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(54) Title: STEM CELL-PRODUCED MICROVESICLES FOR TREATING TENDON PATHOLOGIES

(57) Abstract: Microvesicles produced by stem cells grown on a silk scaffold for enhancing stem cell self-renewal/proliferation and promoting tenogenesis in damaged tendons, and their use in tendon wound repair and tendinopathy treatment. Also provided are compositions containing the microvesicles and devices for obtaining them.

STEM CELL-PRODUCED MICROVESICLES FOR TREATING TENDON PATHOLOGIES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 62/378,392, filed August 23, 2016, the contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] The disclosures of all references and all patents, patent application publications and books referred to herein are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

[0003] It takes a long time for a tendon injury to heal, even where it is possible. In most cases, the ruptured tendon does not heal, but forms scar tissue at the injury site. The impaired healing leads to the wounded tendon having impaired mechanical properties and reduced function, and often requires revision surgery. In addition, tendinopathies (chronic tendon degenerative disorders) are also associated with tendon pain and impaired function. Tendinopathy in most cases is due to tendon overload which leads to microscopic collagen fiber failure and a failed healing response, often resulting in tendon rupture. There is currently no cure for tendinopathy.

[0004] The present invention address the need for improved tendon rupture and tendinopathy treatments.

SUMMARY OF THE INVENTION

[0005] The present invention can enhance self-renewal of the resident stem cells in tendons, and modify the impaired local stem/progenitor cell fate and function to promote tenogenesis and tendon regeneration to improve the disease condition and its associated pain.

[0006] A method is provided of treating a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to treat a damaged tendon.

[0007] Also provided is a method of reducing scar formation in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to treat reduce scar formation.

[0008] Also provided is a method of accelerating healing of a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to accelerate healing of a damaged tendon.

[0009] Also provided is a method of enhancing tenogenesis in a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to enhance tenogenesis.

[0010] Also provided is a pharmaceutical composition for treating a damaged tendon comprising a sterile pharmaceutically acceptable carrier and an amount of purified microvesicles derived from mammalian mesenchymal stem cells.

[0011] Also provided is a method of producing microvesicles derived from mammalian mesenchymal stem cells comprising obtaining adipose-derived stem cells from a mammalian subject by a cannula; placing the adipose-derived stem cells into a sterile culture system comprising a scaffold of longitudinal fibers under tension and under conditions permitting growth of the adipose-derived stem cells on the scaffold of longitudinal fibers; and recovering microvesicles secreted from the cultured adipose-derived stem cells under sterile conditions.

[0012] Also provided is a device comprising a 3D scaffold of plurality of fibers substantially aligned in a parallel manner along their longitudinal axes and held under at least 15N of tension in a longitudinal direction of the fibers.

[0013] Additional objects of the invention will be apparent from the description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Fig. 1A-1D. Gene expression profile analysis of micro-vesicles (1A) Gene expression profile that indicates TSPCs treated with Micro-vesicles-S exhibit increased expression of tendon markers ((1A) Scx, Tnmd) and reduced levels of stem cell ((1B) Oct-4, Nst), chondrogenesis ((1C) Sox9), and adipogenesis markers ((1D) PPARγ, LPL), with no change in osteogenic marker (Runx, not shown). Scale bar=100nm. Veh: Vehicle, MV-C: Micro-vesicles-C, MV-S: Micro-vesicles-S. Results represent triplicates of TSPCs treated with micro-vesicles from 37, 42, or 59 y.o. patients.

[0015] Fig. 2A-2G. Therapeutic effect of Micro-vesicles-S injec tendinopathy in rats. The animals were subjected to overuse treadmill running for 6 weeks. At two weeks after the start of treadmill running, rats were injected with Micro-vesicles-S [MV-S], Microvesicles-C [MV-C], or placebo [P]. The animals maintained in cages served as a naïve controls. Histology/immunohistochemistry, mechanical property testing, and pain behavior assessment were carried out in the animals at the end of the experiment. Compared to placebo and exosome control-treated rats, Micro-vesicles-S-treated tendinopathy rats had tendons with (2A) collagen-fiber alignment similar to naïve controls, as shown in H&E stained histology sections. The images were representative at sites most susceptible to tendinopathy, at the bone-tendon insertion and midsubstance site of tendon. The improved cellular alignment was supported by quantification of (2B) cell alignment and (2C) cell shape. A comparable reduction in (2D) cleaved collagen and an (2E) increase in collagen I/III were detected in Micro-vesicles-S-treated tendinopathy rats and naïve controls, compared to control treatments. (2F) Micro-vesicles-S-treated tendinopathy rats traveled a longer distance in an open field within a 10 minute measurement. (2G) Supraspinatus tendons from Micro-vesicles-S-treated tendinopathy rats exhibited higher maximum load and maximum stress. *$p<0.05$, one-way ANOVA with Tukey post-hoc test.

[0016] Fig. 3A-3C. MSC attachment after culture on a novel silk scaffold. (3A) Image of scaffold. (3B) Scanning electron microscope image of scaffold. (3C) Immunocytochemistry image of MSCs grown on a silk scaffold for 7 days (blue = DAPI, green = β-actin).

[0017] Fig. 4A-4D. MSC differentiation toward tenocytes on rope-like silk scaffold. (4A) Relative mRNA expression of Scx at 0, 4, and 12 h, and 1 day and 7 days after seeding MSCs onto the silk scaffold. (4B) Relative mRNA expression levels of Scx, Tnmd, Nst, Oct4, Sox9, Col2a1, Runx2, and Alp in MSCs grown on silk-coated plates or silk scaffolds at 7 days after MSCs were seeded. (4C) Immunocytochemistry staining (blue = DAPI, red = Scx or Tnmd) and (4D) western blot of Scx and Tnmd in cells that were seeded on silk-coated plates and silk scaffolds. Data represent three independent experiments per assay. *$P < 0.05$ compared to MSCs grown on silk-coated plates. Scale bar = 50 μm.

[0018] Fig. 5A-5G. Cytochalasin D disrupted actin cytoskeletal arrangement in cells attached to scaffold fibers and abolished the elongated cell body [(5A) vs (5B)] and associated nuclear shape change [(5C) vs (5D)]. (5E) shows the quantitative analysis of the cell body and nuclear shape changes. Cytochalasin D treatment also diminished the

expression of tendon phenotypic markers Scx and Tnmd in these ce
immunocytochemistry (blue = DAPI, red = Scx or Tnmd). (5G) Relative mRNA and (5H) western blot of Scx and Tnmd expression with (i) relative mRNA expression of the housekeeping genes Hrpt1 and Pgk1 as an internal control. Data represent at least three independent experiments per assay. *P < 0.05 compared to MSCs seeded on a scaffold without cytochalasin D. Scale bar = 25 μm.

[0019] Fig. 6A-6C. Disruption of Rho/Rock Pathway signaling and stress fiber formation are required for tendon lineage determination. (6A) Immunocytochemistry of β-actin in MSCs grown either with no treatment or treated with Latrunculin A, Y-27632 and Blebbistatin for 7 days (blue = DAPI, green = β-actin). *P < 0.05 for all treatments. (6B) mRNA expression and protein expression of Scx and mRNA expression of (6C) Col1a1 and Nfatc4 Nst in MSCs grown on silk scaffolds following treatment with Latrunculin A (LatA), Y-27632 (Y) and Blebbistatin (Bleb) for 24 h. Data represent three independent experiments per assay.

[0020] Fig. 7A-7B. Morphology and size distribution of micro-vesicles grown on a novel silk scaffold detected by TEM (7A) and light scattering (7B), respectively.

DETAILED DESCRIPTION OF THE INVENTION

[0021] This invention is based on the discovery that microvesicles produced by stem cells under specific conditions, such as growing on a novel silk scaffold, enhance stem cell self-renewal/proliferation and promotes tenogenesis in vitro, and exert therapeutic effects on tendon wound repair and tendinopathy in vivo.

[0022] Mesenchymal stem cells ("MSC", e.g. adipose-derived MSC, bone-marrow-derived MSC, bursa-derived MSC, tendon stem/progenitor cells) at passage 0 (P0) to P2 are cultured on a novel silk rope-like scaffold, which results in the cells to differentiate towards the tenogenic lineage. Within a special window of time, these cells produce and extracellularly secrete microvesicles. This microvesicles can modulate stem cells by enhancing their self-renewal/proliferation and by promoting tenogenesis and accelerate wound or disease healing. The microvesicles can be made by autologous cells so as to provide an autologous therapy for tendon injuries and tendinopathy. In an embodiment, microvesicles are collected from cultured MSC obtained from fat tissue from a subject, e.g. an athlete, or an active and/or aged individuals who is at high risk for, or suffering from, a tendon injury or tendinopathy. In an embodiment the microvesicles are banked before

injury and available for administration via, for example, local inject
diseased tendon site of the patient when the injury or disease occurs.

[0023] A method is provided of treating a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to treat a damaged tendon.

[0024] As used herein, microvesicles "derived from" or "isolated from" mammalian mesenchymal stem cells are microvesicles made by mammalian mesenchymal stem cells and then obtained. Such microvesicles are usually secreted extracellularly by the cells and are collected from, for example, a culture medium in which the mammalian mesenchymal stem cells are present.

[0025] As used herein "treating" a damaged tendon means ameliorating one or more signs or symptoms of tendon damage. For example, amelioration of damaged tendon tissue structure disruption by restoration (full or partial) of tendon cellular and matrix composition, amelioration of damaged tendon mechanical and functional performance by restoration (full or partial) mechanical properties including strength and elasticity of tendon tissue, amelioration of damaged tendon pain, are each examples of treating a damaged tendon.

[0026] Also provided is a method of reducing scar formation in a damaged or "repaired" tendon tissue in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to reduce scar formation. Scar formation or fibrosis in tendon results of non- or incomplete tendon repair or regeneration of damaged tendon tissues. Scar tissues are composites with fibroblasts instead of tenocytes, which produce non-tendon like matrix components with much weaker mechanical property and often lead to re-injury. As used herein "reducing scar formation" in a damaged tendon means ameliorating one or more signs or symptoms of scar formation. For example, reducing the extent of scar formation in a tendon as compared to the extent of scar formation in an equivalent untreated damaged tendon.

[0027] Also provided is a method of accelerating healing of a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to accelerate healing of a damaged tendon. As used herein "accelerating healing" of a damaged tendon means increasing the rate of healing of a damaged tendon as compared to the rate of healing in an equivalent untreated damaged tendon or reducing the time taken to achieve a predetermined

point in the healing process as compared to the time taken predetermined point in an equivalent untreated damaged tendon.

[0028] Also provided is a method of enhancing tenogenesis in a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to enhance tenogenesis. As used herein "enhancing tenogenesis" in a damaged tendon means increasing the rate and/or amount of tenogenesis in a damaged tendon as compared to the rate and/or amount of tenogenesis, respectively, in an equivalent untreated damaged tendon.

[0029] In an embodiment of the methods, the microvesicles have been secreted from mesenchymal stem cells from sources including, but not limited to unless specified, bone marrow, adipose tissue and various tendon tissues. MSCs derived from tendon tissues such as human rotator cuff, and long head of biceps, can be isolated based on plastic adhesion in culture and are phenotypically characterized by flow cytometry with cell surface markers with CD90+, CD146+, CD 44+ and CD34-, CD117-, CD18, CD45-.

[0030] In an embodiment of the methods, the mesenchymal stem cells have been cultured on a specifically defined silk scaffold prior to the microvesicles being derived from the mesenchymal stem cells.

[0031] In an embodiment of the methods, the mesenchymal stem cells within passages (P)1 to P3 following passage 0, which is derived from the the cell population isolated via enzymatic digestion of human tissue specimen such as tendon and based on plastic adherence in cell culture have been cultured on a silk scaffold.

[0032] In an embodiment of the methods, the mesenchymal stem cells (MSC) are adipose-derived MSC, bone marrow-derived MSC, bursa-derived MSC, or are tendon progenitor cells.

[0033] In an embodiment of the methods, the mesenchymal stem cells (MSC) are from the same species as the subject. In an embodiment of the methods, the mesenchymal stem cells (MSC) are from the same subject that is being treated.

[0034] In an embodiment of the methods, the damaged tendon is damaged as a result of a tendinopathy. In an embodiment of the methods, the damaged tendon is damaged as a result of physical injury to the tendon. In an embodiment of the methods, the damaged tendon is a ruptured tendon.

[0035] In an embodiment the tendon has been treated conservatively with rest, analgesia (such as non-steroidal anti-inflammatory drugs), physiotherapy, and/or surgery repair. For

example, for proximal biceps tendon rupture, patients may benefit i
inflammatory drugs and physiotherapy, while early surgical repair yields the best results for complete quadriceps tendon ruptures. However, surgery must be performed early in order to avoid scarring of the biceps. With delayed treatment, the biceps may be attached to the brachialis.

[0036] In an embodiment of the methods, the amount of microvesicles is administered locally into the tendon. In an embodiment of the methods, the amount of microvesicles is administered by injection.

[0037] In an embodiment of the methods, the amount of microvesicles has been cryopreserved prior to administration. In an embodiment of the methods, the amount of microvesicles has been cryopreserved below -50°C. In an embodiment of the methods, the amount of microvesicles has been cryopreserved below -75°C. In an embodiment of the methods, the amount of microvesicles has been cryopreserved at -80°C or below.

[0038] In an embodiment of the methods the microvesicles can be administered a plurality of times over a series of time. For example, microvesicles in 0.9% NaCl is administrated via injection to the wounded tendon, weekly or used as a single dose treatment.

[0039] In one embodiment where microvesicles are being administered, an effective amount is a single administered dose. In one embodiment where microvesicles are being administered, an effective amount will be administered over a series of individual doses.

[0040] In an embodiment, the routes of administration is local, e.g. by direct injection. For example, the microvesicles (e.g. naked or in a carrier) can be injected directly into the damaged tendon. Other embodiments of the routes of administration encompassed by the methods of the invention include, but are not limited to, each of the following individual routes, and any subset thereof, auricular, buccal, conjunctival, cutaneous, subcutaneous, endocervical, endosinusial, endotracheal, enteral, epidural, via hemodialysis, interstitial, intrabdominal, intraamniotic, intra-arterial, intra-articular, intrabiliary, intrabronchial, intrabursal, intracardiac, intracartilaginous, intracaudal, intracavernous, intracavitary, intracerebral, intracisternal, intracorneal, intracoronary, intradermal, intradiscal, intraductal, intraepidermal, intraesophagus, intragastric, intravaginal, intragingival, intraileal, intraluminal, intralesional, intralymphatic, intramedullary, intrameningeal, intramuscular, intraocular, intraovarian, intraepicardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrasinal, intraspinal, intrasynovial, intratendinous, intratesticular, intrathecal, intrathoracic, intratubular, intratumor, intratympanic, intrauterine, intravascular,

intravenous, intraventricular, intravesical, intravitreal, laryngea
ophthalmic, oral, oropharyngeal, parenteral, percutaneous, periarticular, peridural, rectal, inhalationally, retrobulbar, subarachnoid, subconjuctival, sublingual, submucosal, topically, transdermal, transmucosal, transplacental, transtracheal, ureteral, uretheral, and vaginal administration.

[0041] Preferred administration is direct, local administration. In an embodiment, the administration is injection into the tendon. In an embodiment, the administration is injection into the subject within up to 2cm from the tendon.

[0042] In an embodiment, the mammalian subject is a human.

[0043] Also provided is a pharmaceutical composition for treating a damaged tendon comprising a sterile pharmaceutically acceptable carrier and an amount of purified microvesicles derived from mammalian mesenchymal stem cells. In an embodiment, the pharmaceutically acceptable carrier comprises a buffered saline. In an embodiment, the pharmaceutically acceptable carrier comprises phosphate-buffered saline (PBS), optionally containing 1mM EGCG.

[0044] Also provided is a method of producing microvesicles derived from mammalian mesenchymal stem cells comprising obtaining adipose-derived stem cells from a mammalian subject by a cannula; placing the adipose-derived stem cells into a sterile culture system comprising a scaffold of longitudinal fibers under tension and under conditions permitting growth of the adipose-derived stem cells on the scaffold of longitudinal fibers; and recovering microvesicles secreted from the cultured adipose-derived stem cells under sterile conditions.

[0045] In an embodiment, recovering the microvesicles comprises obtaining culture medium conditioned by the cultured adipose-derived stem cells and ultracentrifuging the conditioned medium under sterile conditions so as to obtain a pellet comprising the microvesicles. In an embodiment, the culture medium is a serum-free medium. In an embodiment, the method further comprises aliquoting the microvesicles for therapeutic use. In an embodiment, the method further comprises freezing the microvesicles for future use. In an embodiment, the ultracentrifuging comprises 1) 300 x g, for 5-15 mins; 2) 2000 x g, for 5-15 mins; 3) 10,000 x g, for 25-35 mins; 4) 100,000 x g, for 65-75 mins, with the supernanatant being collected after each step, and then washing the pellet (e.g. in phosphate buffered saline (PBS)) an ultracentrifuging once more (100,000 x g, for 65-75 mins), to yield the microvesicles. In an embodiment, the microvesicles are isolated by differential

ultracentrifugation as follows, with the supernatant being collected a
g, 10 minute; 2) 2000 x g, 10 minute; 3) 10,000 x g, 30 minute; 4) 100,000 x g, 70 min. The resulting pellet is washed in PBS, and subjected to one more centrifugation step (100,000 x g, 70 min), which yields the microvesicles.

[0046] In an embodiment, the microvesicles obtained have an average diameter of 40nm - 100nm as measured by negative staining transmission electron microscopy.

[0047] In an embodiment, the longitudinal fibers are silk fibers. In an embodiment, longitudinal fibers are substantially aligned in a parallel manner along their longitudinal axes and held under at least 15N of tension in a longitudinal direction of the fibers.

[0048] Also provided is a device comprising a 3D scaffold of plurality of fibers substantially aligned in a parallel manner along their longitudinal axes and held under at least 16.5N to 19.8N of tension in a longitudinal direction of the fibers.

[0049] In an embodiment, the fibers are held under at least 16.5N to 19.8 of tension in a longitudinal direction of the fibers. In an embodiment, the fibers have an average diameter of 11 uM to 15 uM. In an embodiment, the 3D scaffold is contained within a sterile container.

[0050] In an embodiment, the device further comprises an amount of mammalian mesenchymal stem cells adhered to or growing along the fibers. In an embodiment, the silk fibers are made of Raw Bombyx mori 20122 density silk fiber with chemical modification (Maharam et al. 2015, *Bone Research*, **3**, 15015). The fibers also can be made of other biodegradable and cell growth-friendly surfaces with similar physical properties (such as 11 uM to 15 uM diameter, under 16.5N to 19.8 tension) .

[0051] All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0052] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

Experimental Results

Introduction

[0053] Unlike current concepts and techniques of stem cell-based therapies, the therapeutic treatment disclosed herein uses microvesicles secreted by stem cells, but not the stem cells themselves. Thus, different from cell-based therapy, there are no live-cell-related hurdles or concerns during product production, storage, transportation, and administration. In particular, this product can be cryopreserved (for example at -80 °C or in liquid nitrogen) for years. One-time production of the microvesicles can be used for multiple treatments. If desired, the microvesicles can even be made while an individual is healthy and stored for later use. These unique microvesicles are produced when stem cells grow on a scaffold and are reprogrammed through physical means. There are no special exogenous chemicals or genetic modifications required to reprograms the cells. Furthermore, the microvesicles can be produced by stem cells which are derived from the subject's own tissues such as fat, bone marrow, and bursa, which are all easy to obtain with no harm, and easy to administer without immune-rejection concerns to the subject.

Examples

[0054] I. Exogenous micro-vesicles derived from MSCs grown on a novel scaffold enhances TSPC tenogenic gene expression in vitro: Human primary mesenchymal stem cells (105 cells), such as tendon stem/progenitor cells (TSPCs as an example of MSCs), isolated from the biceps tendon of patients undergoing shoulder replacement surgery (37, 42, 59 y.o.) were cultured on a novel scaffold or regular 2D culture. After 2 days of culture, 2ml of the 4ml of conditioned medium was harvested and the micro-vesicles within the medium were collected by ultracentrifugation. Micro-vesicles isolated from the conditioned medium of MSCs grown on the novel scaffold are termed: "Micro-vesicles-S". Micro-vesicles isolated from the conditioned medium of MSCs grown in 2D culture are termed: "Micro-vesicles-C".

[0055] TSPCs cultured with exogenous Micro-vesicles-S (10μg/ml) resulted in the following gene expression profile changes in TSPCs: 1) increased expression of tenogenic markers scleraxis (Scx) and tenomodulin (Tnmd) (Fig 1A), 2) decreased expression of stem cell markers Oct-4 and Nst (Fig 1B), chondrogenesis marker Sox9 (Fig 1C), and adipogenesis markers PPARγ and LPL (Fig 1D), compared to Micro-vesicles-C or vehicle

control (cell culture media). Micro-vesicles-S isolated from a 69
similar effect as Micro-vesicles-S from the 37-59 y.o. patients (data not shown).

[0056] II. Micro-vesicles-S exerts therapeutic efficacy in an overuse-induced tendinopathy rat model: The potential efficacy of Micro-vesicles-S for treating tendinopathy was tested using the overuse-induced tendinopathy model in rats. Micro-vesicles-S or Micro-vesicles-C (20μg in 10μl phosphate buffered saline [PBS]) produced by TSPCs from a 69 y.o. patient were injected into the supraspinatus tendons of adult nude rats (5-6 months-old, Charles River) two weeks after starting a total 6-week overuse-induced tendinopathy protocol (10° decline treadmill running for 17 m/min, 1 hour/day, 5 days a week). Other control animals included a placebo (injected with PBS) and naïve (normal cage activity without injection). Four weeks after injection, the animals were sacrificed and the supraspinatus tendons were harvested and subjected to immunohistochemistry and mechanical property testing.

[0057] In contrast to placebo and Micro-vesicles-C, the diseased Achilles tendons in rats treated with Micro-vesicles-S exhibited tissue morphology with cell alignment and cell shape similar to that in naïve controls as revealed in H&E staining (Fig 2A), and quantitatively characterized by cell alignment (angle between individual cell axis and tissue longitudinal axis) (Fig 2B) and cellular shape (cell length / cell width) (Fig 2C). The diseased Achilles tendons in rats treated with Micro-vesicles-S also exhibited a comparable reduction in cleavage of collagen type I (Fig 2D), the predominant collagen in native mature tendons, increased intensity of type I collagen (Col I), decreased intensity of Col III, a collagen subtype often associated with scar or immature tendon (Fig 2E), and a higher number of cells expressing Scx and Tnmd (not shown). The Micro-vesicles-S group was not statistically different from naïve animals ($p>0.05$). Furthermore, Micro-vesicles-S-treated tendinopathy rats exhibited less pain as suggested by an increased total distance traveled in an open field assay (Fig 2F), and had tendons with stronger mechanical properties (Fig 2G). These results are consistent with the efficacy of Micro-vesicles-S produced from MSCs of young and middle-aged groups (37, 42, 59 y.o.) based on preliminary studies (not shown).

[0058] III. MSC Culture

[0059] 1. MSCs cultured on the novel scaffold elongate along the scaffold fibers. The custom-made silk scaffold (LxWxD, 30mm x 1mm x 1mm) consists of well-aligned silk fibers held in 19.6N of tension. This scaffold allowed the MSCs to attach and elongate

along the fibers, remain viable, and differentiate on the fibers inductive stimulus. Figure 3A shows an image of the custom-made parallel-fiber scaffold, and Figure 3B is a scanning electron microscope image of the scaffold. Seven days post seeding, the C3H10T-1/2 MSCs were able to attach to the scaffold and aligned along the scaffold fibers (Fig. 3C).

[0060] 2. MSCs growing on the novel silk scaffold, but not on the silk protein coated surface, express tendon phenotypic markers Scleraxis and Tenomodulin. To determine the tenogenic potential of these MSCs seeded on the silk scaffolds, relative gene expression of Scleraxis (Scx), an early marker of tendon differentiation, was assayed. Scx expression increased from 4 hours to 24 hours post seeding, and began to plateau at 24 hours and maintained the same level until 7 days (Fig 4A). To test whether the tenocyte differentiation promoted by the silk scaffold was in response to the topography of the silk fibers rather than the silk protein itself, the expression of tenogenic phenotypic-related markers and stem cell markers was analyzed in MSCs seeded onto silk scaffolds compared to MSCs cultured on silk-coated culture dishes. Cells cultured on the silk scaffold had significantly higher expression of Scx and tenomodulin (Tnmd), a late marker of tendon formation, compared to MSCs cultured on silk-coated plates (Fig 4B), indicating tenogenic differentiation was induced due to the physical properties of the silk fibers but not the chemical components of the silk. Cells seeded on the silk scaffold also expressed significantly reduced nucleostemin (Nst), and octamer-binding transcription factor 4 (Oct4), markers for undifferentiated MSCs, when compared to MSCs grown on the silk coated plates (Fig. 4B). These data suggest that MSCs cultured on silk-coated plates remain largely undifferentiated, while cells cultured on the scaffold were differentiating towards a tenogenic lineage. Furthermore, mRNA expression of chondrogenic markers Sox9 and Col2a1 were unchanged or undetected, respectively, and mRNA expression of osteogenic markers Runt-related transcription factor 2 (Runx2) and alkaline phosphatase (Alp) were undetected, in MSCs seeded either on a silk coated surface or a silk scaffold (Fig 4B), which may indicate differentiation specifically towards the tenogenic lineage. Immunocytochemistry (Fig 4C) and Western blot (Fig 4D) confirmed a lack of Scx and Tnmd expression in MSCs cultured on silk protein-coated plates while expression of Scx and Tnmd was detected in MSCs grown on the silk scaffold. These results strongly suggest that topography (i.e. the elongated-related physical property) of the silk fibers, but not the

silk protein/chemical components, is a determining factor for the t
of the MSCs in this context.

[0061] 3. Actin polymerization is required for scaffold induced stem cell elongation and expression of tenogenesis markers Scx and Tnmd. To determine the role of cytoskeletal organization in the differentiation of MSCs into tenocytes when MSCs are grown on silk, the effect of cytochalasin D, which inhibits actin polymerization by capping the barbed ends of F-actin polymers was examined on silk-fiber induced MSC teno-differentiation. Differences in cell shape and cell nuclei were quantified by measuring the lengths of the cells that were growing in parallel with the fibers and were compared to the maximal widths of the cells, which were measured perpendicular to the fibers. Cells that were treated with cytochalasin D did not retain an elongated shape, as demonstrated by the smaller length-to-width ratios of their cytoplasms (Fig. 5A and 5C) and nuclei (Fig. 5B and 5D) compared to untreated MSCs. Significant differences were found when quantifying the cytoplasmic ($P < 0.05$) and nuclear ($P < 0.05$) ratios (Fig. 5E).

[0062] To determine whether actin polymerization in elongated cells is required for tenogenic differentiation, the effect of cytochalasin D was examined on tenogenic marker gene and protein expression in MSCs that were grown on silk scaffolds for 7 days. Compared to untreated controls, cytochalasin D treatment abolished the elongated phenotype of the cells and was found to be associated with a loss of Scx and Tnmd immunocytochemistry staining (Fig. 5F), which was confirmed by real-time PCR (Fig 5G) and western blot (Fig. 5H). To determine whether the loss of Scx and Tnmd expression was due to specific effects produced by cytochalasin D on actin polymerization and cell morphology, rather than due to general toxicity related to gene expression, the expression levels of two housekeeping genes, Hypoxanthine-guanine phosphoribosyltransferase

[0063] 1 (Hprt1) and phosphoglycerate kinase 1 (Pgk1), were analyzed. The results revealed that the expression levels of both genes remained unchanged in the presence of cytochalasin D (Fig. 5I), suggesting that the loss of cell elongation and tenogenic gene expression observed previously was due to changes in actin polymerization.

[0064] 4. Disruption of stress fiber formation by blocking Rho/Rock Pathway signaling abolished silk scaffold induced MSCs tendon lineage differentiation. To determine the role of stress fibers in mediating silk fiber-induced MSC tenogenic differentiation, the impact of cell signaling on the regulation of stress fiber formation was examined. RhoA and ROCKII are two small GTPase proteins that have been shown to

regulate the actin cytoskeleton during the formation of stress fi
following Rho/Rock inhibiting reagents were used to dissect the potential effects of the Rho/Rock pathway on stress fiber formation during the differentiation of MSCs (grown on silk scaffolds) into tenocytes: (1) Latrunculin A, which binds to monomeric G-actin and prevents its polymerization into F-actin and stress fibers; (2) Y-27632 dihydrochloride, which inhibits ROCK activity; and (3) blebbistatin, which selectively inhibits the ATPase function of nonmuscle myosin II.

[0065] Compared to untreated cells, cells that were exposed to the above treatment conditions lost their elongated phenotype (Fig 6A). Additionally, the expression levels of Scx mRNAs and SCX proteins were significantly decreased after treatment with Latrunculin A, Y-27632, and blebbistatin (Fig. 6B), which supports that the Rho/Rock pathway plays a role in tenocyte differentiation through its regulation of stress fiber formation. In addition to Scx, the Nfatc4 and Col1a1 genes have been suggested to be a part of the regulatory network that is involved in the differentiation of MSCs into tenocytes. Therefore, the expression of Nfatc4 and Col1a1 following treatment with Latrunculin A, Y-27632, and blebbistatin was also examined. It was found that the expression levels of both Nfatc4 and Col1a1 were significantly decreased in response to all three of the treatments (Fig 6C). Collectively, these findings suggest that the organization of actin stress fibers is a critical determinant of MSC teno-differentiation.

[0066] Taken together, these studies show the novel scaffold, via physical but not chemical means, induced the stem cells to elongate. These cell shape changes generated stress fiber formation that led to the generation of mechanical tension which triggered a pathway leading to increased tenogenesis-related gene expression and morphological changes towards tenocyte differentiation.

[0067] IV. Isolation and characterization of the micro-vesicles derived from stem cells such as tendon stem/progenitor cells (TSPCs) grown on novel scaffold: TSPCs isolated from biceps tissue (48-year-old female) were cultured using the above-mentioned custom-designed silk scaffold in serum-free medium in the presence of 1% penicillin/streptomycin. After 3 days of culture, 2/3 of the conditioned medium was harvested and replaced with fresh medium every other day for 6 days in this study. The micro-vesicles were isolated by differential ultracentrifugation. Briefly, the supernatant was collected after every following step: 1) 300 x g, 10 minute; 2) 2000 x g, 10 minute; 3) 10,000 x g, 30 minute; 4) 100,000 x g, 70 min. The resulting pellet was washed in PBS, and subjected to one more

centrifugation step (100,000 x g, 70 min), which yielded the micro-v
based on a series of centrifugation steps and can be carried out in a custom designed sterile device without expose to the air, therefore allowing to avoid air or chemical contamination. Micro-vesicles isolated from the conditioned medium of MSCs grown on the novel scaffold are termed: "Micro-vesicles-S". Micro-vesicles isolated from the conditioned medium of MSCs grown in 2D culture are termed: "Micro-vesicles-C". Approximately 200µg of Micro-vesicles-S can be harvested within 48 hours. The Micro-vesicles-S had a diameter from 40-100nm, as revealed by negative staining transmission electron microscopy (TEM) (Fig 7A) and dynamic light scattering (Dynopro Nanostar) (Fig 7B).

What is claimed is:

1. A method of treating a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to treat a damaged tendon.

2. A method of reducing scar formation in a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to treat reduce scar formation.

3. A method of accelerating healing of a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to accelerate healing of a damaged tendon.

4. A method of enhancing tenogenesis in a damaged tendon in a mammalian subject comprising administering to the subject an amount of microvesicles derived from mammalian mesenchymal stem cells effective to enhance tenogenesis.

5. The method of Claim 1, 2, 3 or 4, wherein the microvesicles have been secreted from mesenchymal stem cells.

6. The method of any of Claims 1 to 5, wherein the mesenchymal stem cells have been cultured on a silk scaffold prior to the microvesicles being derived from the mesenchymal stem cells.

7. The method of Claim 6, wherein the mesenchymal stem cells at P1 to P3 have been cultured on a silk scaffold at.

8. The method of any of Claims 1 to 7, wherein the mesenchymal stem cells (MSC) are adipose-derived MSC, bone marrow-derived MSC, bursa-derived MSC, or are tendon progenitor cells.

9. The method of any of Claims 1 to 8, wherein the mesenchymal stem cells (MSC) are from the same species as the subject.

10. The method of any of Claims 1 to 9, wherein the mesenchymal stem cells (MSC) are from the same subject that is being treated.

11. The method of any of Claims 1 to 10, wherein the damaged tendon is damaged as a result of a tendinopathy.

12. The method of any of Claims 1 to 10, wherein the damaged tendon is damaged as a result of physical injury to the tendon.

13. The method of any of Claims 1 to 12, wherein the damaged tendon is a ruptured tendon.

14. The method of any of Claims 1 to 13, wherein the amount of microvesicles is administered locally into the tendon.

15. The method of any of Claims 1 to 14, wherein the amount of microvesicles is administered by injection.

16. The method of any of Claims 1 to 15, wherein the amount of microvesicles has been cryopreserved prior to administration.

17. The method of any of Claims 1 to 16, wherein the amount of microvesicles has been cryopreserved below 50°C.

18. The method of any of Claims 1 to 17, wherein the amount of microvesicles has been cryopreserved below 75°C.

19. The method of any of Claims 1 to 18, wherein the amount of microvesicles has been cryopreserved below 80°C.

20. A pharmaceutical composition for treating a damaged tend
pharmaceutically acceptable carrier and an amount of purified microvesicles derived from mammalian mesenchymal stem cells.

21. The pharmaceutical composition of Claim 20, wherein the pharmaceutically acceptable carrier comprises a buffered saline.

22. A method of producing microvesicles isolated from mammalian mesenchymal stem cells comprising obtaining adipose-derived stem cells from a mammalian subject by a cannula; placing the adipose-derived stem cells into a sterile culture system comprising a scaffold of longitudinal fibers under tension and under conditions permitting growth of the adipose-derived stem cells on the scaffold of longitudinal fibers; and recovering microvesicles secreted from the cultured adipose-derived stem cells under sterile conditions.

23. The method of Claim 22, wherein the recovering the microvesicles comprises obtaining culture medium conditioned by the cultured adipose-derived stem cells and ultracentrifuging the conditioned medium under sterile conditions so as to obtain a pellet comprising the microvesicles.

24. The method of Claim 23, further comprising aliquoting the microvesicles for therapeutic use.

25. The method of Claim 23, further comprising freezing the microvesicles for future use.

26. The method of any of Claims 22-25, wherein the microvesicles obtained have an average diameter of 40nm -100nm as measured by negative staining transmission electron microscopy.

27. The method of any of Claims 22-26, wherein the longitudinal fibers are silk fibers.

28. The method of any of Claims 22-27, wherein longitudinal
aligned in a parallel manner along their longitudinal axes and held 16.5N to 19.8N of tension in a longitudinal direction of the fibers.

29. A device comprising a 3D scaffold of plurality of fibers substantially aligned in a parallel manner along their longitudinal axes and held under 16.5N to 19.8N of tension in a longitudinal direction of the fibers.

30. The device of Claim 29, wherein the fibers are held under at least 19N of tension in a longitudinal direction of the fibers.

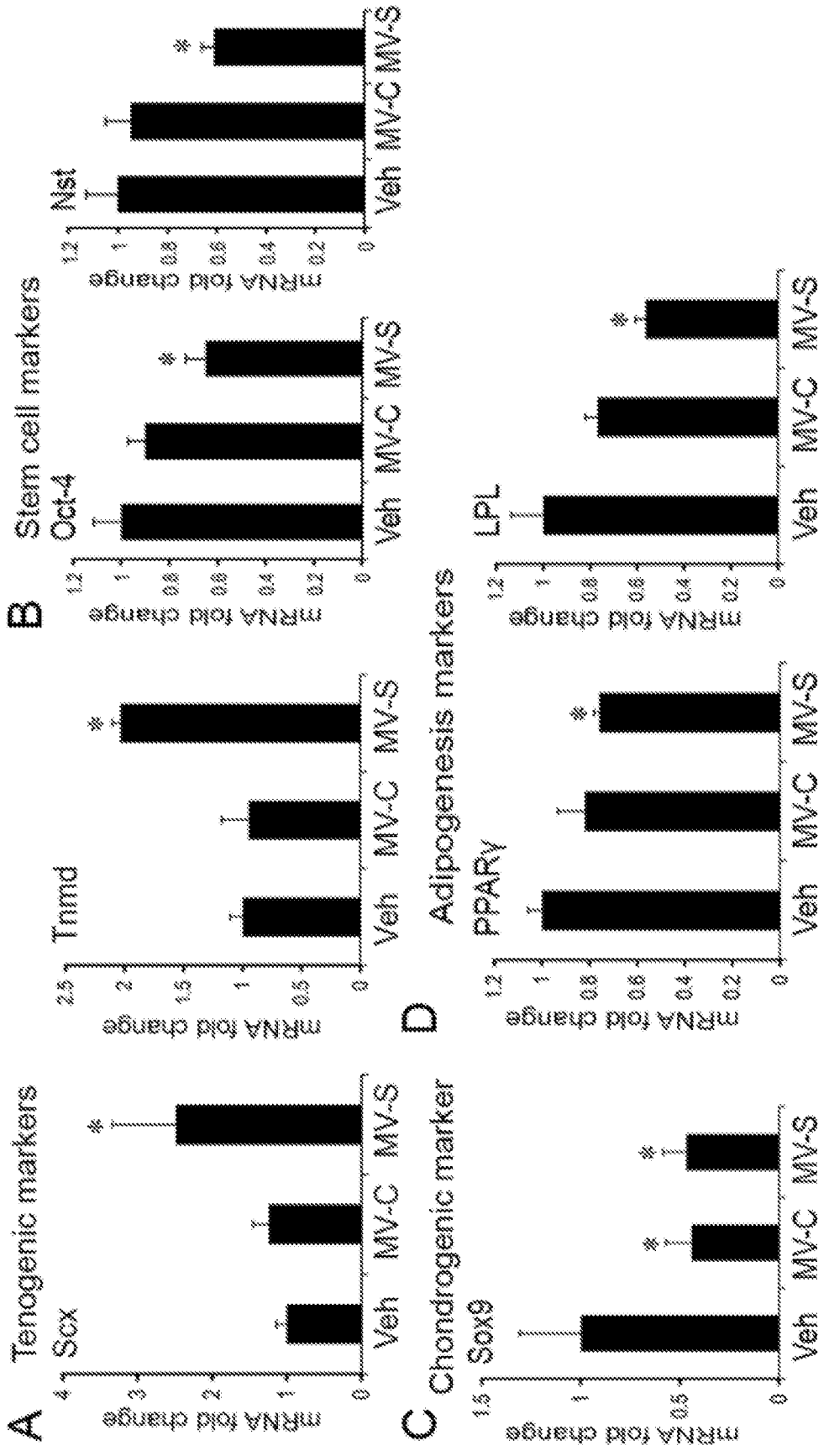
31. The device of Claim 29 or 30, wherein the fibers are held 16.5 to 19.8N of tension in a longitudinal direction of the fibers.

32. The device of any of Claims 29 to 31, wherein the fibers have an average diameter of 11 to 15 uM.

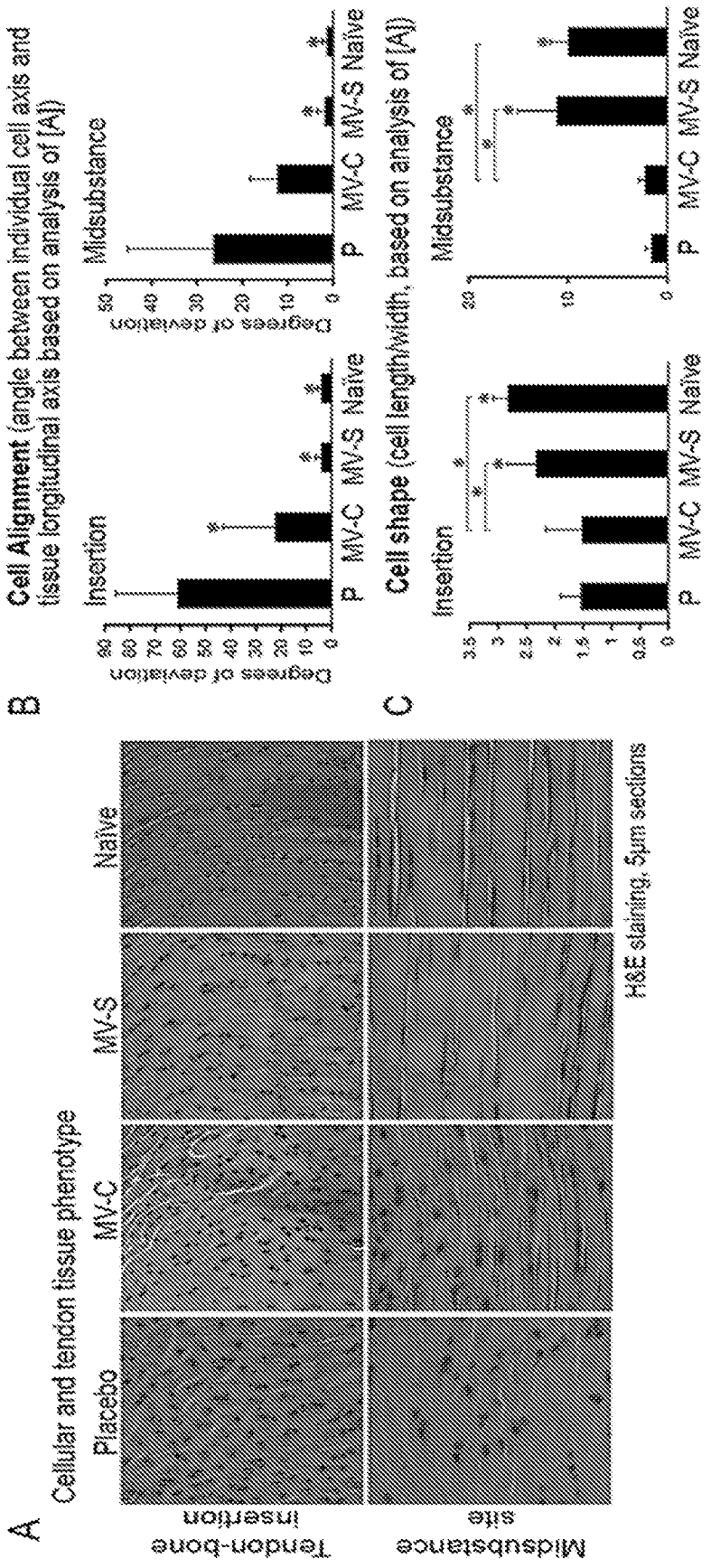
33. The device of any of Claims 29 to 32, wherein the 3D scaffold is contained within a sterile container.

34. The device of any of Claims 29 to 33, further comprising an amount of mammalian mesenchymal stem cells adhered to or growing on at least one of the fibers.

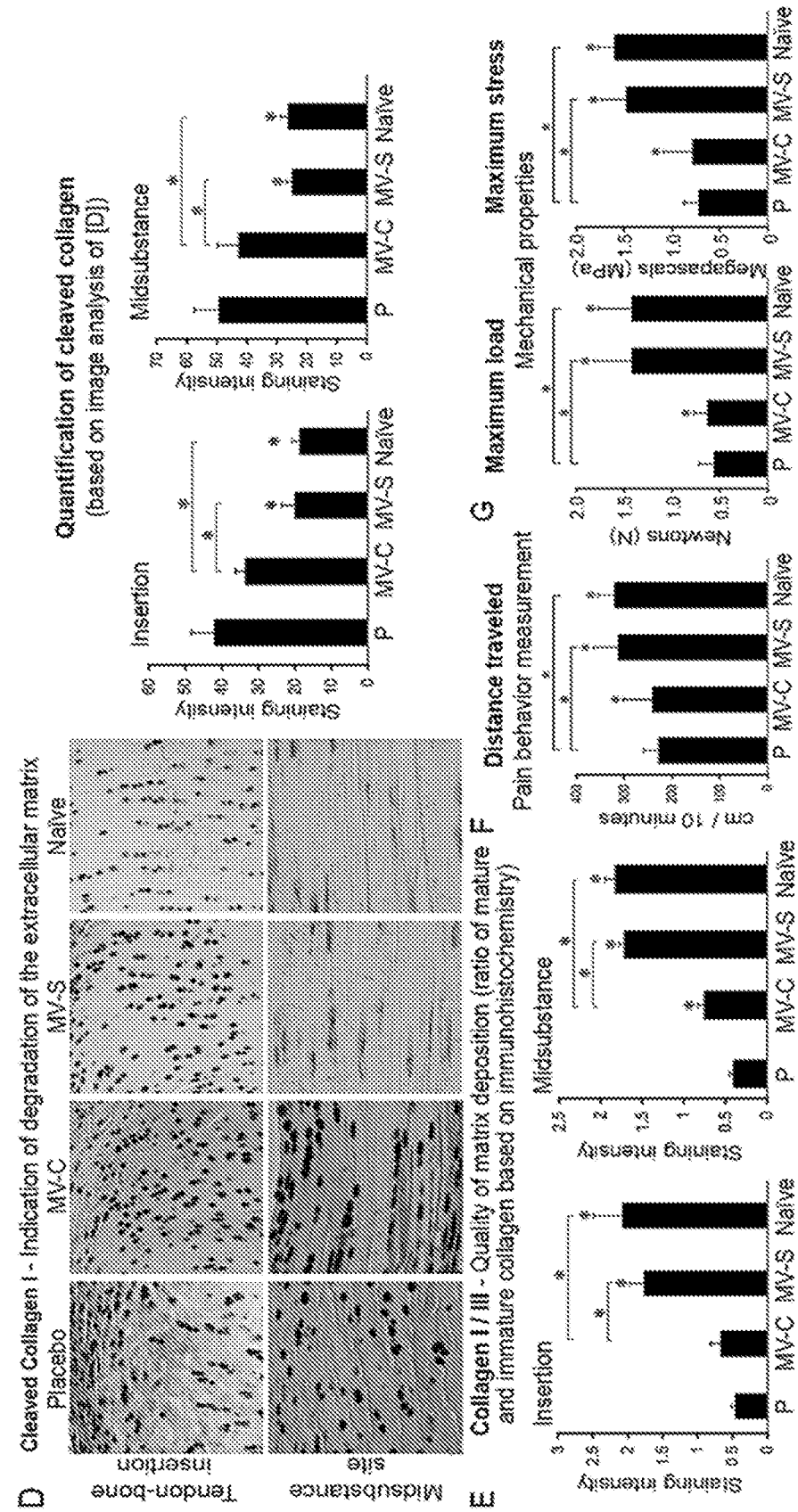
35. The device of any of Claims 29 to 34, wherein the fibers are silk fibers.


A
Tenogenic markers
Scx
Tnmd
B
Stem cell markers
Oct-4
Nst
C
Chondrogenic marker
Sox9
D
Adipogenesis markers
PPARγ
LPL
mRNA fold change
Veh
MV-C
MV-S

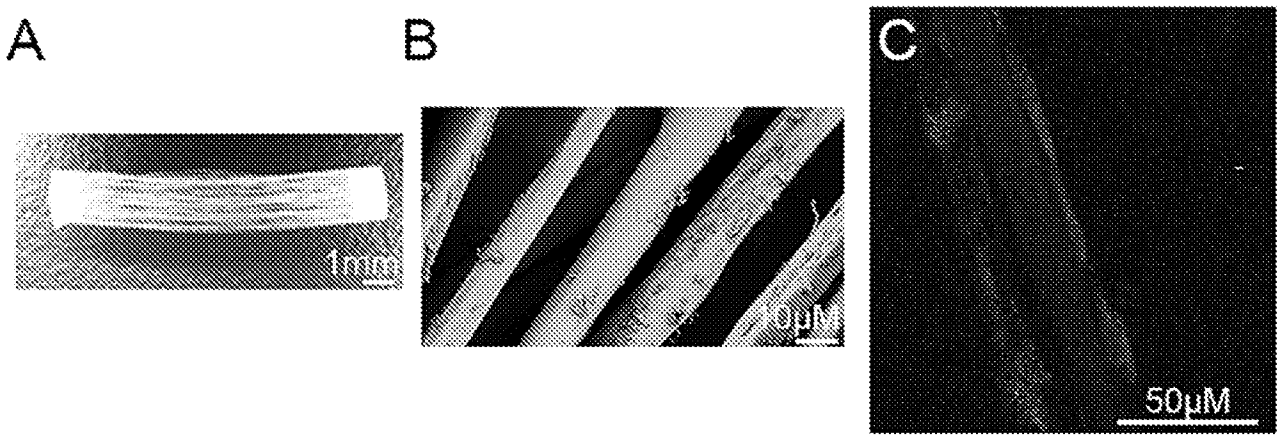
FIGS. 1A - 1 E


A
Cellular and tendon tissue phenotype
Placebo
MV-C
MV-S
Naïve
Tendon-bone insertion
Midsubstance site
H&E staining, 5μm sections
B
Cell Alignment (angle between individual cell axis and tissue longitudinal axis based on analysis of [A])
Insertion
Midsubstance
Degrees of deviation
P MV-C MV-S Naïve
C
Cell shape (cell length/width, based on analysis of [A])
Insertion
Midsubstance
P MV-C MV-S Naïve

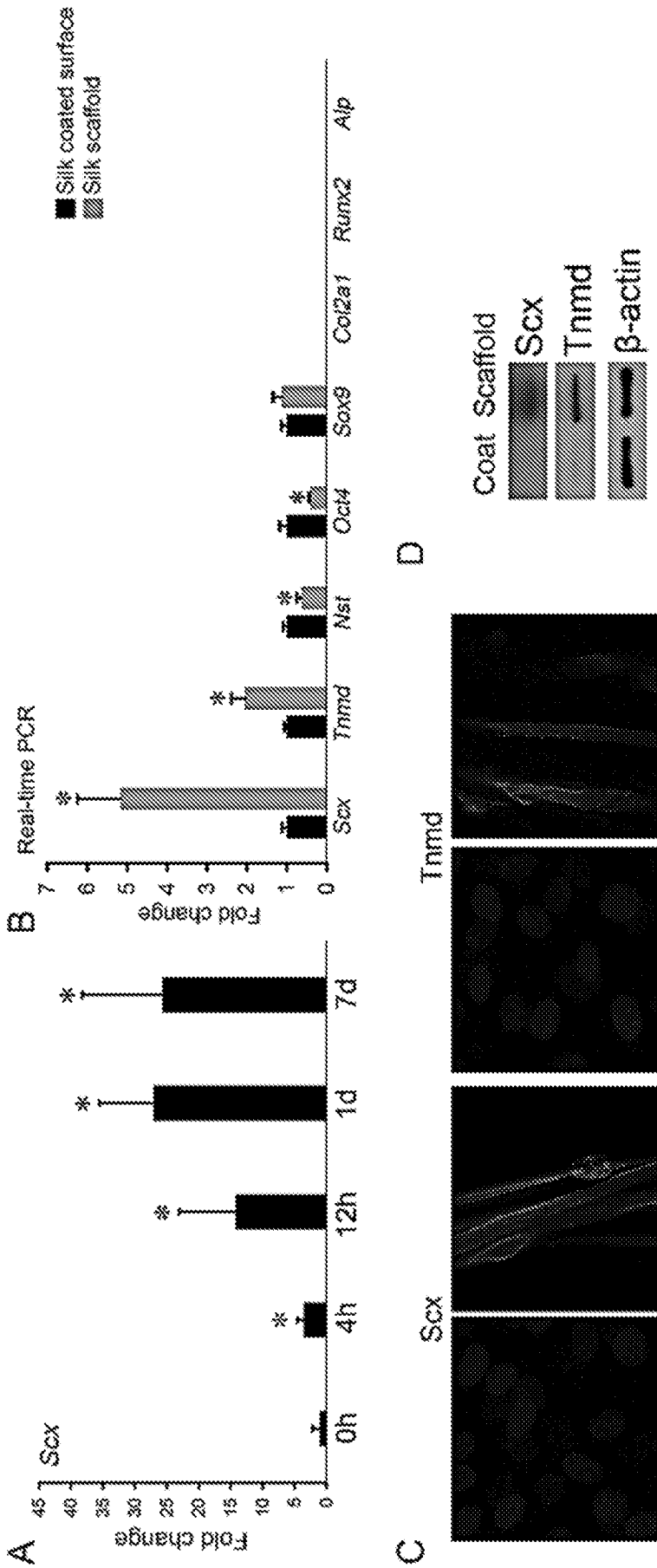
FIGS. 2A - 2C


D
Cleaved Collagen I - Indication of degradation of the extracellular matrix
Placebo
MV-C
MV-S
Naive
Tendon-bone insertion
Midsubstance site
Quantification of cleaved collagen
(based on image analysis of [D])
Insertion
Midsubstance
Staining intensity
P MV-C MV-S Naive
E
Collagen I / III - Quality of matrix deposition (ratio of mature and immature collagen based on immunohistochemistry)
F
Distance traveled
Pain behavior measurement
cm / 10 minutes
G
Maximum load
Maximum stress
Mechanical properties
Newtons (N)
Megapascals (MPa)

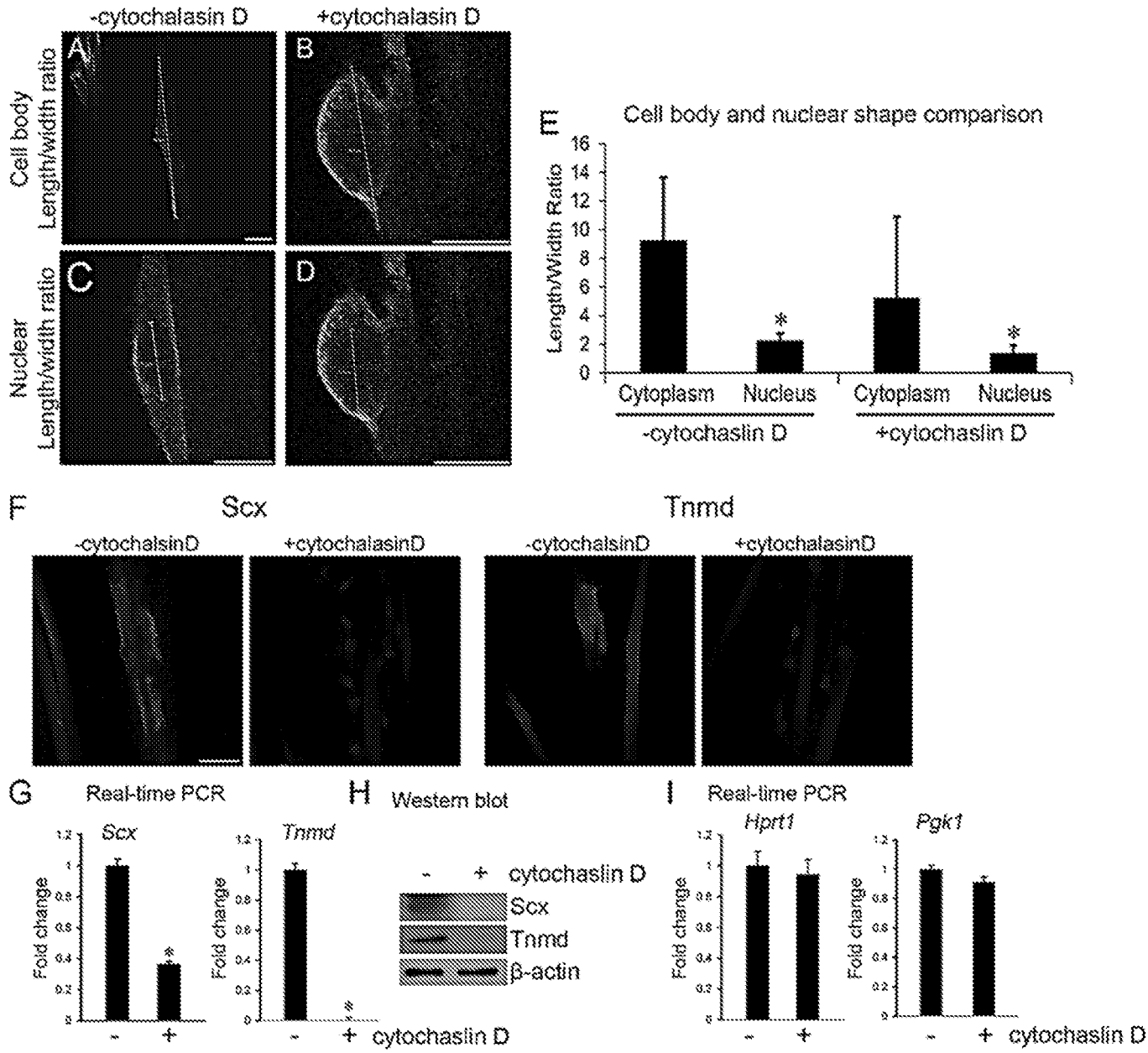
FIGS. 2D - 2G



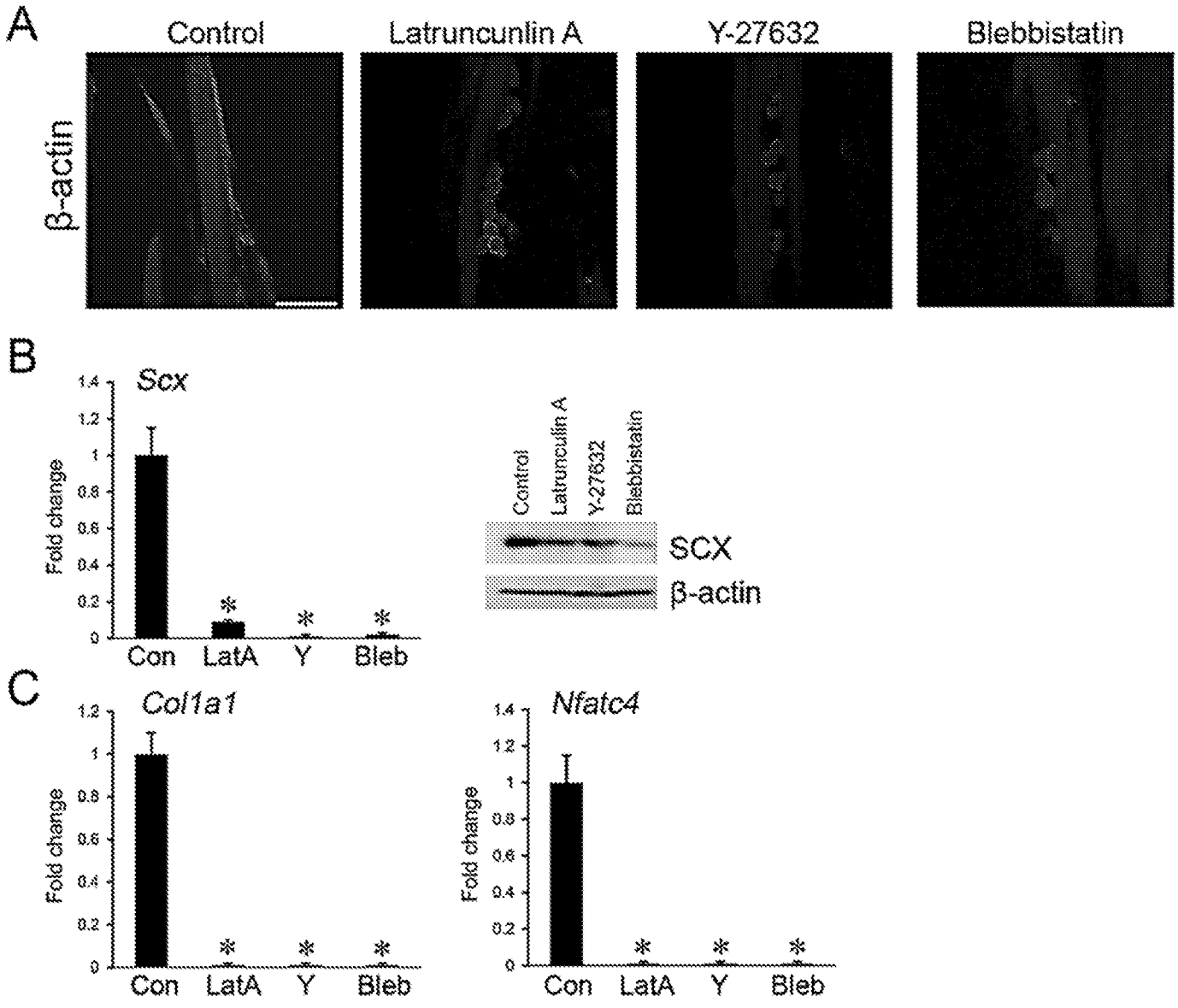
FIGS. 3A - 3C


A
Scx
Fold change
0h
4h
12h
1d
7d
B
Real-time PCR
Silk coated surface
Silk scaffold
Fold change
Scx
Tnmd
Nst
Oct4
Sox9
Col2a1
Runx2
Alp
C
Scx
Tnmd
Silk Coated Surface
Silk Scaffold
Silk Coated Surface
Silk Scaffold
D
Coat Scaffold
Scx
Tnmd
β-actin

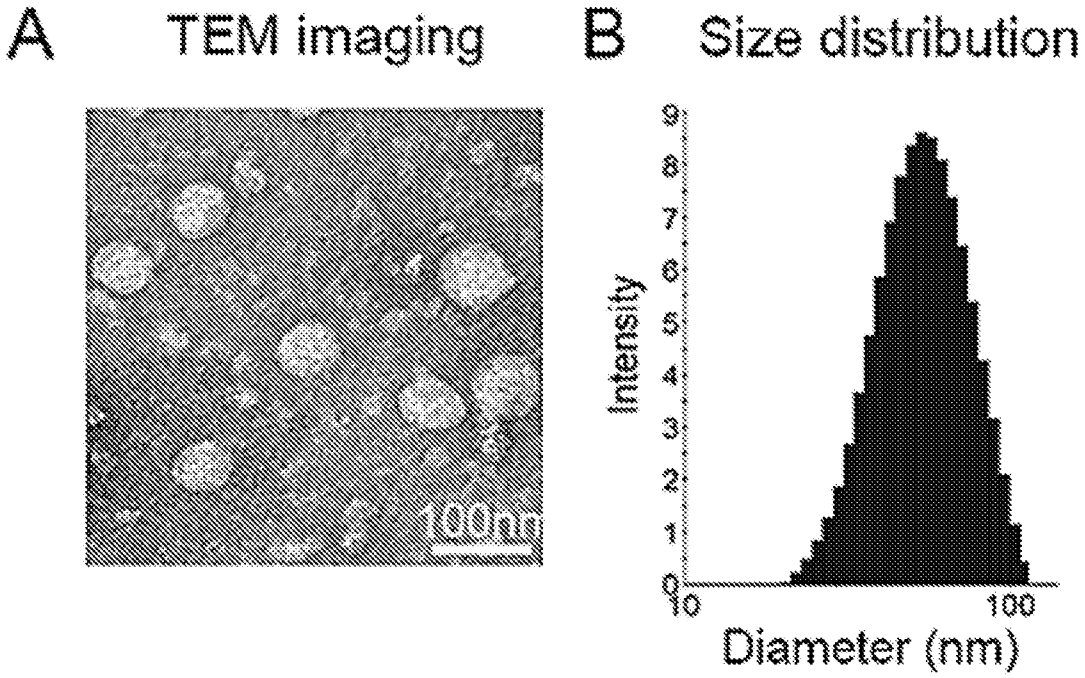
FIGS. 4A - 4D


-cytochalasin D
+cytochalasin D
A
B
C
D
Cell body Length/width ratio
Nuclear Length/width ratio
E
Cell body and nuclear shape comparison
Length/Width Ratio
Cytoplasm
Nucleus
-cytochaslin D
+cytochaslin D
F
Scx
Tnmd
-cytochalsinD
+cytochalasinD
G
Real-time PCR
Scx
Tnmd
Fold change
H
Western blot
cytochaslin D
Scx
Tnmd
β-actin
I
Real-time PCR
Hprt1
Pgk1

FIGS. 5A - 5I


A
Control
Latruncunlin A
Y-27632
Blebbistatin
β-actin
B
Scx
Fold change
Con
LatA
Y
Bleb
Control
Latruncunlin A
Y-27632
Blebbistatin
SCX
β-actin
C
Col1a1
Fold change
Con
LatA
Y
Bleb
Nfatc4
Fold change
Con
LatA
Y
Bleb

FIGS. 6A - 6C


A
TEM imaging
100nm
B
Size distribution
Intensity
0
1
2
3
4
5
6
7
8
9
10
100
Diameter (nm)

FIGS. 7A - 7B