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Supplementary Materials for

Single-cell analysis reveals a nestin$^+$ tendon stem/progenitor cell population with strong tenogenic potentiality

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Supplementary Materials

Supplementary Materials and Methods

Cell isolation and culture

We obtained human adult Achilles tendon samples from individuals (male, age 38-45 years) that had undergone leg amputation surgery. We cut the tendon tissue into 1–2 mm^3 pieces, and washed these three times with PBS. The tissue fragments were digested with 0.25% collagenase (Life technologies) and 1mg/ml dispase overnight at 37°C. The digest was centrifuged, and the pellet was suspended in Dulbecco's modified Eagle's medium (DMEM, low glucose; Gibco) with 10% (v/v) fetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin-streptomycin (Gibco). At 80-90% confluence, cells were trypsinized, centrifuged, resuspended in growth medium as passage 1 cells, and incubated in 5% CO_2 at 37°C, with fresh medium changes every 3 days. For nestin knock-down experiments and transplantation study in vivo, polyclonal TSPCs were used at passage 2.

Monoclonal selection

The cells were cultured in DMEM supplemented with 1% (v/v) penicillin–streptomycin and 20% (v/v) FBS. Most of the cells in culture appeared fibroblast-like after two passages and were then seeded at very low density (2 cells/cm^2) to form colonies. After 10–12 days, the colonies formed were stained with 1% (w/v) crystal violet (Sigma) in methanol for 10 min. The number and size of all colonies with diameters >2 mm were counted. For single cell clonal isolation, the cells were plated by serial dilution in 96 well plates. Clones could be detected by microscopy after 4 to 5 days. Each well was checked and wells that contain just a single colony were marked. These colonies can then be sub-cultured from the marked wells into a 12 well plate. 24 single colonies were selected for sub-culture and their gene expression was analyzed. The top six and bottom six colonies with the highest and lowest nestin expression respectively, were selected for use in further studies. And this experiment was performed using two different individual human origin cells.

Inhibition of nestin expression by RNA interference

To stably knock down endogenous nestin expression, we utilized lentiviral e expression vector pGLVH1/GFP+Puro encodingshRNA (purchased from GenePharma, Shanghai, China) to infect cells. Target cells were infected with the lentivirus for 24–48 hrs according to the manufacturer's instruction in the presence of 10 ng/ml polybrene (Sigma, Saint Louis, MO). The cells were then treated with 1ug/ml puromycin for 10 days to obtain the nestin knockdown cell lines. The RNAi oligonucleotide sequence utilized to knockdown endogenous nestin expression is as follows: ShRNA-Nes, CAAGGAAAGUCAAAGGAAU; and ShRNA-Nes-1, GAUCUAAACAGGAAGGAAA. One scrambled shRNA (utilized asa negative control [mock]) with the following sequence was used, NC: TTCTCCGAACGTGTCACGT.

Quantitative PCR

Total cellular RNA was isolated by lysis in TRIzol (Invitrogen). PCR was performed using a Brilliant SYBR Green QPCR Master Mix (TakaRa) on a Light Cycler apparatus (ABI 7900HT). The PCR cycling consisted of 40 cycles of amplification of the template DNA with primer annealing at 60°C. The relative expression level of each target gene was then calculated using the 2-ΔΔCt method. The amplification efficiencies of primer pairs were validated to enable quantitative comparison of gene expression. RNA samples were prepared from at least 5 independent samples and analyzed at least 3 times.

Single-Cell Data Processing

A background Ct of 28 was used for all real-time signals. Samples with low GAPDH expression level are outliers of normal distribution and are excluded from the analysis. Hierarchical clustering (HC), principal component analysis (PCA), violin plots, analysis of variance, co-expressed gene identification were performed using the SINGuLAR Analysis Toolset 2.1, which is designed specifically for single-cell gene expression data analysis. The unbiased HC was performed on gene expression data and visually present it as a heat map with a dendrogram by using SINGuLAR. HC algorithms connect data points to form clusters based on distance, with a cluster being described largely by the maximum distance needed to connect parts of the cluster. The PCA used in SINGuLAR utilizes successive orthogonal transformations to convert data into a series of PCs that explain variance in the data. It permits a look at variance in single-cell samples and in the genes used in the single-cell data set. One-way ANOVA using SINGuLAR autoAnalysis includes three steps: (1) Retrieve the experimental data along with the sample list or gene list supplied by the user. A PCA will be performed automatically. (2) Select the Find the top genes… option and supply 10 autoAnalysis will return the top 10 PCA genes and trim the expression data with those top genes. All subsequent analysis will be performed with the trimmed gene list. (3) Supply sample group information, autoAnalysis will perform ANOVA and create a violin plot of differentially expressed genes ranked by p-values The SPADE algorithm was applied to analyze these single-cell expression profiles in order to identify cellular hierarchy . SPADE analysis was performed on http://cytobank.org/. Briefly, the SPADE algorithm contains four steps. (1) The cells were down-sampled in order to enrich for stem cells and progenitors; (2) The down-sampled cells were partitioned into clusters of similar gene expression signature by agglomerative clustering; (3) A minimal spanning tree was constructed joining different clusters; (4) Each remaining cell was mapped to the cluster to which it was most similar. The final results were visualized as a tree, in which the size and color of each node represent the number of cells and median gene expression level, respectively.

Immunofluorescence

Briefly, cells were fixed in 4% (w/v) paraformaldehyde for 10 min at room temperature, permeabilized, and blocked for 30 min with 1% (w/v) bovine serum albumin, and then permeabilized with 0.1% (v/v) Triton X-100. Fixed cells were washed and incubated with a primary antibody againsteither SCX (Abcam Inc.), nestin (Millipore), or control IgG (BD) at 4°C overnight. The cells were then

incubated with Alexa fluor 488-conjugated secondary antibody (Invitrogen) for 2 hrs and the nuclei were stained with DAPI. TRITC-phalloidin staining was used to visualize the cytoskeleton. Imaging was performed with confocal microscopy (Zeiss LSM-510). For quantification of cell morphology, a minimum of twelve cells for each sample were selected randomly as ROI (Regions of Interest), n=3 cell samples per group. The area radius ratio, radius (min) and radius (max) were then quantified using the Image-Pro Plus software.

Tendon injury and repair animal model

Eighteen Sprague Dawley male adult rats (8 weeks, body weight of 250-300g) were used in this study. To create the tendon defect, the central one-third of the patellar tendon (~1 mm in width) was removed from the distal apex of the patella to the insertion of the tibia tuberosity according to our well-established protocol in our previous work. The operated rats were divided into 2 groups: (a) TSPCs infected by ShRNA-Nes lentivirus formed cell sheet and (b) TSPCs infected by scramble ShRNA lentivirus formed cell sheet. The engineered tendon tissue was placed in the tendon defect and sutured to the patellar bone and tibia tuberosity using Ethicon 6-0 suture. The animals were allowed to have free-cage activity until euthanasia. We did Patellar tendon injury model on both right and left sides, so each experimental group at each time point at least have 6 samples. Upon sacrifice, six limbs from each experimental group were used for histological evaluation and gene expression analysis at the 2- and 4-week time points. Six limbs were used for mechanical testing, and imaged by transmission electron microscopy (TEM) 4 weeks after surgery.

Twenty female homozygous transgenic mice of C57BL/6 strain that express GFP under the control of the nestin promoter (Nes-GFP) weighing 25–30 g were utilized. The Zhejiang University Institutional Animal Care and Use Committee approved the study protocol. The Achilles tendon was exposed through a lateral incision under general anesthesia. For each leg, a gap wound model (half in width and 2 mm in length) of the Achilles tendon was performed by using micro-operating instruments. The wound was then irrigated and skin was closed with suture. Post-operatively, mice were allowed free cage activity at constant temperature with a 12-h dark–light cycle, together with unrestricted access to a standard diet and water. At 1, 2, and 3 weeks post-injury, five mice were sacrificed for histological evaluation at each time point. The other five mice without surgery were used as control.

Histological examination

The harvested specimens were immediately fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 24 h. For hematoxylin and eosin staining, the samples were dehydrated through an alcohol gradient, cleared, and embedded in paraffin blocks. Histological sections (7 μm) were prepared using a microtome and subsequently stained with hematoxylin and eosin (H&E). Additionally, Masson's trichrome staining was performed according to standard procedures to examine the general appearance of the collagen fibers. The stained sections were photographed digitally under a microscope. Polarizing microscopy was used to identify mature collagen fibrils. General histological scoring was performed using hematoxylin and eosin staining. Histological sections were prepared from at least 5 independent samples and performed by three independent observers who were blinded to the annotated

histological type. Six parameters (fiber structure, fiber arrangement, rounding of nuclei, inflammation, vascularity, cell population) were semi-quantitatively assessed. These six parameters were semi-quantitatively graded on a four-point scale (0–III), with 0 being normal and 3 being maximally abnormal. Therefore, a normal tendon would score 0, while a maximally abnormal tendon would score 18.

Mechanical Testing

Mechanical testing was performed using an Instron tension/compression system with Fast-Track software (Model 5543, Instron, Canton, MA). The hindlimbs were wrapped in gauze soaked in saline and frozen at -80°C for later testing. Before testing, the hindlimbs were gradually thawed at room temperature, and all soft tissues spanning the knee, except for the center of the patellar tendon, were rigorously transected. Measurements of the tendon cross-sectional area were performed using aqueous rapid curing alginate dental impression paste, digital photography and computerized image analysis as previously described . The femur-patellar tendon-tibia complex (FPTC) was then rigidly fixed to custom-made clamps. After applying a preload of 0.1 N, each FPTC underwent pre-conditioning by cyclic elongation of between 0 and 0.5 mm for 20 cycles at 5 mm/min. This was followed bya load to failure test at an elongation rate of 5 mm/min. The load–elongation behavior of the FPTCs and failure modes were recorded. The structural properties of the FPTC were represented by stiffness (N/mm), ultimate load (N), energy absorbed at failure (mJ) and stress at failure. For each FPTC, the greatest slope in the linear region of the load–elongation curve over a 0.5 mm elongation interval was utilized to calculate stiffness.

Transmission Electron Microscopy

Tissue specimens were fixed by standard procedures for TEM to assess collagen fibril diameters and alignment. Briefly, samples were pre-fixed with 2% (w/v) glutaraldehyde for 2 h at 4°C and then washed twice with PBS at 4°C followed by post-fixation with 1% (w/v) osmic acid for 2 h at 4°C. After two washes in PBS, the samples were dehydrated with an ethanol gradient and dried to a critical point. Then the samples were mounted and sputter-coated with gold for viewing under TEM (Quanta 10 FEI). About 500 collagen fibrils were measured for each sample to obtain an accurate representation of the fibril diameter distribution.

Supplementary Figures

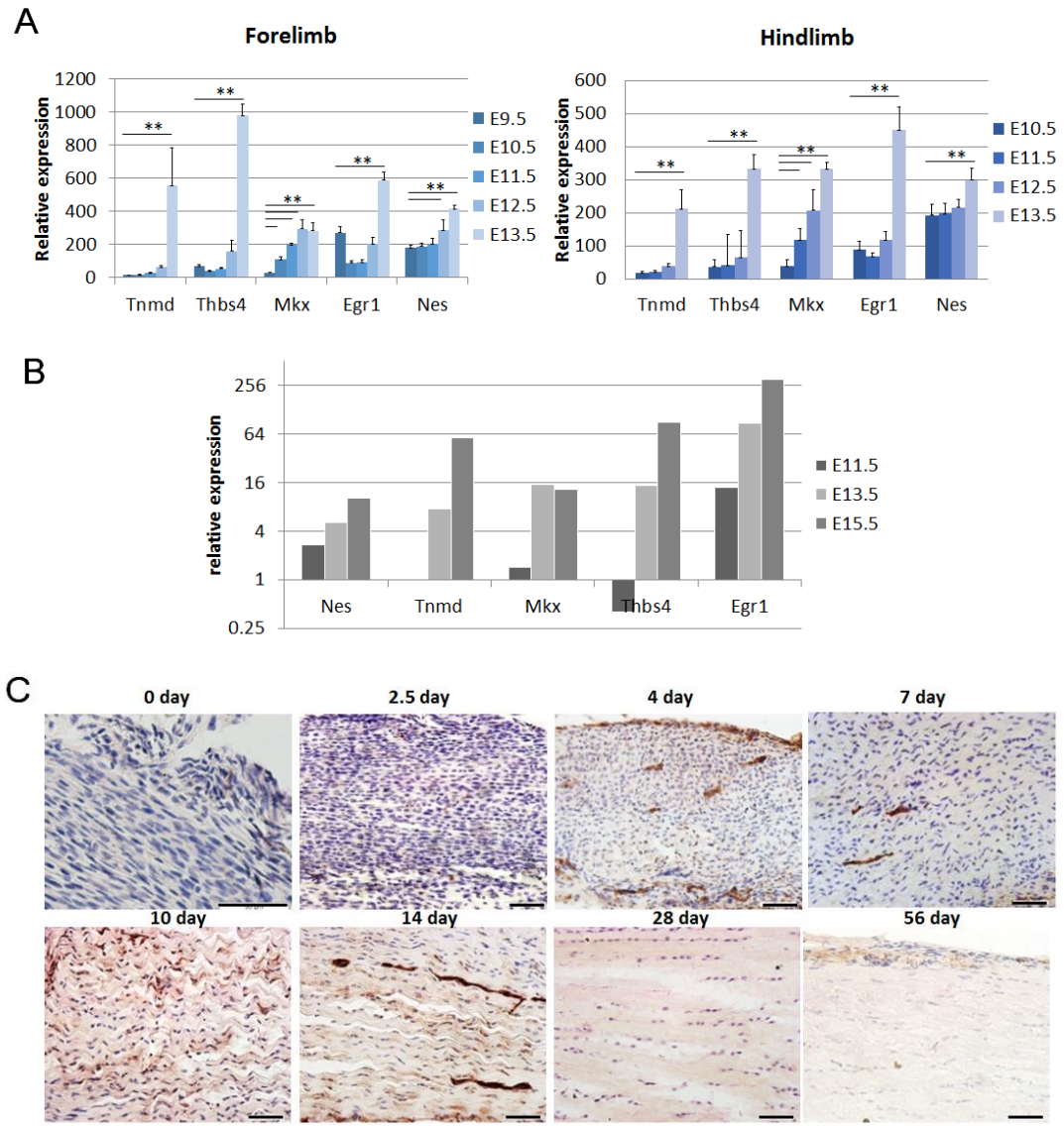

fig. S1. Nestin expression in the developing tendon. (**A**) Expression of nestin and teno-lineage marker genes obtained from ScxGFP forelimb and hindlimb tendons during mouse embryogenesis. (**B**) Expression of nestin and teno-lineage marker genes obtained from ScxGFP hindlimb tendons during mouse embryogenesis. (**C**) The Immunohistochemical staining of nestin protein expression in the developing rat Achilles tendon between postnatal day 0 and 56.

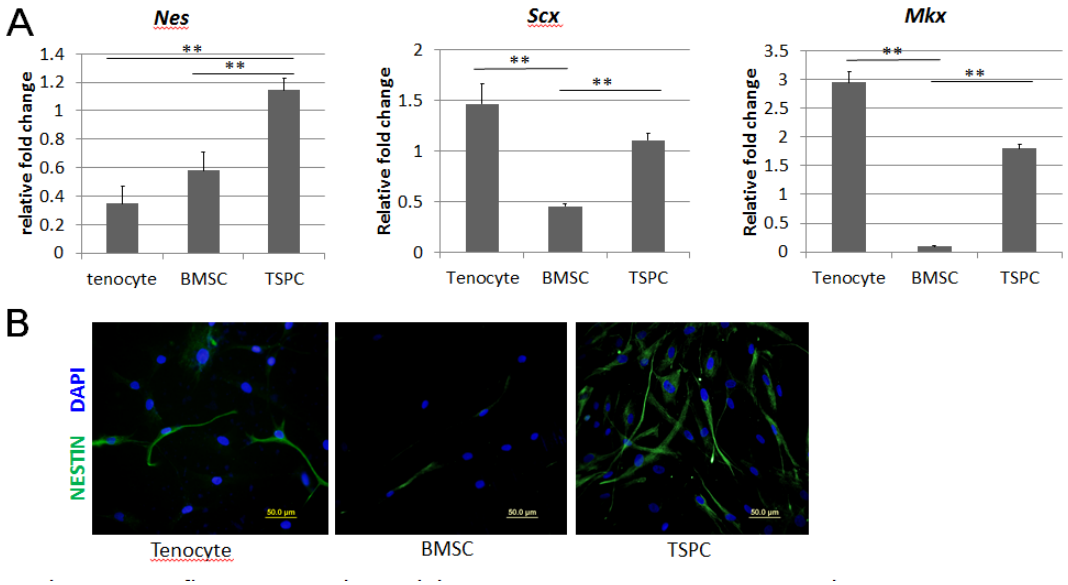

fig. S2. Comparison of nestin expression in tenocytes, BMSCs, and TSPCs. (**A**) The QPCR results of *nestin* and tendon lineage gene expression in tenocyte, mesenchymal stem cells from bone marrow (BMSC) and tendon stem/progenitor cells (TSPC). (**B**) The immunofluorescence showed the nestin expression in tenocytes, BMSC and TSPC.

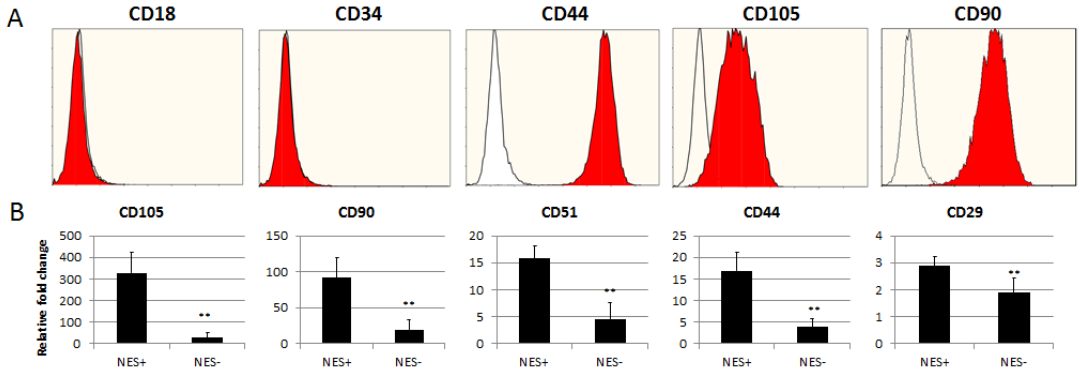

fig. S3. Cell surface marker expression in TSPCs. (**A**) Flow cytometry analysis of the expression of cell surface in isolated TSPC. (**B**) QPCR analyses of stem/progenitor cell related surface marker gene expression in selected Nestin^{+} and nestin^{-} colony formation TSPCs (n=6, **p <0.01).

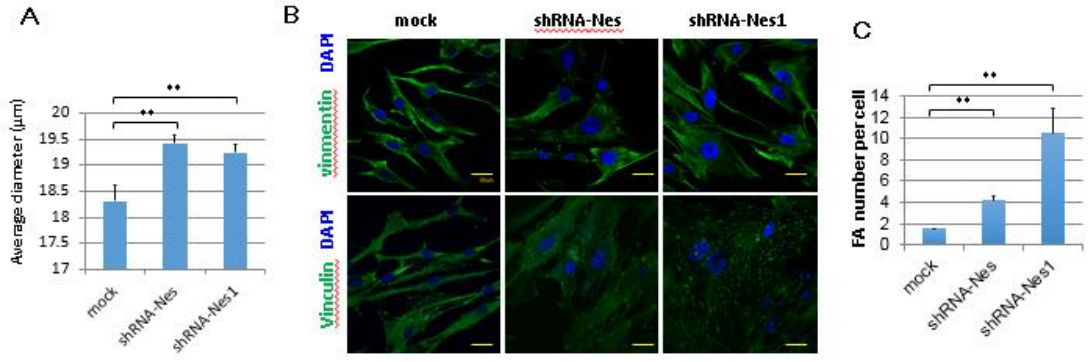

fig. S4. Morphological changes of TSPC infected with RNAi against nestin. (**A**) The average diameter of TSPCs in suspension. (**B**) Immunofluorescence staining of vinmentin and vinculin expression in TSPCs infected with RNAi against nestin (shRNA-Nes/shRNA-Nes1) or a control scrambled sequence (mock). (**C**)The number of focal adhesions in TSPCs infected with RNAi against nestin (shRNA-Nes/shRNA-Nes1) or a control scrambled sequence (mock). *p <0.05, **p <0.01 (One-way analysis of variance (ANOVA)). Results are presented as mean ± SD. Scale bars, 30 μm.

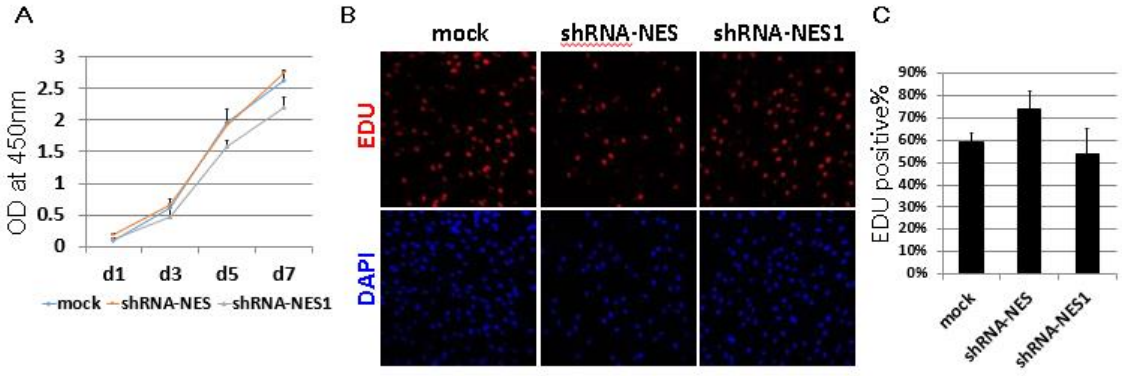

fig. S5. Proliferation capability of TSPC infected with RNAi against nestin. (**A**) The proliferation rate of TSPCs infected with RNAi against nestin (shRNA-Nes/shRNA-Nes1) or a control scrambled sequence (mock). (**B**) Immunofluorescence staining of Edu and DAPI expression in TSPCs. (**C**)The number of Edu positive cells in TSPCs infected with RNAi against nestin (shRNA-Nes/shRNA-Nes1) or a control scrambled sequence (mock).

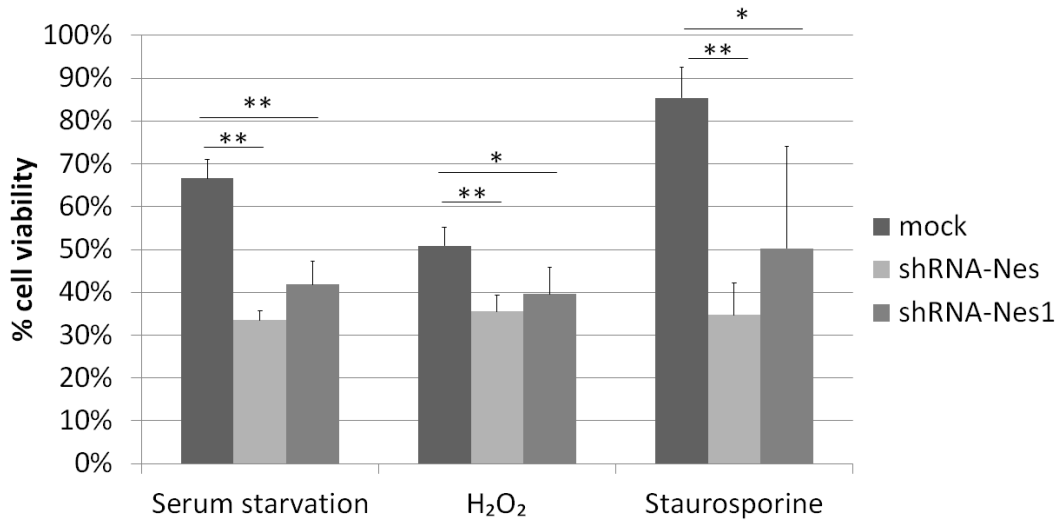

fig. S6. Cell viability tests of TSPC infected with RNAi against nestin.
Downregulation of *nestin* sensitizes TSPCs to serum starvation-induced cell death, oxidant-induced cell death and staurosporine-induced apoptosis. N=4, *p <0.05, **p <0.01 (One-way analysis of variance (ANOVA)).

table S1. Primers used in single-cell gene analysis.

Target	For Preamplification		For QPCR detection	
Collagen I	forward	GGGCTCTAATGATGTTGAACTTGT	forward	GGGCTCTAATGATGTTGAACTTGT
	reverse	ATGATTGTCTTTCCCCATTCATTT	reverse	ATGATTGTCTTTCCCCATTCATTT
Scx	forward	CGAGAACACCCAGCCCAAAC	forward	ACAGATCTGCACCTTCTGCC
	reverse	CTCCGAATCGCAGTCTTTCTGTC	reverse	GAATCGCTGTCTTTCTGTCGC
Collagen 14	forward	AAGGATTGCCCTCCGACTACAC	forward	GACACTCCACAGGAGCCATT
	reverse	CTGATGCGTTCATTGCCTTCTC	reverse	TCTCACCCACTTGCTTGCAG
Elastin	forward	GGCTTCGGATTGTCTCCCATTT	forward	GAGCTTCCTAGGACCCCTGA
	reverse	CCCTCTTGTTTCCTTGCCCTGT	reverse	GGCATGGGATGGGGTTACAA
Bgn	forward	GATGGCCTGAAGCTCAA	forward	GAGGACCTGCTTCGCTACTC
	reverse	GGTTTGTTGAAGAGGCTG	reverse	CCCTGGCCAACTTGTTGTTG
Fmod	forward	AGAACCTCTACCTCCAAGGCAATA	forward	GTGGTGGACGTCGTGAACTT
	reverse	AAAGCCAAACCAAACCATCAAG	reverse	CCATCAAGCCAAATGCCACG
Epha4	forward	TGTGGGCTGTGACAATCTGGAATA	forward	AACGTTCACACTTCGCTCCT
	reverse	CATTTAGACGGAACTGAGGAGGGT	reverse	GGGCTGTGAGGCCAAGATAG
Thbs4	forward	AACCTGAGACCATTGAATTGAGG	forward	GGTGAGAGGCTCACTGTTCC
	reverse	CTGCCGGTTAAAGTCCCCTG	reverse	CGGTTAAAGTCCCCTGTGCC
Six1	forward	GGAGTTATTGTTTTCGGTGTTC	forward	GGAGTTATTGTTTTCGGTGTTC
	reverse	TGTCCTGCGGGAGTGGTA	reverse	TGTCCTGCGGGAGTGGTA
Tnc	forward	TCTCTGCACATAGTGAAAAACAATACC	forward	TCTCTGCACATAGTGAAAAACAATACC
	reverse	TCAAGGCAGTGGTGTCTGTGA	reverse	TCAAGGCAGTGGTGTCTGTGA
Comp	forward	TGCCTTCAATGGCGTGGACTTC	forward	TGCCTTCAATGGCGTGGACTTC
	reverse	CCAATACGTTTGCTCCATCTGCTT	reverse	CCAATACGTTTGCTCCATCTGCTT
Eya2	forward	AGGGCAGGAATTAGTTGAGT	forward	CTCCCAAAAGGGAGACCTGG
	reverse	CGTTGGTGGGTTGATAGG	reverse	GCACATTGACACAGTTGGGC
Tnmd	forward	TGGGTGGTCCCTCAAGTGAAAGT	forward	ACGCCAGACAAGCAAGTGAG
	reverse	CTCGACGGCAGTAAATACAACAATA	reverse	CTCATCCAGCATGGGATCAAA
Mkx	forward	GAAGGCAACTTTGTCTATCGCA	forward	GAAGGCAACTTTGTCTATCGCA
	reverse	TGATCTCCTTCCAATACGTGTC	reverse	TGATCTCCTTCCAATACGTGTC
Hoxa11	forward	CAGCCCCGAGTCGTCTTCCG	forward	CAGCCCCGAGTCGTCTTCCG
	reverse	GTAGACGCTGAAGAAGAACTCC	reverse	GTAGACGCTGAAGAAGAACTCC
KITLG	forward	GTATCAACACTGTTACTTTCG	forward	CTGTCTGGAGTGACAGACCAC
	reverse	TAAATGAGACCCAAGTCCCG	reverse	AGTCCTGCTCCATGCAAGTT
Sca-1	forward	TCGTCATGCAATACGCCGAC	forward	TCGTCATGCAATACGCCGAC
	reverse	TACGGGTGAGGAACCGACT	reverse	TACGGGTGAGGAACCGACT
ALP	forward	AGCCCTTCACTGCCATCCTGT	forward	AGCCCTTCACTGCCATCCTGT
	reverse	GACTGCGCCTGGTAGTTGTTGT	reverse	GACTGCGCCTGGTAGTTGTTGT

Oct-4	forward	CACCATGGCTGGACACCTGGCTTC	forward	CACCATGGCTGGACACCTGGCTTC
	reverse	TTAACCCCAAAGCTCCAGGTTCTC	reverse	TTAACCCCAAAGCTCCAGGTTCTC
Nanog	forward	CCCAGCCTTTACTCTTCCTACCAC	forward	GTCCCAAAGGCAAACAACCC
	reverse	CATCTTCACACGTCTTCAGGTTGC	reverse	GCTGGGTGGAAGAGAACACA
Sox9	forward	GGCGGAGGAAGTCGGTGAAGAA	forward	GGCGGAGGAAGTCGGTGAAGAA
	reverse	GCTCATGCCGGAGGAGGAGTGT	reverse	GCTCATGCCGGAGGAGGAGTGT
CXCR4	forward	CTCCTGCTGACTATTCCCGAC	forward	CGTCAGTGAGGCAGATGACA
	reverse	GATAAGGCCAACCATGATGTGC	reverse	AAGGCCAACCATGATGTGCT
Nestin	forward	GCAGCTGGCGCACCTCAAGAT	forward	AAGATGTCCCTCAGCCTGGA
	reverse	GGAGCAAAGATCCAAGACGCCG	reverse	GAGGGAAGTCTTGGAGCCAC
Nestin’	forward	CAGCTGGCGCACCTCAAGAT	forward	AAGATGTCCCTCAGCCTGGA
	reverse	GTGCTGGCCAAGGTAGGGGTA	reverse	GAGGGAAGTCTTGGAGCCAC
CD29	forward	TTCAGTGAATGGGAACAACGA	forward	AGTGAATGGGAACAACGAGGT
	reverse	ATGCAAGGCCAATAAGAACAA	reverse	TGATGTCTGGACCAGTGGGA
CD 200	forward	ACGTCTGTTACCAGCATCCTC	forward	GCATCCTCCATATCAAAGACCC
	reverse	CTTAAAGTCGGTCACAGTCCC	reverse	AAGTCGGTCACAGTCCCCA
CD 44	forward	TGCCTTTGATGGACCAATTACC	forward	TGCCTTTGATGGACCAATTACC
	reverse	GGACTGTCTTCGTCTGGGATGG	reverse	GGACTGTCTTCGTCTGGGATGG
CD 274	forward	GGACAAGCAGTGACCATCAAG	forward	GACCACCACCACCAATTCCA
	reverse	CCCAGAATTACCAAGTGAGTCCT	reverse	GCCAGAGGTAGTTCTGGGATG
CD 146	forward	AGAGCCAACAGCACCTCCACA	forward	GGCTGTGATTGTGTGCATCC
	reverse	CTGGGAGCTTATCTGACTTAACTTC	reverse	GATCTCCTGCTTCCCTGAGC
CD24	forward	CCCACGCAGATTTATTCCAGT	forward	TGGAACTTCAAGTAACTCCTCCC
	reverse	GCCTTGGTGGTGGCATTAGTT	reverse	CCTTGGTGGTGGCATTAGT
CD302	forward	GATGATGCGAGTTTCAAGTGGT	forward	TGATGCGAGTTTCAAGTGGTT
	reverse	AGCACAGGTGTCAACTAAATCC	reverse	ATCATCATCTTGGTCTGTCCACT
CD51	forward	GGCTGCATATTTCGGATTTTCTG	forward	GGCTGCATATTTCGGATTTTCTG
	reverse	CCATTCAGCTTTGTCGTCTGG	reverse	CCATTCAGCTTTGTCGTCTGG
CD 90	forward	ATGAAGGTCCTCTACTTATCCGC	forward	GCCTTCACTAGCAAGGACGA
	reverse	GCACTGTGACGTTCTGGGA	reverse	TGTGACGTTCTGGGAGGAGA
CD 31	forward	TCCCCCTGTCCCCTCTATGA	forward	TCTATGACCTCGCCCTCCACAAA
	reverse	TTGTGCGTTGCCTGAATGAA	reverse	GAACGGTGTCTTCAGGTTGGTATTTCA
CD 34	forward	GGGAAGGATGCTGGTCCG	forward	GCGCTTTGCTTGCTGAGT
	reverse	TGGGTAGGTAACTCTGGGGT	reverse	GGGTAGCAGTACCGTTGTTGT
CD 45	forward	TCAGATGTCCCAGGAGAGAGG	forward	ACAGCCAGCACCTTTCCTAC
	reverse	ATCTGAGGTGTTCGCTGTGA	reverse	GTGCAGGTAAGGCAGCAGA
CD271	forward	TGGCCTACATAGCCTTCAAGA	forward	GCTGCAAGCAGAACAAGCAA
	reverse	GAGATGCCACTGTCGCTGT	reverse	CGCTGTGGAGTTTTTCTCCC
CD105	forward	CAACATGCAGATCTGGACCAC	forward	GCTCCCAGACACACCTCAAG

	reverse	CTTTAGTACCAGGGTCATGGC	reverse	CACGAAGGATGCCACAATGC
Runx2	forward	GTGATAAATTCAGAAGGGAGG	forward	CAGAAGGGAGGAGATGTGTGT
	reverse	CTTTTGCTAATGCTTCGTGT	reverse	ATTCTGGGCCAGTCCCTTTC
Agn	forward	CTGCAGACCAGGAGGTATGTGA	forward	CTGCAGACCAGGAGGTATGTGA
	reverse	GTTGGGGCGCCAGTTCTCAAAT	reverse	GTTGGGGCGCCAGTTCTCAAAT
PAX7	forward	GACGACGGCGAAAAGAAGG	forward	GACGACGGCGAAAAGAAGG
	reverse	GTAGTGGGTCCTCTCAAAGGC	reverse	GTAGTGGGTCCTCTCAAAGGC
LepR	forward	CTTGTGTGCAGTCTATGCTGT	forward	CTATGCTGTTCAGGTGCGCT
	reverse	CCATGACAACTGTGTAGGCTG	reverse	TGTAGGCTGGATTGCTCCAA
PDGFRa	forward	TTGAAGGCAGGCACATTTACA	forward	TTGAAGGCAGGCACATTTACA
	reverse	GCGACAAGGTATAATGGCAGAAT	reverse	GCGACAAGGTATAATGGCAGAAT
PDGFRb	forward	TGATGCCGAGGAACTATTCATCT	forward	TGATGCCGAGGAACTATTCATCT
	reverse	TTTCTTCTCGTGCAGTGTCAC	reverse	TTTCTTCTCGTGCAGTGTCAC
GAPDH	forward	TGACGCTGGGGCTGGCATTG	forward	TGACGCTGGGGCTGGCATTG
	reverse	GGCTGGTGGTCCAGGGGTCT	reverse	GGCTGGTGGTCCAGGGGTCT
PPAP2B	forward	AGCCAGATCAACTGCTCTGAA	forward	GTCCAGGAAGCCAGGAAGTC
	reverse	GGCTCCTCGCCAAGTGAAG	reverse	GCCTGCAGGTATAGCACCAA
PCDH18	forward	TCCAGATGAGGGCGCTAATG	forward	GATGAGGGCGCTAATGGGAA
	reverse	TGGACCCAAATCTTGAGCCTG	reverse	GGACCCAAATCTTGAGCCTGA