35 nucleotides, from 12 to 30, from 12 to 25, from 12 to 22, from 15 to 35, from 15 to 30, from 15 to 25, from 15 to 22, from 18 to 22, or about 19, 20 or 21 nucleotides. The antisense oligonucleotide according to the invention may for example comprise or consist of 12 to 50 consecutive nucleotides, e.g. 12 to 35, from 12 to 30, from 12 to 25, from 12 to 22, from 15 to 35, from 15 to 30, from 15 to 25, from 15 to 22, from 18 to 22, or about 19, 20 or 21 consecutive nucleotides of a sequence complementary to the mRNA of SEQ ID NO: 2. In some embodiments, the antisense oligonucleotide of the present invention is further modified, preferably chemically modified, in order to increase the stability and/or therapeutic efficiency of the antisense oligonucleotide in vivo. In particular, the antisense oligonucleotide used in the context of the invention may comprise modified nucleotides. Chemical modifications may occur at three different sites: (i) at phosphate groups, (ii) on the sugar moiety, and/or (iii) on the entire backbone structure of the antisense oligonucleotide. For example, the antisense oligonucleotide may be employed as phosphorothioate derivatives (replacement of a non-bridging phosphoryl oxygen atom with a sulfur atom) which have increased resistance to nuclease digestion. 2'-methoxyethyl (MOE) modification (such as the modified backbone commercialized by ISIS Pharmaceuticals) is also effective. Additionally or alternatively, the antisense oligonucleotide of the present invention may comprise completely, partially or in combination, modified nucleotides which are derivatives with substitutions at the 2' position of the sugar, in particular with the following chemical modifications: O-methyl group (2'-O-Me) substitution, 2-methoxyethyl group (2'-O-MOE) substitution, fluoro group (2'-fluoro) substitution, chloro group (2'-Cl) substitution, bromo group (2'-Br) substitution, cyanide group (2'-CN) substitution, trifluoromethyl group (2'-CF₃) substitution, OCF₃ group (2'-OCF₃) substitution, OCN group (2'-OCN) substitution, O-alkyl group (2'-O-alkyl) substitution, S-alkyl group (2'-S-alkyl) substitution, N-alkyl group (2'-N-alkyl) substitution, O-alkenyl group (2'-O-alkenyl) substitution, S-alkenyl group (2'-S-alkenyl) substitution, N-alkenyl group (2'-N-alkenyl) substitution, SOCH₃ group (2'-SOCH₃) substitution, SO₂CH₃ group (2'-SO₂CH₃) substitution, ONO₂ group (2'-ONO₂) substitution, NO₂ group (2'-NO₂) substitution, N₃ group (2'-N₃) substitution and/or NH₂ group (2'-NH₂) substitution. Additionally or alternatively, the antisense oligonucleotide of the present invention may comprise completely or partially modified nucleotides wherein the ribose moiety is used to produce locked nucleic acid (LNA), in which a covalent bridge is formed between the 2' oxygen and the 4' carbon of the ribose,

fixing it in the 3'-endo configuration. These constructs are extremely stable in biological medium, able to activate RNase H and form tight hybrids with complementary RNA and DNA. Accordingly, in a preferred embodiment, the antisense oligonucleotide used in the context of the invention comprises modified nucleotides selected from the group consisting of LNA, 2'-OMe analogs, 2'-phosphorothioate analogs, 2'-fluoro analogs, 2'-Cl analogs, 2'-Br analogs, 2'-CN analogs, 2'-CF₃ analogs, 2'-OCF₃ analogs, 2'-OCN analogs, 2'-O-alkyl analogs, 2'-S-alkyl analogs, 2'-N-alkyl analogs, 2'-O-alkenyl analogs, 2'-S-alkenyl analogs, 2'-N-alkenyl analogs, 2'-SOCH₃ analogs, 2'-SO₂CH₃ analogs, 2'-ONO₂ analogs, 2'-NO₂ analogs, 2'-N₃ analogs, 2'-NH₂ analogs and combinations thereof. More preferably, the modified nucleotides are selected from the group consisting of LNA, 2'-OMe analogs, 2'-phosphorothioate analogs and 2'-fluoro analogs. In some embodiments, the antisense is a Tricyclo-DNA antisense. The term "tricyclo-DNA (tc-DNA)" refers to a class of constrained oligodeoxyribonucleotide analogs in which each nucleotide is modified by the introduction of a cyclopropane ring to restrict conformational flexibility of the backbone and to optimize the backbone geometry of the torsion angle γ as (Ittig D, et al., *Nucleic Acids Res*, 2004, 32:346-353; Ittig D, et al., Prague, Academy of Sciences of the Czech Republic. 1:21-26 (Coll. Symp. Series, Hocec, M., 2005); Ivanova et al., *Oligonucleotides* 2007, 17:54-65; Renneberg D, et al., *Nucleic Acids Res*, 2002, 30:2751-2757; Renneberg D, et al., *Chembiochem*, 2004, 5:1114-1118; and Renneberg D, et al., *JACS*, 2002, 124:5993-6002). In detail, the tc-DNA differs structurally from DNA by an additional ethylene bridge between the centers C(3') and C(5') of the nucleosides, to which a cyclopropane unit is fused for further enhancement of structural rigidity. See e.g. WO2010115993 for examples of tricyclo- DNA (tc-DNA) antisense oligonucleotides. The advantage of the tricyclo-DNA chemistry is that the structural properties of its backbone allow a reduction in the length of an AON while retaining high affinity and highly specific hybridization with a complementary nucleotide sequence.

The inhibitor of expression may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the the inhibitor of expression and preferably in cells expressing ephrin-B1, and more particularly in cardiomyocytes. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources. Viral vectors are a preferred type of vector. In some embodiments, the vector is an adeno-associated virus (AAV) vector. As used herein, the term "AAV vector" means a vector derived from an adeno- associated virus serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, and mutated forms thereof. AAV vectors can have one

or more of the AAV wild-type genes deleted in whole or part, preferably the rep and/or cap genes, but retain functional flanking ITR sequences. Preferably, the AVV vector is an AAV9 vector.

By a "therapeutically effective amount" of the inhibitor of the present invention as above described is meant a sufficient amount of the inhibitor for promoting cardiac regeneration at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the inhibitors and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific inhibitor employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific inhibitor employed; the duration of the treatment; drugs used in combination or coincidental with the specific inhibitor employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the inhibitor at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the inhibitor of the present invention for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the inhibitor of the present invention, preferably from 1 mg to about 100 mg of the inhibitor of the present invention. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

The inhibitor of the invention may be used or prepared in a pharmaceutical composition. Typically, the inhibitor of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. "Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. In the pharmaceutical compositions of the present invention for oral, sublingual, intramuscular, intravenous, local or

rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, intraperitoneal, intramuscular, intravenous and intranasal administration forms and rectal administration forms. Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Solutions comprising inhibitors of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The inhibitor of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can

be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatine. Sterile injectable solutions are prepared by incorporating the active inhibitors in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. In addition to the inhibitors of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. Ephrin-B1 deletion promotes cardiac regeneration following myocardial infarction through adult CM proliferation. a, Schematic of the myocardial infarction (MI)

protocol using control- (mCherry) or shmi*Refnb1*-AAV9 in 2-month-old mice. **b**, Left Ventricle Ejection Fraction (LVEF) measured by echocardiography 3 days post-MI (Sham n=10; MI/control n=12; MI/shmi*Refnb1* n=16). **c**, kinetics of mice survival. Groups compared by Log-rank (Mantel-Cox) Test. **d**, LVEF measured by echocardiography 56 days post-MI (Sham n=10; MI/cherry n=12; MI/ shmi*Refnb1* n=16). **e**, Cardiac fibrosis quantification (MI/mCherry n=5; MI/ shmi*Refnb1* n=7). **f**, Heart weight/tibia length ratios (MI/mCherry n=12; MI/ shmi*Refnb1* n=16). **g**, CM size in situ (~50 CMs/mouse; Sham n=4; MI/mCherry n=5; MI/ shmi*Refnb1* n=8). **h-i**, *In situ* quantification of CM mitosis (**h**) (Aurkb⁺/DAPI⁺/cTnT⁺ and PCM1⁺/DAPI⁺/Aurkb⁺) and cytokinesis (**i**) (Aurkb⁺ cleavage furrows/cTnT⁺) in the left ventricle remote zone (Sham n=4; MI/mCherry n=5, MI/shmi*Refnb1*; n=7). Values presented as means ± s.e.m.; Statistical significance was assessed using Student's *t*-test for 2 group comparisons or one-way ANOVA with Tukey post-hoc test for 4 group comparisons * *P*<0.05, ** *P*<0.01, *** *P*<0.001.

EXAMPLE:

Material & Methods

Animal models. Global (KO) and CM-specific (cKO) *efnb1* KO mice have already been described¹⁵. *Efnb1* WT and KO mice were kept in a mixed S129/S4 × C57BL/6 background. For AAV9 experiments, adult C57Bl/6 mice (8 weeks, Envigo laboratories) were used. All studies were performed on male and age-matched mice. Experimental animal protocols were carried out in accordance with the French regulation guidelines for animal experimentation and were approved by the French CEEA-122 ethical committee.

Isolation and culture of adult cardiomyocytes. Isolation of adult CMs from mice was performed using the Langendorff perfusion method as previously described¹⁵. After isolation, dissociated CMs were plated on 10 µg/ml laminin-coated culture dishes. After 15 min, plating medium was changed to culture medium containing MEM, 1 % penicillin-streptomycin-glutamine, 1 % insulin-transferrin-selenium, 4 mM NaHCO₃, 10 mM Hepes, 0.2 % BSA and 25 µM blebbistatin which was refreshed every day.

***In vitro* adult CM proliferation assay.** Isolated adult CMs were cultured in their cell culture medium in the presence or not of 100 ng/ml neuregulin-1 (NRG-1, EGF-like domain, amino acids 176–246; R&D Systems, 396-HB-050) for 8 days. The medium and NRG-1 were refreshed every day. For detection of DNA synthesis or pharmacological inhibition of cdc42, 5-bromo-2-deoxyuridine labeling reagent (BrdU, 1:100 dilution, Life technologies, 00-0103) or Casin (0.1 µM, Xcess Biosciences Inc., San Diego, CA, USA) were supplemented to the culture medium every day from the second day of cell culture.

Cardiomyocyte nucleation. Isolated ventricular CMs were fixed in 4 % paraformaldehyde (PFA) and co-stained with 4',6'-diamidino-2-phenylindole (DAPI, Sigma Aldrich, 32670) and Oregon green® 488 conjugated Wheat-Germ-Agglutinin (WGA, Life technologies, W6748). Images were acquired on a Zeiss Observer Z.1 microscope and the nucleation profile was manually quantified using Axio Vision Rel 4.8 software.

H9C2 cell line culture and transfection. H9C2 rat cardiomyoblasts were used in this study because they phenocopy Ephrin-B1 expression/localization seen in neonatal CMs within the tissue. Neonatal primary culture of CMs couldn't be used because of their loss of *in situ* phenotype after purification (loss of Ephrin-B1 and Yap1). H9C2 (ATCC) were cultured in complete medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2 mM glutamine, 4.5 g/l D-glucose, pyruvate, 10 % fetal calf serum and 1 % penicillin-streptomycin). For Ephrin-B1 silencing experiments, H9C2 cells were transfected twice at a 24 h interval using Lipofectamine RNAiMAX transfection Reagent (Life technologies) alone (Mock) or with 40 nM final concentration of ON-TARGET plus SMARTpool siRNA oligonucleotides targeting rat *efnb1* (Dharmacon, L-087677-02-0005). Ephrin-B1 silencing was confirmed by real-time quantitative PCR (Each reaction was run with *Actb* as a reference gene (β -actin encoding gene) and all data were normalized based on *Actb* expression levels).

For adhesion assays, H9C2 cells were harvested two days after the last siRNA transfection and 15 000 cells were seeded in 12-well-plates and cultured in complete medium during 6 hours. Then, cells were fixed in 4% PFA and co-stained with Oregon green® 488 conjugated Wheat-Germ-Agglutinin (WGA, Life technologies, W6748) and DAPI (Sigma Aldrich, 32670). The density of adherent cells was quantified by using a home-made Fiji macro on images acquired from a digital slide scanner *NanoZoomer* (Hamamatsu).

For proliferation assays, 2 days after the last siRNA transfection, cells were serum starved for 24 hours and then cultured in 10 % serum-complete medium during 24 hours. Cell density was calculated by manual counting of H9C2 cells co-stained with Oregon green® 488 conjugated Wheat-Germ-Agglutinin (WGA, Life technologies, W6748) and DAPI (Sigma Aldrich, 32670) on images acquired from a digital slides scanner *NanoZoomer* (Hamamatsu). The impact of siRNA on cell proliferation was evaluated by the percentage of cell density quantified after 24 hours of 10% serum culture relative to the cell density quantified after serum starvation.

Immunocytochemistry. After ice-cold methanol fixation, permeabilization (0.1 % Triton X-100) and blocking (1% bovine serum albumin), CMs were immunostained using standard protocols with the following primary antibodies (all diluted in 0.1 % BSA / 0.1 %

Tween 20 / 0.5 % Triton X-100 PBS) incubated overnight at 4°C: mouse anti- α -actinin (Sigma Aldrich, A7732), rat anti-BrdU (Abcam, ab6326), rabbit anti-aurora kinase B (Abcam, ab2254), rabbit anti-histone H3 phosphorylated at serine 10 (pH3, Cell signaling, 9701S), rabbit anti-acetyl-histone H3 (H3K9/14Ac, Millipore, 06-599), rabbit anti-tri-methyl-K9 histone H3 (H3K9me3, Abcam, ab8898), goat anti-ephrin B1 (R&D, AF473), rabbit anti-Yap1 (Cell signaling, 14074). Cells were washed and then incubated with the corresponding secondary antibodies conjugated to specific fluorophores (Alexa-Fluor 488 goat anti-rabbit Ig-G, Texas-red goat anti-mouse IgG or Alexa-fluor 488 donkey anti-rat IgG, Life technologies). Nuclei were visualized with DAPI (Sigma Aldrich, 32670). Fluorescent images were acquired on a Zeiss Observer Z.1 microscope with *Apotome* optical sectioning using Axio Vision Rel 4.8 software (Carl Zeiss). For Yap1 expression studies in isolated adult CMs, images were acquired in XYZ on a Zeiss LSM 780 confocal microscope using Zen 2011 software (Carl Zeiss).

Quantification of nuclear to cytosolic Yap1 immunofluorescence ratio in cultured CMs was conducted using a Fiji home-made macro relying on both Yap1 and DAPI immunofluorescence detected in different z-stacks / cell. For each cell, only z-stacks in which nuclei were visible have been quantified individually. The results represent the ratio between quantification of nuclear Yap1 (sum of all quantified z-stacks) to cytosolic Yap1 (nuclear Yap1 subtracted to total Yap1 calculated from the sum of all quantified z-stacks). Quantifications have been selectively performed on binucleated CMs (major CM population) only to avoid bias.

Histology. Hearts were excised and immediately fixed in 10 % formalin (24 h), 4 % formalin (48 h) and then embedded in paraffin and sectioned at 6 μ m intervals. Except for ageing studies (transversal sections), all other heart sections were longitudinal.

Cardiac fibrosis was quantified on Masson's trichrome-stained paraffin-embedded heart sections. For apectomy, the apical cardiac fibrosis (3-4 sections spaced out of 200 μ m/heart) was quantified using NIS Element Basic Research version 2.31 (Nikon imaging software) in the all apex area (between the above subventricle and bottom apex). Similar analysis was conducted for quantification of myocardial fibrosis in the ageing mouse models. For myocardial infarction/AAV9 study, quantification of global cardiac fibrosis was performed. Briefly, 24 longitudinal sections spaced out of 100 μ m (almost all heart) were quantified with ImageJ software from images acquired from a digital slide scanner *NanoZoomer* (Hamamastu). The experimenter was blinded to the mouse genotype.

Immunohistochemistry. Formalin-fixed paraffin-embedded or frozen tissues were used for immunohistochemical analysis. For paraffin-embedded sections, samples were deparaffinized, rehydrated and subjected or not to heat-induced epitope retrieval depending on

the antibodies. For cryosections, samples were fixed with acetone or 4 % PFA. Heart sections were then permeabilized (0.5 % Triton X100-PBS), blocked (Dako Protein Block, Dako, X0909 or 10 % normal goat serum, Dako, X0907 or 0.5% BSA/PBS), and stained over night at 4°C with the following primary antibodies all diluted in 0.1 % BSA / 0.1 % Tween 20 / 0.5 % Triton X-100-PBS: rabbit anti-troponin I (Santa cruz, sc-15368), mouse anti-troponin T (ThermoFischer scientific, MA5-12960), rat anti-BrdU (Abcam, ab6226), rabbit anti-aurora B kinase (Abcam, ab2254) , rabbit anti-Yap1 (Abcam, ab39361), rabbit anti-PCM1 (Santa Cruz biotechnology, 50-164). Secondary fluorescent antibodies used in this study were as follows: Alexa-Fluor 488 goat anti-rabbit IgG, Texas-red goat anti-rabbit IgG, Texas-red goat anti-mouse IgG, Alexa-fluor 488 donkey anti-rat IgG, Alexa-fluor 594 donkey anti-rabbit IgG, Alexa-Fluor 488 donkey anti-rabbit IgG (Life technologies). Nuclei were visualized with DAPI (Sigma Aldrich, 32670). Cell membranes were stained with Texas Red- or Alexa-fluor 633-conjugated Wheat Germ Agglutinin (WGA, Life technologies, W21404). Endothelial cells were stained with OG488-conjugated Griffonia simplicifolia isolectin-B4 (Life technologies, I21411).

Localization of Cdc42 activity in situ was evaluated by cryosection staining with GST-WASP-crib fusion protein³⁵. After fixation in 4% PFA in buffer A (5 mM MgCl₂ / PBS), cryosections were washed in buffer A, permeabilized in 0.5% Triton-X-100-buffer A during 20 minutes and PFA neutralized with 100 mM glycine -buffer A. After blocking (Dako protein block), cryosections were incubated with 30 µg GST-WASP-crib diluted in buffer A overnight at 4°C. After washing in buffer A, cryosections were fixed in PFA 2 %, rinsed and incubated with anti-GST antibody (Santa Cruz Biotechnology), followed by secondary Alexa-fluor 488-labeled goat anti-mouse antibody staining and co-labeling of cell membranes with Alexa-fluor 633-conjugated WGA (Life technologies).

All Images were acquired on Zeiss LSM 780 confocal microscope using Zen 2011 Software (Carl Zeiss).

For *in situ* quantification of cardiomyocyte surface area and density, deparaffinized slides were stained with Texas Red-conjugated-WGA (Life technologies, W21405) and CM area and density were measured in transversal (aged mice) or longitudinal (apectomy and myocardial infarction) heart cross-sections by manually tracing the cell contour on images of whole hearts acquired on a digital slide scanner *NanoZoomer* (Hamamastu) using Zen 2011 software (Carl Zeiss). The experimenter was blinded to groups and genotypes.

Stereology. Stereology analysis was conducted on 50-60 µm-thick paraffin-embedded heart sections. After deparaffinization and rehydration, sections were permeabilized with

proteinase K (S3020, 3 minutes, room temperature), blocked (Protein Block, Dako, X0909) and incubed overnight at 4°C with Oregon green® 488 conjugated Wheat-Germ-Agglutinin (WGA, Life technologies, W6748) to visualize all cell borders. Z-stack images were then acquired on a Leica TCS SP8 confocal microscope (63x oil immersion objective) up to 40-60 µm with an optimal step of 0.3 µm applying compensation of intensity loss during the z-stack acquisitions. Stereological analysis was performed using Image J software on z-stacks and brightness inversion of the AF488-WGA staining to provide clearer delimitation of CM surface. The number of CMs was counted manually (point function on Image J) on each acquired image (~150 z-stacks). CM density was estimated as the global acquisition volume relative to the CM number. To estimate the CM volume, we proceeded first to image binarization and manual cleaning so to exclude non CM cells. The average CM volume was then estimated as the global CM volume within the image relative to the CM number. The total CM number in the heart was estimated as previously described³⁶ by using $N_v \times V_{REF}$ method (N_v is the estimated CM density and V_{REF} is the reference volume of the heart). The reference volume was calculated using the tissue density of the myocardium (1.06 g/cm^3)³⁶.

Analysis of cardiomyocyte cell cycle. Cell cycle was evaluated on purified CM nuclei. Thus, isolated ventricular CMs were first fixed in ice-cold 70 % ethanol and intact nuclei were obtained from 1 mg/ml pepsin / 0.2 M HCl digestion (15 min, 37°C). After purification, nuclei were stained with 200 µg / mL propidium iodide (Life technologies, P1304MP) / 12 µg / mL RNase A (Roche)-PBS and further proceeded for FACS experiments (BD Accuri™ C6 flow cytometer and BD Accuri C6 software).

Total RNA isolation and real-time quantitative RT-PCR. Total RNA extraction from isolated CMs was performed using Trizol according to the manufacturer's instructions (TRI Reagent®, Molecular Research Center). RNA quality and quantification of extracted RNA were assessed by an Experion automated electrophoresis system (Biorad). First-strand cDNA was synthesized using the superscript II RT-PCR system (Invitrogen) with random hexamers. Negative controls without reverse transcriptase were conducted to control the absence of genomic DNA contamination. Fifteen nanograms of cDNA from RT reaction were then mixed with specific primers and EVA green mix (Euromedex). Real-time PCR was performed in 96-well plates using an ABI 7900 Fast (Applied Biosystems). Geometric mean values of GAPDH and HPRT housekeeping genes were used for normalization, as previously described¹⁵. Melting curve analysis was performed to ensure a single PCR product and a specific amplification. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method¹⁵.

Lentivector constructs, transduction and evaluation of gene down-regulation.

Three shRNA constructs targeting Ephrin-B1 (*shefnb1*) were generated by Sigma-Aldrich with the pLKO.1-puro vector. The sequences of the cassettes are listed in supplementary table 2 (shB1-44, -45 and -47, respectively). The *shefnb1* and the control shRNA vectors (targeting luciferase, *shluc*, SHC007, Sigma-Aldrich) were produced using the tri-transfection procedure with the plasmids pLvPack and pLvVSVg (Sigma-Aldrich, Saint-Quentin Fallavier, France), in HEK-293FT cells. They were evaluated for their ability to transduce the C2C12 myoblast cell lines (ATCC, Catalog No. CRL-1775TM) and adult CMs, both naturally expressing Ephrin-B1 in the nuclei. The efficiency of cell transduction *in vitro* was checked in parallel by FACS analysis using the pTRIP-GFP lentivector³⁷. The efficiency was at least 40 % for C2C12 cells and 99 % for primary CMs. C2C12 cells (4 x 10⁴/well) were plated into 6-well plate and transduced overnight in Dulbecco's Modified Eagle's Medium (DMEM) with 20 % fetal calf serum at 37°C with 5 % CO₂ in the presence of purified concentrated lentiviral vector. Cells were transduced with 5.10⁵ transduction unit (TU) of the three *shenfb1* lentivectors per well or with 5.10⁵TU of the *shluc* lentivector as control. Primary CMs were transduced using the same protocol but in their culture medium. The efficiency of the *shefnb1* was evaluated by real-time quantitative RT-PCR (specific primers are listed in supplementary table 1) and promoted ~76 % *efnb1* gene downregulation. For that purpose, total cellular RNA was extracted from C2C12 cells and purified using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. A total of 1 µg RNA was used to synthesize cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Villebon s/ Yvette, France). Ephrin-B1 expression was investigated using SsoFast Eva Green Supermix (Bio-Rad) and performed on a StepOne sequence detection system (Applied Biosystems). Each reaction was run with HPRT as a reference gene and all data were normalized based on HPRT expression levels. The efficiency of the shB1 on the downregulation of Ephrin-B1 expression was evaluated by immunofluorescence in isolated adult CMs and was ~87 %.

shmiR construction and rAAV production. In the knock-down experiments *in vivo*, we used shmiRs as they have been reported to produce siRNAs with more efficiency than shRNAs³⁸⁻⁴⁰. Indeed shmiRs undergo a better processing and less toxicity than shRNAs. Design and construction of the shmiR targeting Ephrin-B1 was adapted from the BLOCK-iTTM PolIII Mir RNAi expression system (Stratagene, Massy, France): two complementary oligonucleotides mEB1-47 Top and mEB1-47 Bottom containing the sequence targeting mouse Ephrin-B1 with flanking regions derived from miR-155, were annealed, phosphorylated and cloned between the XbaI and BglII sites of the plasmid pAAV-MCS (Stratagene). The resulting

recombinant AAV vector, rAAV-shmi*Refnb1* and the control rAAV-*mCherry* (pAAV-MCS, Stratagene) were produced as previously described³⁷ by calcium phosphate tri-transfection procedure of HEK293 cells (pAAV-shmi*Refnb1* or pAAV-*mCherry* and pAAV2/9 and pHelper (stratagène)). The titers for the recombinant AAV9-*mCherry* and AAV9-shmi*Refnb1* were
 5 9.10¹² vg/ml and 1.10¹³ vg/ml respectively.

MicroRNA isolation and quantification. For miR-195 quantification, RT was performed on 500 ng of total RNA (Trizol extraction) using the miScript (Qiagen) kit. For the real-time RT-PCR reaction, the resultant cDNA was diluted 1:100. Each RT step was performed in duplicate and the qPCR in triplicate for each reaction. U6 RNA was used as an endogenous
 10 control (primer sequences listed in supplementary table 1) and relative miR-195 (Qiagen) quantity calculated by the $2^{-\Delta\Delta CT}$ method. MicroRNA expression was quantified by PCR using a Bio-Rad thermal-cycler. miR-195 primer sequences were purchased from Qiagen and used according to the manufacturer's protocol.

Apical resection (Apectomy). Two-month-old adult mice were anesthetized
 15 (intraperitoneal injection with 125 mg/kg ketamine plus 10 mg/kg xylazine for induction and 0.5 % O₂ / 1 % isoflurane mixture inhalation for anesthesia maintenance), placed on a heating mat and subjected to artificial ventilation through endotracheal cannulation. The animals were positioned on their right side and a thoracotomy was performed on the sixth or the seventh intercostal space. After exposition of the heart apex, apical resection was controlled using a 3/8
 20 circle needle and 8.0 polypropylene thread and performed with microscissors following the curvature of the thread to ensure surgery reproducibility. Further homogenization of apex resection was controlled by apex weighing. After hemostasis control, the intercostal space was closed in two separate points using a 6.0 nylon monofilament, Ethnor), followed by skin surface suture. Then, isoflurane was stopped and subcutaneous buprenorphine (100 µg/kg) was
 25 administered. Mice remained air ventilated until the return of peripheral reflexes. The whole procedure was performed using an operating microscope (Zeiss OPMI 1 FC). Sham animals underwent exactly the same surgical procedure without apex resection. One day after surgery, mice that had undergone apectomy were indistinguishable from sham-operated ones. Hearts were collected 21 days following apectomy. For quantification of CM replication in cardiac
 30 tissue, BrdU was administered by intraperitoneal injection on days 1, 7 and 14 following apectomy (0.01 ml BrdU solution/g; Life technologies, 00-103).

Myocardial infarction and AAV injections. Myocardial infarction (MI) was induced in 2-month-old male C57Bl/6J mice. In brief, mice were anaesthetized (intraperitoneal injection with 125 mg/kg ketamine plus 10 mg/kg xylazine for induction and 0.5 % O₂ / 1.5 % isoflurane

mixture inhalation for anesthesia maintenance), placed on a heating mat and subjected to artificial ventilation through endotracheal cannulation. The heart was exposed by performing a thoracotomy through the fourth or fifth intercostal space and MI was induced by permanent ligation of the left anterior descending coronary artery with a 9.0 silk suture. Post-surgery analgesia was reached through buprenorphine injection (0.01 mg/kg body weight). Mice were then warmed until recovery. Sham-operated mice were treated similarly, except that the ligature around the coronary artery was not tied. Three days after surgery, transthoracic echocardiography was performed on mice lightly anesthetized (0.5-1% isoflurane) using a Vivid7 device and a 14 MHz transducer (i13L, GE Healthcare). Images were transferred and analysed off line with EchoPAC (GE Healthcare). Only mice within 30-40 % ejection fraction were included for the following protocol. The next day, AAV9-*mCherry* or AAV9-shmiR-*efnb1* were injected by retro-orbital injection with 100 μ L of 3×10^{11} viral genomes/ mL physiological serum solution.

Echocardiography. For experiments on aged mice, animals were anesthetized by intraperitoneal injection of 10 μ g/g etomidate and underwent noninvasive transthoracic echocardiography using a General Electric Vivid 7® (GE Medical System) equipped with a 14 MHz linear probe. Cardiac ventricular dimensions were measured in M-mode images at least 5 times for each animal. Left ventricle ejection fraction (LVEF) was calculated using the Teichholz formula. For apectomy and NRG-1 *in vivo* experiments, animals were anesthetized with a 0.5 % O₂ / 1 % isoflurane mixture and underwent noninvasive transthoracic echocardiography using a Vevo® 2100 (VisualSonics). LVEF was measured in long-axis B-mode at least three times for each animal. Global longitudinal strain was measured from parasternal long-axis images using speckle-tracking-based imaging to evaluate global cardiac performance. All measurements were obtained by an examiner blinded to the genotype of the animals.

Immunoprecipitation and Western-blotting. For immunoprecipitation (IP) experiments, isolated CMs were lysed in CHAPS buffer (140 mM NaCl / 2 mM EDTA / 25 mM TrisBase, pH 7.4 / 1.5% CHAPS) while for CM protein analysis, isolated CMs were lysed in Triton buffer (150 mM NaCl / 1 mM EDTA / 50 mM TrisBase, pH 7.4 / 1% Triton X-100) both supplemented with complete protease and phosphatase inhibitors (Roche). Protein extracts were immunoprecipitated (1 mg) with anti-Ephrin-B1 (R&D, AF473) or anti-Yap1 (Cell signaling, 4912) antibodies, or directly subjected (50 μ g) to SDS-PAGE and transferred into nitrocellulose membranes. Proteins were detected with primary antibodies followed by Horse Radish Peroxydase-conjugated secondary antibodies to goat, mouse, or rabbit IgG (GE

Healthcare) using enhanced chemoluminescence detection reagent (GE Healthcare). Protein quantification was obtained by densitometric analysis using ImageJ software and was normalized to GAPDH expression and expressed in arbitrary units (A.U.).

Mass spectrometry analysis. Protein samples obtained from isolated CMs (2-month-old-WT mice) and after immunoprecipitation using or not (IP-, control) the anti-Ephrin-B1 antibody were concentrated in a single SDS-PAGE gel band, digested using trypsin and extracted peptides were analyzed by nanoLC-MS with an UltiMate 3000 RSLCnano system coupled to a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) using a data-dependent acquisition mode. After desalting onto a C-18 pre-column (300 μ m ID x 5 mm), peptides were separated on an analytical column (75 μ m ID x 50 cm, Reprosil C18) using a 105-min gradient from 8.75% to 42.5% acetonitrile containing 0.2% formic acid. Survey scan mass spectra were acquired in the Orbitrap at a resolution of 60,000. The 20 most intense ions per survey scan were selected for CID fragmentation. Dynamic exclusion was used within 60 s. Raw MS files were processed with MaxQuant software (version 1.6.0.1). Data were searched against the SwissProt database (release june 2017, Mouse taxonomy, 16902 entries) with the Andromeda search engine. The following parameters were used for data analysis: enzyme specificity: tryptic, 2-missed cleavages; fixed modification: carbamidomethylation of Cys; variable modifications: oxidation at Met, acetylation at protein N-terminus; mass tolerance: 4.5ppm (precursor), 0.5 Da (fragment); minimum peptide length: 7 amino acids. Results were validated by the target-decoy approach with a reverse database at both a peptide and protein FDR of 1%. The “match between runs” option was enabled.

For each protein, a mean LFQ intensity value was computed from two technical replicates nanoLCMS runs. Missing protein intensity values were replaced by a constant noise value.

Enrichment ratios between Ephrin-B1 immuno-purified samples and control samples were calculated from the mean protein intensities derived from three independent biological replicate experiments. Potential Ephrin-B1 interactors were selected based on an enrichment ratio > 1.5 and a Student's t test $P < 0.01$.

Bioluminescence Resonance Energy Transfer (BRET). For BRET experiments, mouse Ephrin-B1 cDNA encoding plasmid was a generous gift from A. Davy (Toulouse, France) and was C-terminally fused in frame to GFP2 in pGFP2-N3 (Perkin). Human Cdc42 and human Cip4 cDNA were a generous gift from F. Gaits/J. Viaud (Toulouse, France) and mouse Yap1 cDNA was obtained from Dharmacon (IMAGE:4239820). Then, Cdc42, Cip4 and Yap1 were fused in frame at the N-terminus region to *Renilla* Luciferase (RLuc8) or to GFP2

in modified vectors derived from pEGFP-C3 (Clontech). All plasmids were fully sequenced. BRET² was measured between *RLuc8*- and GFP2-fusion proteins following transient transfection of appropriate vectors in living HEK293T cells and in real time as previously described⁴¹.

Statistical analysis. The *n* number for each experiment and analysis is stated in each figure legend. All bar graphs represent means $\pm$ s.e.m.. Statistical analyses were performed using Prism v5 software. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ were considered statistically significant; ns: not statistically significant.

Results and discussion:

In the recent years, reinitiating the proliferative activity of differentiated resident cardiomyocytes (CMs) has emerged as an exciting avenue for cardiac regenerative medicine^{4,5}. Theoretically, this challenging concept provides the opportunity to yield sufficient CMs to replace their profuse loss after myocardial damages. Several evidences support the natural self-renewal potential of mammalian adult CMs but this mechanism remains too weak to efficiently repair the heart¹⁻³. Thus, strategies now focus on boosting this regenerative process pharmacologically^{6, 7, 10} or genetically by exploiting factors involved in the postnatal loss of CM proliferation^{8, 9, 11-13}. However, while most of these studies have successfully extended the neonatal regenerative window, they demonstrated only modest impact on the adult CM proliferation and most target manipulations were associated with concomitant cardiac defects, precluding them as ideal candidates for future regenerative therapies. Thus, the characterization of the molecular mechanisms specifically governing the post-mitotic state of adult CMs remains a crucial issue for future therapeutics in human cardiac regenerative medicine.

We recently identified the transmembrane protein Ephrin-B1, a member of the large family of Eph receptors/Ephrin ligands¹⁴, as a new constituent of the adult CM lateral membrane expressed in CMs from all major heart compartments (data not shown). This protein stabilizes adult CM rod-shape and is essential for overall cardiac tissue cohesion in mice¹⁵. However, while global knockout (KO) and CM-specific (cKO) deletions of *efnb1* were associated with a general architectural disorganization of the contractile machinery and intercalated disk together with a focal loss of the typical CM rod-shape within the tissue, 2-month-old KO mice did not exhibit cardiac failure in homeostatic conditions¹⁵. Here, in the continuity, we investigated the long-term consequences of *efnb1* deletion, by examining the cardiac phenotype of aged (12-month-old) *efnb1* KO mice. At the tissue level, the absence of Ephrin-B1 promoted a general disorganization of cardiac tissue architecture, including the loss of the rod-shape of adult CMs (data not shown) in all compartments (data not shown) with the

presence of whorled CMs in some tissue areas (data not shown) but without fibrosis (data not shown). Both wild-type (WT) and KO aged mice similarly reactivated the fetal gene program (BNP, β -MHC encoding genes) as gold standard indicator of cardiac hypertrophy remodeling (data not shown), most probably reflecting compensatory mechanism for ageing stress as suggested by the specific and dramatic breakdown of TGF β 3 inflammatory gene expression in both aged genotypes (data not shown) in agreement with heart senescence¹⁶. Intriguingly, while only a modest septal hypertrophy could be detected in aged WT mice (data not shown), KO mice compensated through marked septum (data not shown) and left ventricle (data not shown) hypertrophy, without alteration of the contractile function for both genotypes (data not shown).

Accordingly, aged KO mice exhibited a mild but significant increase in heart/body weight ratio compared to age-matched WT mice (data not shown). At the cellular level, unexpectedly, 3D-stereology-based analysis showed that aged KO mice exhibited a significant increase in CM density (data not shown) with conversely a reduction in CM volume (data not shown), in agreement with 2-D heart cross section analysis (data not shown), and demonstrating that adult KO mice compensated for ageing stress through atypical CM hyperplasia thereby evoking proliferation. The specific capability of adult CMs to proliferate during ageing in Ephrin-B1 absence was more directly confirmed both *in situ* and *in vitro*. First, immunofluorescence for specific CM markers (cardiac Troponin T, cTnt; or pericentriolar material protein, PCM1¹⁷, despite CM proliferation undervaluation using this marker, data not shown) indicated co-staining with mitosis (aurora kinase B (Aurkb)⁺/DAPI⁺) or cytokinesis (aurora kinase B (Aurkb)⁺/cleavage furrows⁺) markers (data not shown) in the cardiac tissue. Secondly, highly pure CMs (data not shown) demonstrated notable induction of cell cycle gene expression from all phases and specifically involved in CM proliferation^{18, 19} only in aged KO mice (data not shown), while flow cytometry analysis of propidium iodide-stained isolated CM nuclei showed a significant number of aged KO CMs in the DNA replicative S-phase (data not shown; 13.50 ± 3.05 %), compared to the 2- and 12-month-old WT or 2-month-old KO mice that were blocked in the G0/G1- and G2/M-phases, as expected due to their post-mitotic state²⁰. These results were confirmed in the aged CM-specific-KO mice (cKO, data not shown; 7.20 ± 0.91 %), thus precluding the contribution of other cell types in this process and clearly validating adult CM proliferation in the absence of Ephrin-B1. Noteworthy, events from replication, mitosis or cytokinesis cell cycle were not detected in CMs from 2-month-old KO mice (data not shown), indicating a specific and progressive adaptation of *efnb1* KO mice over time to compensate for ageing stress, as already observed at 6-months of age (data not shown), and not a constitutive CM proliferation phenotype of the *efnb1* KO mice at birth. To get further

insight into the capability of these CMs to proliferate over age, we examined their nucleation profile (data not shown) since several studies have demonstrated that mononucleated CMs have a higher proliferation propensity than binucleated CMs^{6, 9, 11, 21}. In agreement with the aged *efnb1* cKO-CM phenotype, at young adulthood (P60, 2-month-old), cKO hearts harbored increased number of mononucleated CMs with conversely relatively less binucleated but also an increased number of tri- and quadri-nucleated CMs than in WT, thus suggesting higher proliferative potential. This basal defect in CM nucleation from cKO was even more evident when examining the postnatal cardiac maturation (postnatal day 10 and 20; P10 and P20) during which CMs progressively lose their proliferative potential (data not shown). Finally and in agreement with proliferation occurring specifically during ageing, the CM multinucleation profile of 12 month-old cKO mice changed and reached that of WT. Indeed, the percentage increase in bi-nucleated CMs ($13.6 \% \pm 1.9$) correlated exactly with the percentage decrease in the number of mono-, tri- and quadri-nucleated CMs ($13.6 \% \pm 0.7$) compared to 2-month-old mice, thus highly suggesting that not only mononucleated as expected, but also tri- and quadri-nucleated CMs participated in the proliferation in aged cKO mice to generate bi-nucleated CMs. These results thus confirmed the intrinsic potential of *efnb1* cKO CMs to proliferate at least during ageing. This proliferative potential at resting state is further reinforced by the dramatic decrease in miR-195 expression in CMs from 2-month-old cKO mice (data not shown) which is consistent with findings from Sadek's group demonstrating the role of miR-195 as a specific mitotic blocker of adult CMs¹².

We next determined whether proliferative potential background of young adult *efnb1* cKO CMs could be further monopolized and manipulated under exogenous mitogenic stimulus neuregulin-1 (NRG-1), a growth factor promoting adult CM proliferation⁶. *In vitro*, 8 days-culture of 2-month-old WT and cKO CMs in the presence of recombinant human NRG-1 resulted in $0.17 \pm 0.07 \%$ bromodeoxyuridine (BrdU) uptake in WT CMs (data not shown), in agreement with a previous report⁶, while the percentage of replicative CMs considerably increased in cKO (data not shown; $9.57 \pm 0.70 \%$ BrdU uptake). Consistent with NRG-1-induced CM proliferation, CMs from cKO hearts exhibited a substantially higher frequency of mitosis (pH3⁺, data not shown) and cytokinesis (aurora kinase B⁺ cleavage furrows, data not shown) events than WT. Proliferation signature of NRG-1 in *efnb1* KO CMs came also from i/ morphological observations showing myofibril delocalization at the CM periphery as previously reported^{6, 11, 20}, and additional nuclei rounding and delocalization in numerous cKO CMs (data not shown) but also from ii/ epigenetic analysis showing DNA-containing foci indicative of heterochromatin (data not shown) confirmed by the expression of gene silencing-

H3K9me3 methylase¹⁸ (data not shown) NRG-1-untreated CMs, contrasting with diffuse DNA-euchromatin (data not shown) and the induction of gene activating-H3K9/14Ac acetylase¹⁸ expression (data not shown) in proliferative NRG-1-treated CMs. NRG-1-induced CM proliferation was finally confirmed *in vivo* since NRG-1 injection in 2-month-old mice (data not shown) that had no impact on cardiac function (data not shown), morphometry (data not shown) or fibrosis (data not shown), significantly induced mitosis (Aurkb⁺/DAPI⁺) of TroponinT⁺ (cTnT⁺) CMs in cKO mice compared to WT (data not shown) (qualitatively confirmed by Aurkb⁺/PCM1⁺ co-labeling, data not shown), but also cytokinesis (Aurkb⁺/Cleavage furrows) (data not shown). Overall, these results demonstrate that resident adult CMs can also proliferate under NRG-1 stimulus in the absence of Ephrin-B1.

To determine whether the absence of Ephrin-B1 in adult CMs could ultimately contribute to cardiac regeneration, a natural process restricted to lower vertebrates^{22, 23}, we first used the surgical model of apectomy^{24, 25} at non-regenerative adulthood (2-month old). WT or cKO mice underwent similar calibrated surgical resection of the left ventricle apex (data not shown). As expected, 21 days after apectomy, analysis of HE- and trichrome-stained heart cross-sections from WT mice clearly showed a significant fibrotic scar replacing the excised tissue area (data not shown) characterized by the deficiency of resident CMs (data not shown) and disorganized vasculature (data not shown). Intriguingly, the resected apex in cKO hearts was replaced by almost normal myocardial tissue with the remarkable presence of a large number of CMs compared to WT (data not shown), considerably reduced fibrosis (~ -50 %, data not shown) and highly organized microvasculature (data not shown). The CMs detected in the resected area of cKO hearts had reentered the cell cycle and undergone cell division, as indicated by the significant increase in BrdU- (data not shown), Aurora kinase B-nuclei- (mitosis; data not shown) and Aurora kinase B-cleavage furrows- (cytokinesis; data not shown) positive CMs (TroponinT⁺ or PCM1⁺ CMs). CM proliferation in cKO mice was not restricted to the resected tissue area since a significant higher number of smaller (data not shown) and BrdU- or Aurora kinase B-positive CMs were identified both at the border of the resected area but also at distance in both ventricles and septum (data not shown), suggesting that the apex resection promoted a global distal tissue compensation most likely for the dynamic ventricular systolic twist defect in which the apex plays a fundamental role²⁶. The cardiac tissue regeneration detected in the *efnb1* cKO mice was consistently associated with a lesser cardiac dysfunction as assessed by echocardiographic left ventricular ejection fraction (data not shown) and global longitudinal strain (data not shown). These results indicate that Ephrin-B1 is a natural blocker of adult CM proliferation *in vivo* that prevents adult cardiac tissue regeneration.

From a better translational therapeutic perspective, we next determined whether *efnb1* deficiency could similarly boost myocardial repair after myocardial infarction (MI). However, we could not use the *efnb1* cKO genetic model since these mice were highly prone to death compared to WT after MI (data not shown), agreeing with an high susceptibility to cardiac mechanical stress as we previously described¹⁵ and with a role for Ephrin-B1 in CM adhesion (data not shown). To bypass this problem and to preserve CMs adhesion during the ischemic shock, we finally deleted Ephrin-B1 only after MI surgery, based on an adeno-associated virus (AAV) serotype 9 strategy with high cardiac tropism and long term expression. Two-month-old C59/BL6 mice underwent permanent left anterior descending coronary artery ligation and were injected intraorbitally with AAV9 vector expressing shmi*Refnb1* or control vector (AAV9-Cherry) 3 days post-MI and immediately after functional selection of mice exhibiting 30-40 % left ventricular ejection fraction (**Fig. 1a, b**). Efficiency of Ephrin-B1 knockdown was successfully validated *in vitro* but also *in vivo* in the heart at the end of the protocol (data not shown). Deletion of *efnb1* considerably favored mice survival after 3-days AAV9 injection and over 56 days post-MI compared to control AAV9 (**Fig. 1c**). Left ventricular ejection fraction was significantly stabilized in infarcted mice injected with AAV-shmi*Refnb1* with some animals exhibiting fully restored LVEF function (**Fig. 1d**). Analysis of whole heart trichrome-stained longitudinal sections showed about 40 % significant fibrosis reduction in AAV9-shmi*Refnb1* injected mice (**Fig. 1e**) indicative of infarct scar size reduction. The increase in heart weight-to-tibia length ratio after MI compared to sham was also significantly higher in these mice, suggesting higher hypertrophy compensation (**Fig. 1f**). However, 56 days after AAV9 vector injection, analysis of the left ventricle remote zone (LV) in MI group indicated the presence of smaller CMs compared to control AAV9 (**Fig. 1g**) and still the presence of mitotic troponin T- (**Fig. 1h, left panel**) or PCM1- (**Fig 1h, right panel**) positive CMs and undergoing cytokinesis (**Fig. 1i**), most likely supporting cardiac compensation through CM hyperplasia. It is noteworthy that trend in CM proliferation could also be measured at distance in the septum (data not shown). In all cases, LVEF in AAV9-injected mice was negatively and significantly correlated with fibrosis and CM area (data not shown). All together, these data indicate that cardiac deletion of Ephrin-B1 immediately after myocardial infarction is beneficial for cardiac function through resident CM proliferation.

We next sought to understand the molecular mechanisms supporting Ephrin-B1 as a repressor of adult CM proliferation. We first confirmed through the use of sh*efnb1* lentiviral vectors that deletion of *efnb1* on isolated adult WT CMs also led to reactivation of the cell cycle and proliferation only following NRG-1 stimulation (data not shown) as observed in the genetic

*efnb1*cKO model. These last years, the effector of the Hippo pathway Yap1 was shown to play a key role in the control of CM proliferation²⁷⁻²⁹. More specifically, a recent study²⁸ demonstrated interplay between the dystroglycan complex (DGC) and Yap1 to inhibit CM proliferation at the CM lateral membrane where Ephrin-B1 is specifically localized. Also, Ephrin-B1 acting independently of the DGC¹⁵, we explored the Hippo pathway in the *efnb1* cKO mice. Yap1 gene or activity were not modified in CMs from cKO or WT mice (data not shown). However, inactive P-Yap1 was delocalized from the CM lateral membrane to the cytosol in cardiac tissue from cKO mice as indicated by the loss of specific lateral immunofluorescent staining (data not shown), confirmed in isolated CMs (data not shown), and conversely increased in heterogeneous cytosolic aggregate staining (data not shown). Noticeably, active Yap1 was absent from CM nuclei (data not shown), in agreement with a lack of CM proliferation in cKO mice at resting state. This effect of *efnb1* deletion on Yap1 delocalization is direct since inactive Yap1 co-immunoprecipitated with Ephrin-B1 in WT but not in cKO CMs (data not shown), as also confirmed by BRET experiments (data not shown). Thus, *efnb1* cKO mice harbor a cytosolic pool of delocalized inactive Yap1, suggesting a potential for proliferation under stress through Yap1 activation and nuclear translocation. We validated this hypothesis both *in vitro* and *in vivo* since NRG-1, ageing or apectomy stimuli all promoted significant Yap1 nuclear translocation (data not shown) and expression of Yap-specific proliferation gene target³⁰ only in CMs from cKO (NRG-1, data not shown). Altogether, these data indicate that Ephrin-B1 sequesters inactive Yap1 at the lateral membrane of adult CM thereby preventing stimulus-promoted Yap-dependent proliferation of the CM.

Since we previously showed that Ephrin-B1 stabilizes the typical rod-shape of the adult CM¹⁵, a morphological process known to occur during the postnatal period and coinciding with the onset of CM proliferation arrest^{19,31} and Yap1 inactivation (data not shown) in agreement with Hippo activity increasing over the course of CM postnatal maturation⁹, we next questioned whether Ephrin-B1 could dually coordinate morphology and proliferation arrest of the adult CM. Mass spectrometry analysis of immunoprecipitated Ephrin-B1 in CMs from WT mice identified Cdc42-interacting protein 4 (Cip4), a specific Cdc42 effector involved in cell polarity-membrane morphology^{32, 33}, as an Ephrin-B1 partner (data not shown), that we confirmed by BRET (data not shown). We then examined the Cdc42-Cip4/Par3/PKC ζ polarity complex³⁴ in the CM of adult WT and cKO mice. Cdc42 (data not shown) or active Cdc42 (data not shown) localized to the lateral membrane of WT CMs similarly to PKC ζ (data not shown) while Par3 was more specifically expressed in the intercalated disk (data not shown). By contrast, we found a specific loss of Cdc42 immunofluorescent staining at the lateral membrane

in the absence of Ephrin-B1 (data not shown), with no spatial modification of PKC ζ (data not shown) or Par3 (data not shown), correlating with loss of the architecture of the CM lateral membrane and of the polarized rod-shape of the CM in *efnb1* cKO mice¹⁵. Western-blot analysis revealed a significant reduced expression of Cdc42 in cKO CMs compared to WT (data not shown). By contrast, while Cip4 also specifically localized to the lateral membrane of WT CMs (data not shown), it delocalized from the lateral membrane to the cytosol in the cKO as indicated by loss of lateral immunofluorescent staining (data not shown) but preserved global Cip4 expression in the cKO CMs (data not shown), most likely suggesting a direct interplay between Ephrin-B1 and Cdc42 and a subsequent Cip4 release in the cytosol. Accordingly, Cdc42 could be immunoprecipitated with Ephrin-B1 in CMs from WT mice (data not shown) and the interaction was confirmed by BRET (data not shown). Noticeably, the BRET signal between Ephrin-B1 and Cdc42 was similar to that detected for the direct Cdc42-Cip4 interaction (data not shown) but much less than that detected between Ephrin-B1 and Cip4, most likely suggesting an indirect interaction between Ephrin-B1 and Cip4 in the Ephrin-B1-Cdc42 complex. Interestingly, Yap1 interacted with Cip4 in CMs from WT mice but also from cKO mice (data not shown), most likely indicating a Yap1-Cip4 complex cytosolic release in the absence of Ephrin-B1. Cdc42 could also be detected in complex with Yap1 (data not shown), most likely indicating that Cdc42-Cip4 are part of the Ephrin-B1-Yap1 complex. In agreement, pharmacological inhibition of Cdc42 with casin in CMs from WT animals phenocopied the *efnb1* cKO phenotype with significant CM mitosis (data not shown), Yap1 nuclearization (data not shown) and induction of Yap-dependent proliferation gene (data not shown) upon NRG-1 stimulation only. Finally, the Ephrin-B1-Cdc42-Cip4-Yap1 complex localization at the lateral membrane is specific of the adult CM since it did not localize to the plasma membrane of CMs from WT neonatal mice (data not shown), despite expression of Ephrin-B1 in neonatal CMs¹⁵ but a role for Ephrin-B1 independent of CM proliferation at this stage (data not shown), in agreement with normal heart morphogenesis and function in 3-week-old *efnb1* KO mice¹⁵. Altogether, these data indicate that Ephrin-B1 forms a complex with both the polarity complex Cdc42-Cip4 and the Hippo effector P-Yap1 at the lateral membrane of the adult rod-shaped CM, thus acting as a hub for the adult CM polarity and proliferation arrest.

Overall, our study reveals for the first time an essential and dual role of Ephrin-B1 in coordinating both the polarization/proliferation of the adult CM by a mechanism relying on a Ephrin-B1-Cdc42 core complex scaffolding both Cip4 and Yap1 during the postnatal period at the lateral membrane and thereby controlling the post-mitotic state of the adult CMs (data not shown). In the future, identification of the signaling networks leading to Ephrin-B1-Cdc42-

Cip4-Yap1 complex formation and targeting to the lateral membrane of adult CMs will be determinant as they could constitute new targets to reactivate the adult CM proliferation. Finally, this study demonstrates that therapeutic targeting of Ephrin-B1 in the adult heart could represent an attractive new strategy in regenerative cardiac medicine using resident CMs for the treatment of post-ischemic patients.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of promoting proliferation of cardiomyocytes in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Ephrin-B1 inhibitor.
- 5 2. A method of promoting cardiac regeneration in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Ephrin-B1 inhibitor.
3. A method of treating cardiac injury in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Ephrin-B1
10 inhibitor.
4. The method of claim 1, 2 or 3 wherein the subject suffers from a cardiac pathology.
5. The method of claim 4 wherein the subject suffers from heart failure or myocardial infarction.
6. The method of claim 4 wherein the subject has one or more signs or symptoms of acute
15 myocardial infarction.
7. The method of claim 6 wherein the Ephrin-B1 inhibitor is administered simultaneously or sequentially (i.e. before or after) with a revascularization procedure performed on the subject.
8. The method of claim 1, 2 or 3 for preventing heart failure in a subject who experienced
20 a myocardial infarction.
9. The method of claim 1, 2 or 3 wherein the Ephrin-B1 inhibitor is an antibody, in particular an antibody having specificity for Ephrin-B1.
10. The method of claim 1, 2, or 3 wherein the Ephrin-B1 inhibitor is an inhibitor of Ephrin-B1 expression.
- 25 11. The method of claim 10 wherein the inhibitor of expression is a siRNA, an endonuclease, an antisense oligonucleotide or a ribozyme.

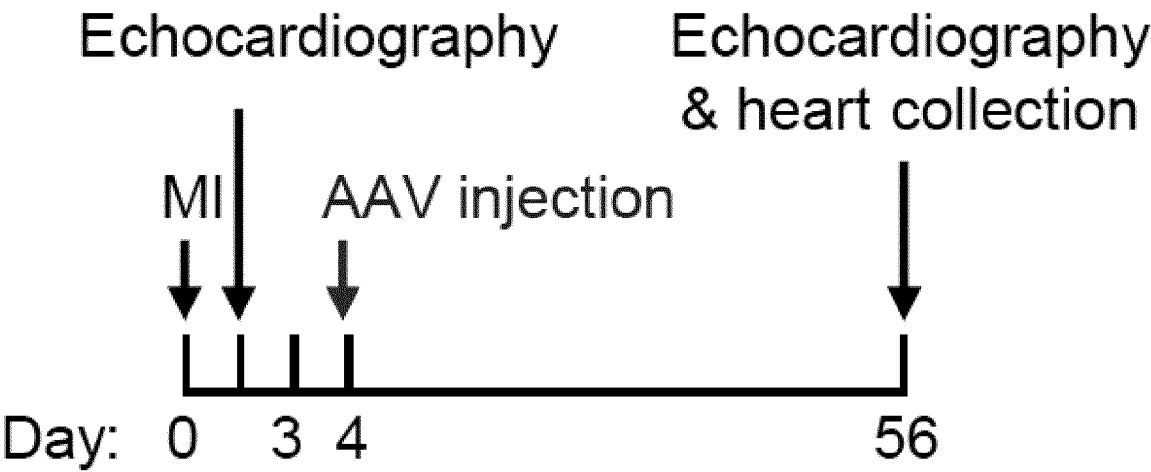


Figure 1a

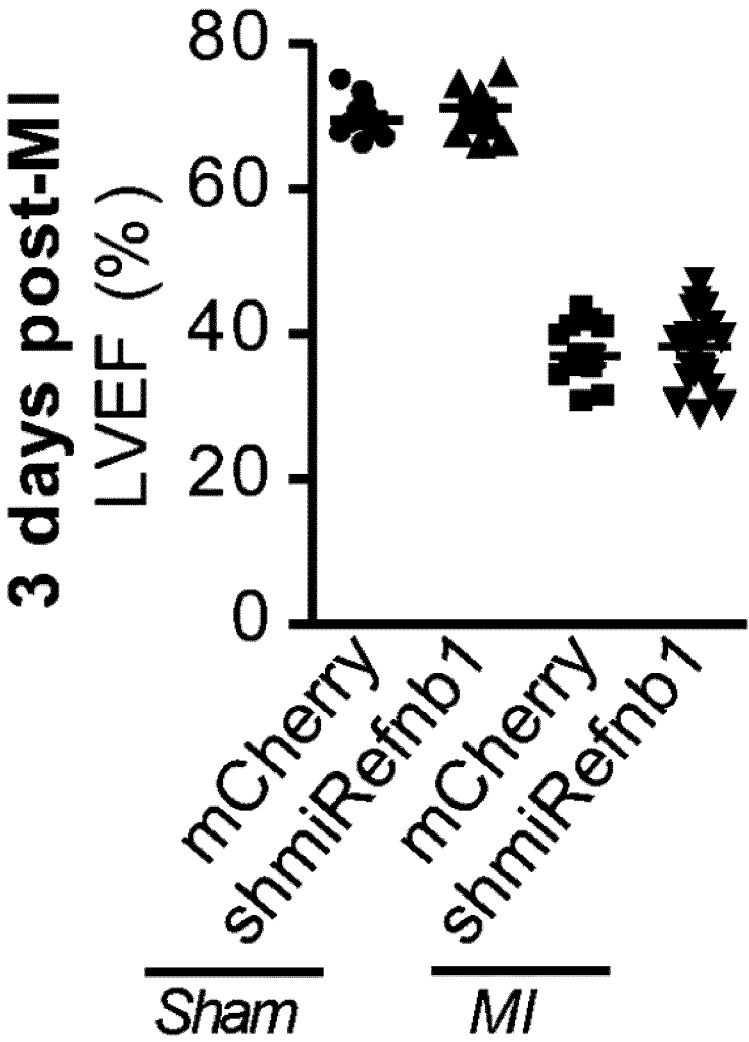


Figure 1b

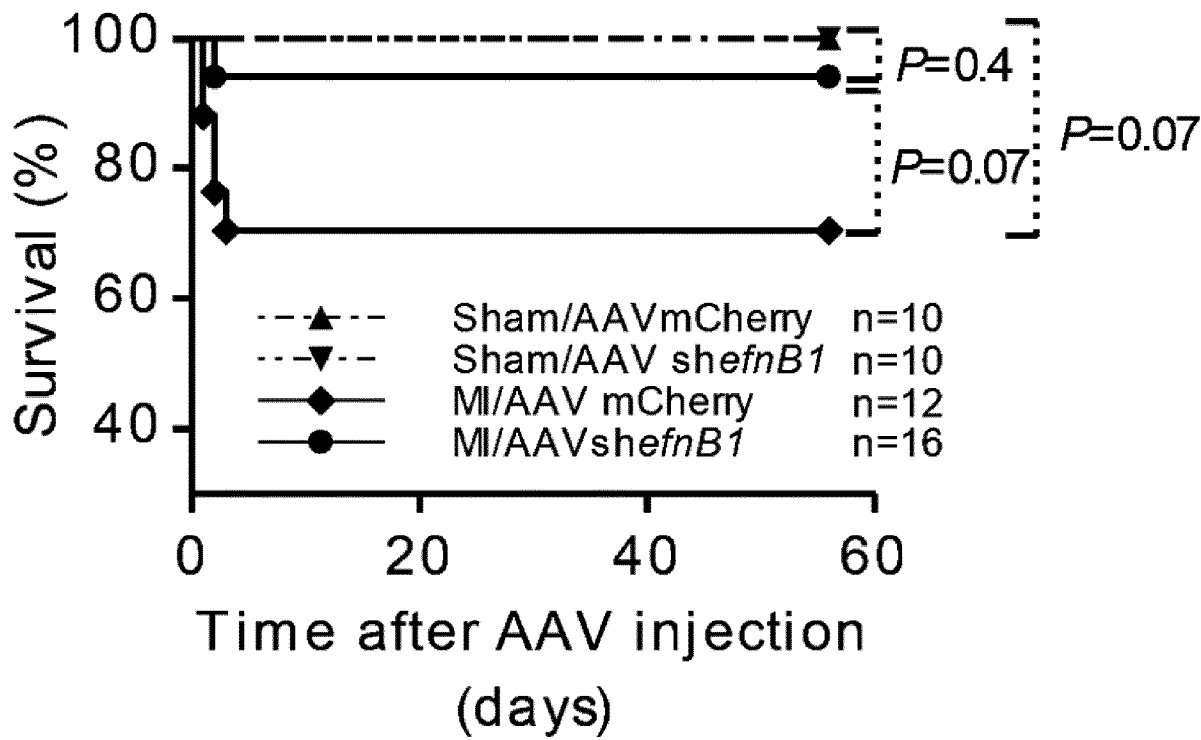


Figure 1c

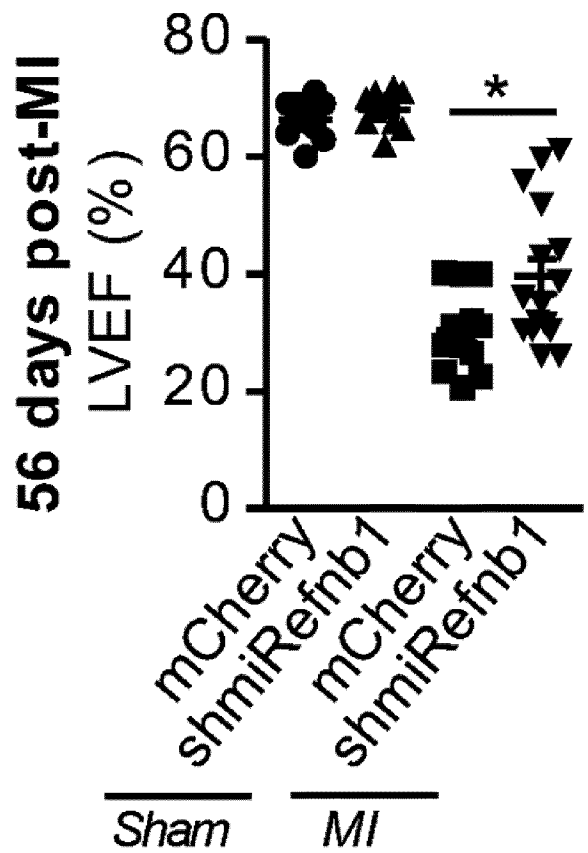


Figure 1d

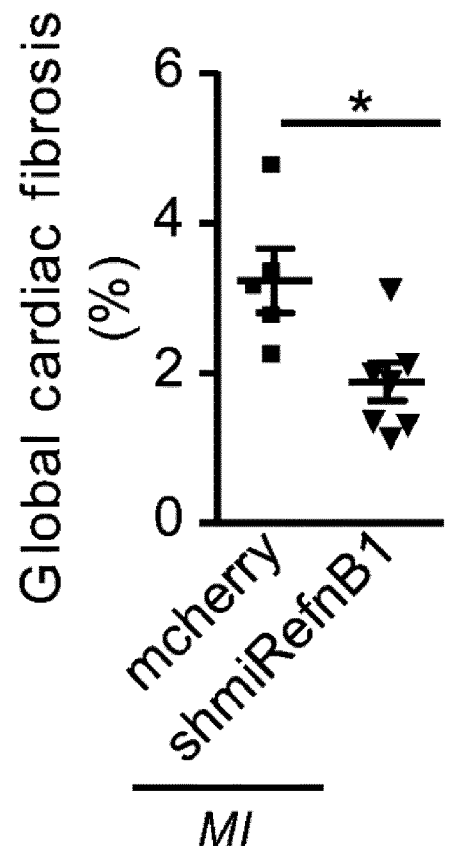


Figure 1e

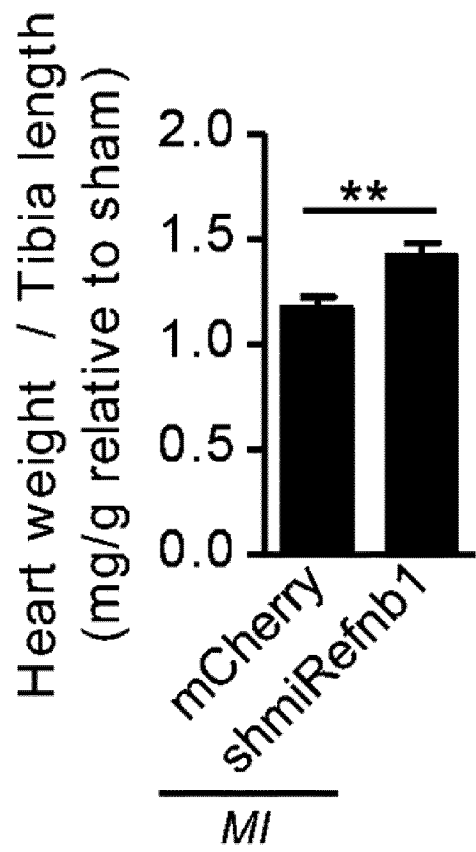


Figure 1f

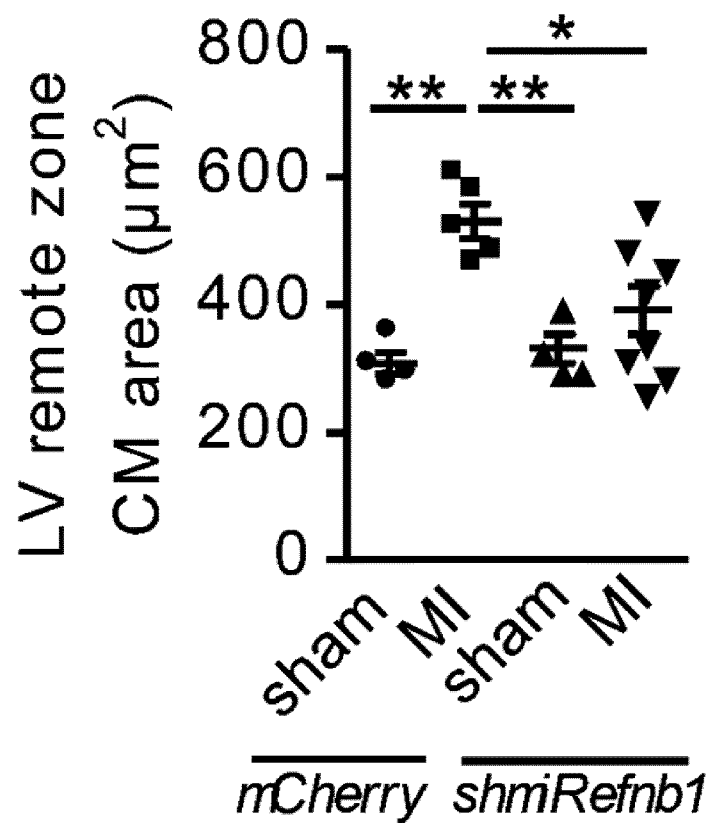


Figure 1g

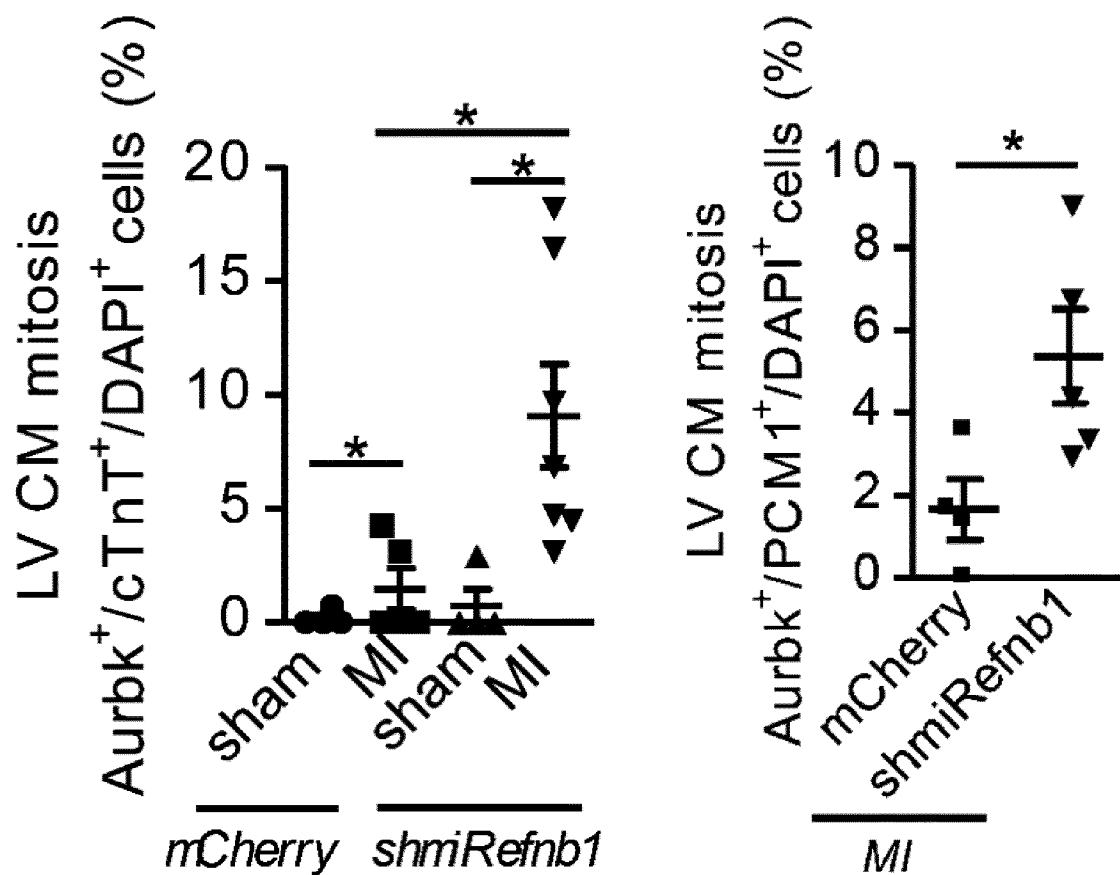


Figure 1h

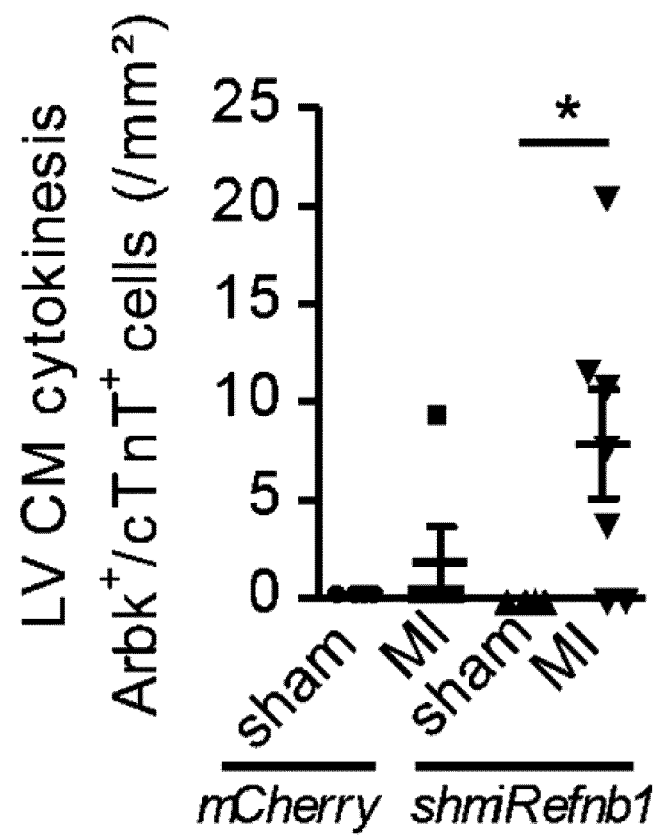


Figure 1i

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/082547

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K38/17 A61K38/19 A61K9/00 C07K14/705 A61P9/10 ADD.											
According to International Patent Classification (IPC) or to both national classification and IPC											
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K C07K A61P Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, Sequence Search, WPI Data											
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>A</td> <td>KULLANDER KLAS ET AL: "Mechanisms and functions of Eph and ephrin signalling", NAT REV MOL CELL B,, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 475-486, XP009152563, ISSN: 1471-0072 the whole document -----</td> <td>1-11</td> </tr> <tr> <td>X</td> <td>KUHN BERNHARD ET AL: "Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair", NATURE MEDICINE, NATURE PUB. CO, vol. 13, no. 8, 1 August 2007 (2007-08-01), pages 962-969, XP002568319, ISSN: 1078-8956, DOI: 10.1038/NM1619 [retrieved on 2007-07-15] abstract ----- -/-</td> <td>1-8,10</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	A	KULLANDER KLAS ET AL: "Mechanisms and functions of Eph and ephrin signalling", NAT REV MOL CELL B,, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 475-486, XP009152563, ISSN: 1471-0072 the whole document -----	1-11	X	KUHN BERNHARD ET AL: "Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair", NATURE MEDICINE, NATURE PUB. CO, vol. 13, no. 8, 1 August 2007 (2007-08-01), pages 962-969, XP002568319, ISSN: 1078-8956, DOI: 10.1038/NM1619 [retrieved on 2007-07-15] abstract ----- -/-	1-8,10
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A	KULLANDER KLAS ET AL: "Mechanisms and functions of Eph and ephrin signalling", NAT REV MOL CELL B,, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 475-486, XP009152563, ISSN: 1471-0072 the whole document -----	1-11									
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☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.											
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Date of the actual completion of the international search		Date of mailing of the international search report									
18 February 2019		07/03/2019									
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer									
		Scheffzyk, Irmgard									

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/082547

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANA EULALIO ET AL: "Functional screening identifies miRNAs inducing cardiac regeneration", NATURE, vol. 492, no. 7429, 5 December 2012 (2012-12-05), pages 376-381, XP055342709, ISSN: 0028-0836, DOI: 10.1038/nature11739 abstract -----	1-8,10, 11
X	Molamma P Prabhakaran ET AL: "Differentiation of embryonic stem cells to cardiomyocytes on electrospun nanofibrous substrates", Journal of Biomedical Materials Research Part B: Applied Biomaterials, 1 April 2014 (2014-04-01), pages 447-454, XP055439070, United States DOI: 10.1002/jbm.b.33022 Retrieved from the Internet: URL:https://core.ac.uk/download/pdf/828317 65.pdf abstract nos. 0202 and 0190 -----	1-11