Engineering Cartilage Graft Using Mesenchymal Stem Cell Laden Polyacrylamide-Galactoxyloglucan Hydrogel for Transplantation

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SI1: Flow cytometry analysis for characterization of stem cells

- The cells were harvested washed, counted, and checked for cell viability using trypan blue assay.
- Cells were resuspended in ice-cold PBS, 10%FBS and 1% sodium azide solution (1-5X10^6 cells/ml).
- To 100µl cell suspension primary antibody at 1µg concentration was added and incubated at room temperature for 30 minutes.
- Cells were washed three times and a secondary antibody was added and incubated at room temperature for 30 minutes.
- Wash the cells and resuspended in ice-cold PBS, 3% BSA, and 1% sodium azide. This was used for flow cytometry analysis.

SI2: Immunofluorescence of differentiation markers

- Fix the cells in 4% formaldehyde/ PBS for 15 mins at 37^0C.
- Remove the fixative and add a permeabilizing buffer to permeate the cell membrane for 5 mins at 37^0C.
- Remove the perm buffer and add 1% BSA/ PBS for 5 mins at 37^0C.
- Remove BSA and add phalloidin/ anti X (Phalloidin is used in 1:500 concentration in PBS/BSA and anti X in 1:50 concentration) incubate at 37^0C for 1 hour (anti X is the antibody of our interest).
- Remove the stain and wash the cells with PBS/0.5% Tween 3 times 5 minutes each.
- Remove tween and add secondary antibody used in concentration 1:50 in PBS/BSA incubate at 37^0C for 1 hour.
- Remove the stain and wash the cells gently in PBS/0.5% Tween 3 times 5 minutes each.
- Finally, add the streptavidin component for 30 minutes at 4^0C.
- Washed the cells and mounted them in DAPI.
- Cells are then viewed under a fluorescent microscope.

SI3: Protocol for preparation of differentiation medium

Below mentioned specific differentiation factors were added to DMEM with 15% FBS to get each differentiation medium.

Osteogenic medium

Dexamethasone 10nMol

Ascorbic acid 100µMol

Adipogenic medium

Indomethacin 100µMol

IBMX 500µmol

Insulin 10µg/ml
Dexamethasone 1µMol
Chondrogenic medium
Ascorbate 2 phosphate 50µg/ml
Dexamethasone 0.1mM
TGFβ 10ng/ml
Sodium pyruvate 100µg/ml
Insulin 5µg/ml

SI4: Protocol for staining of osteo, chondro, and adipogenesis

- **BCIP/NBT staining for osteogenesis**

  Cells were washed in PBS and fixed with formalin for 15 minutes. The cells were washed with wash buffer (0.05% Tween20 in PBS). Cells were stained carefully in BCIP/NBT for 5-10 minutes. Progress was checked every 2-3 minutes. Cells were washed in wash buffer and then in PBS. Cells will be stained in dark blue-violet if alkaline phosphatase activity present (indicative of osteoblast cells).

- **Oil red O staining for adipogenesis**

  Cells were washed and fixed in formalin for 30 minutes to one hour at room temperature. Cells were washed in PBS and cells were incubated with 60% isopropanol for 2-5 minutes. Oil red O working solution was added and kept for 1 minute. Cells were rinsed with tap water and hematoxylin solution was added and incubated for 1 minute and washed and viewed under a phase-contrast microscope.

  - Oil red O stock: 300mg oil red O in 100ml isopropanol
  - Working solution: 30ml stock solution and 20ml distilled water

- **Alcian blue staining for chondrogenesis**

  Cells were fixed with neutral buffered formalin for 60 minutes at room temperature. Washed cells were added with staining solution and incubated overnight at room temperature in dark. Cells were washed using a destaining solution several times. Cartilage cells will stain intense blue after destaining.

  - Staining solution: 10mg alcian blue 8GX in 60ml ethanol and 40ml acetic acid mix
  - Destaining solution: 120ml ethanol and 80ml acetic acid mixed well

SI5: Scanning Electron Microscopy (SEM)

- For SEM cells at a density of $1x10^4$ were seeded onto the scaffold and incubated the plates at 37^0C in a 5% CO_2 atmosphere.
- The period selected for the study was 24 hours and day 7.
- The following table below shows the remaining protocol.

Reaction time	**Procedure**
1-2 hours	Collection in 1.5% glutaraldehyde in 0.1M cacodylate buffer.
10 mins	Wash in the buffer.
60 mins	1% osmium tetroxide in the buffer.
5 mins X 2	Wash in DW.
60 mins	1% tannic acid in the buffer.
5 mins X 2	Wash in DW.
5 mins X 2	30, 50, 70% ethyl alcohol increments.
30 mins	2% uranyl acetate in 70% ethyl alcohol.
5 mins X 2	90% ethyl alcohol.
10 mins X 2	100% ethyl alcohol.
5 mins X 2	Hexamethyldisilazane (HMDS) or Critical Point Drying (CPD)

- After the samples were completely dried out they were coated with gold to make it electron reflective.

SI6: Proteomics analysis using LC-MS to confirm stem cell differentiation

❖ **RapiGest Extraction buffer (Waters, USA)**

- Preparation of the 0.5% (w/v) Rapigest-50mM NH4HCO3/ DNAse/ Protease inhibitor, further referred to as RapiGest Extraction Buffer.
- Prepare 100 mL 50 mM NH_4HCO_3 buffer using analytical-grade (milli-Q) water.

- Add 5µL of Phenyl methane sulfonyl fluoride (PMSF) [Protease inhibitor] from 100mM Stock solution dissolved in isopropanol.
- Add DNAse II, at a concentration of 2 µg/mL. Use the normal bovine DNAse II Boehringer (DRC stock solution 0.4 mg/mL, use at final 5 µL/mL = 1/200).
- To a tube containing 1 mg RapiGestdetergent, add 50 µL of 50 mM NH4HCO3/DNAse/Protease inhibitor buffer and re-suspend vigorously.
- Use the 0.5% (w/v) Rapigest-50mM NH_4HCO_3 buffer always fresh.

❖ **Protein extraction from single cell suspensions using sonication/freeze-thaw/detergent**

- Start from 1.5 x 10^6 cells in isolation medium, 5 mL falcon tube
- Wash 2x with ± 4.5 mL ice-cold PBS in 5 mL falcon tube, 3 min 1200 rpm
- After 2nd wash, transfer pellet in 300 µl to 1.5 mL safe-lock tube, wash original falcon tube, and perform 3rd wash in the 1.5 mL safe-lock tube, spin down vigorously in a microfuge and aspirate till pellet
- Add 50 µLRapiGest Extraction Buffer, resuspend vigorously and sonicate 3x cycles without ice, at room temperature to allow DNAse to be active for ± 10 min. Check with pipette whether DNA has been digested: slimy threads or not? If yes, an additional 10 min at room temp.
- Remove debris: 10 min 14000 rpm ultracentrifugation, collect clear supernatant (± 40 µL) and store -80°C
- The optimal concentration for proteomic analysis: 100µg

Protein expression data was collected using an LC/MS/MS analysis of control (2D cultures cells) and cells grown on scaffolds for 7 days. The pathways were identified using the gene ontology tool (tool.dice-database.org/GOnet)

SI7: H&E staining

H&E staining was performed as follows. Formalin-fixed paraffin-embedded tissues were sectioned using a microtome set at 5µm thickness and collected on coated glass slides. The tissue sections were undergone three rounds of xylene wash for 10 min each. Then hydration was done using a gradient of absolute alcohol. 95%, 70%, 50% ethanol, and distilled water for 5 min each. Hematoxylin was applied for 2 minutes and washed under running tap water for 5 min. 1% acid

alcohol was used to differentiate the slides for 30 sec and washed in running tap water for 20 min. The slides were washed in 95% ethanol for 5 min and eosin was treated for 1min. and dehydration was carried out in three changes of absolutes ethanol for 5 min and three changes of xylene for 5 min each and mounted using DPX mountant.

SI8: Stem cell differentiation on tissue sections using different stains

- **Alcian blue staining**
  - Deparaffinize the slides and hydrate to distilled water
  - Stain in alcian blue (1% in 3% acetic acid)
  - Wash in running tap water for 2 minutes
  - Rinse in distilled water
  - Counterstain with nuclear fast red (0.1% nuclear fast red)
  - Wash in running tap water for 1 min
  - Dehydrate and clear in xylene and mount
- **Alkaline phosphatase activity**
  - Take dried specimens and incubate in BCIP in DMF and NBT in tris buffer and $MgCl_2$ at 37 for 30min
  - Wash and counterstain in nuclear fast red
  - Wash in running tap water
  - Dehydrate and mount
- **Oil red O**
  - Dehydrate the sections
  - Place in absolute propylene alcohol for 2-5 min
  - Stain in oil red o (5% in propylene alcohol) for 8-10 min in 60ºC oven
  - Differentiate in 85% propylene alcohol for 2-5 min
  - Rinse in 2 changes of distilled water
  - Stain in hematoxylin
  - Wash thoroughly in running tap water for 5 min
  - Place in distilled water
  - Mount in glycerin jelly

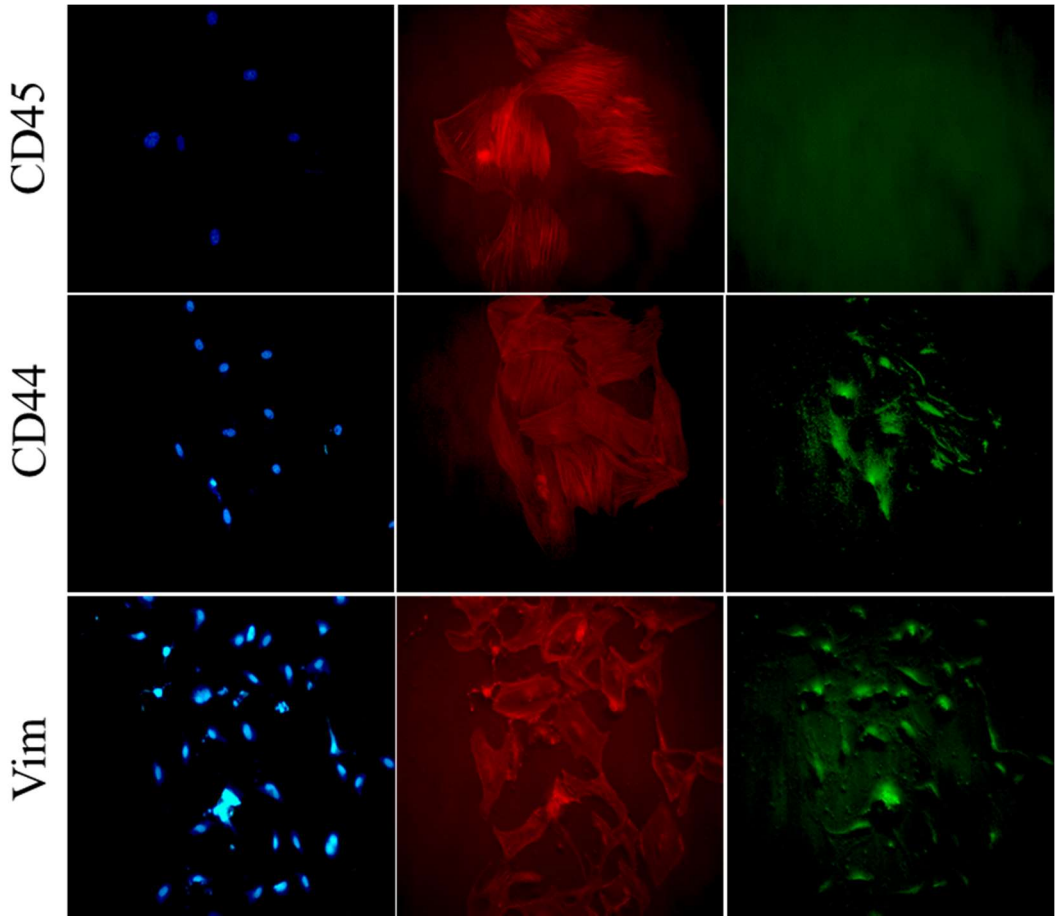
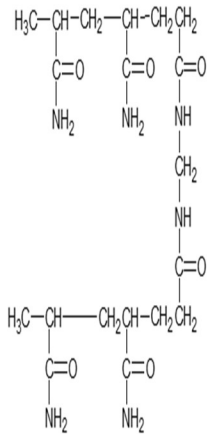


Fig S1: Immunofluorescence analysis of stem cell markers (magnification 40X)

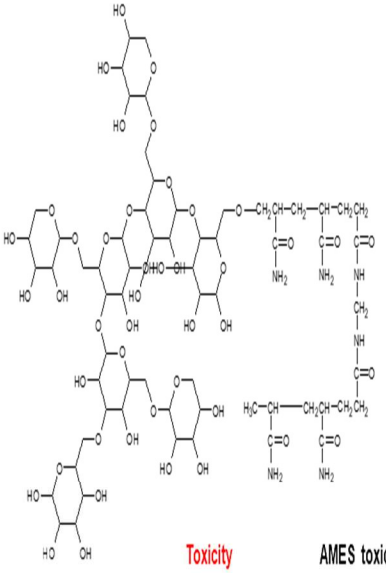
Acrylamide-bis-acrylamide



Descriptor	Value
Molecular Weight	442.517
LogP	-2.0373
#Rotatable Bonds	16
#Acceptors	6
#Donors	6
Surface Area	180.737

Toxicity	AMES toxicity	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose (human)	0.716	Numeric (log mg/kg/day)
Toxicity	hERG I inhibitor	No	Categorical (Yes/No)
Toxicity	hERG II inhibitor	No	Categorical (Yes/No)
Toxicity	Oral Rat Acute Toxicity (LD50)	1.486	Numeric (mol/kg)
Toxicity	Oral Rat Chronic Toxicity (LOAEL)	2.145	Numeric (log mg/kg_bw/day)
Toxicity	Hepatotoxicity	No	Categorical (Yes/No)
Toxicity	Skin Sensitisation	No	Categorical (Yes/No)
Toxicity	*T.Pyriformis* toxicity	0.283	Numeric (log ug/L)
Toxicity	Minnow toxicity	4.373	Numeric (log mM)

PST-Acrylamide- bis-acrylamide



Descriptor	Value
Molecular Weight	1665.566
LogP	-18.554
#Rotatable Bonds	38
#Acceptors	44
#Donors	28
Surface Area	646.402

Toxicity	AMES toxicity	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose (human)	0.438	Numeric (log mg/kg/day)
Toxicity	hERG I inhibitor	No	Categorical (Yes/No)
Toxicity	hERG II inhibitor	Yes	Categorical (Yes/No)
Toxicity	Oral Rat Acute Toxicity (LD50)	2.482	Numeric (mol/kg)
Toxicity	Oral Rat Chronic Toxicity (LOAEL)	5.896	Numeric (log mg/kg_bw/day)
Toxicity	Hepatotoxicity	No	Categorical (Yes/No)
Toxicity	Skin Sensitisation	No	Categorical (Yes/No)
Toxicity	*T.Pyriformis* toxicity	0.285	Numeric (log ug/L)
Toxicity	Minnow toxicity	43.415	Numeric (log mM)

Fig S2: Toxicity analysis using bioinformatics tool pkCSM

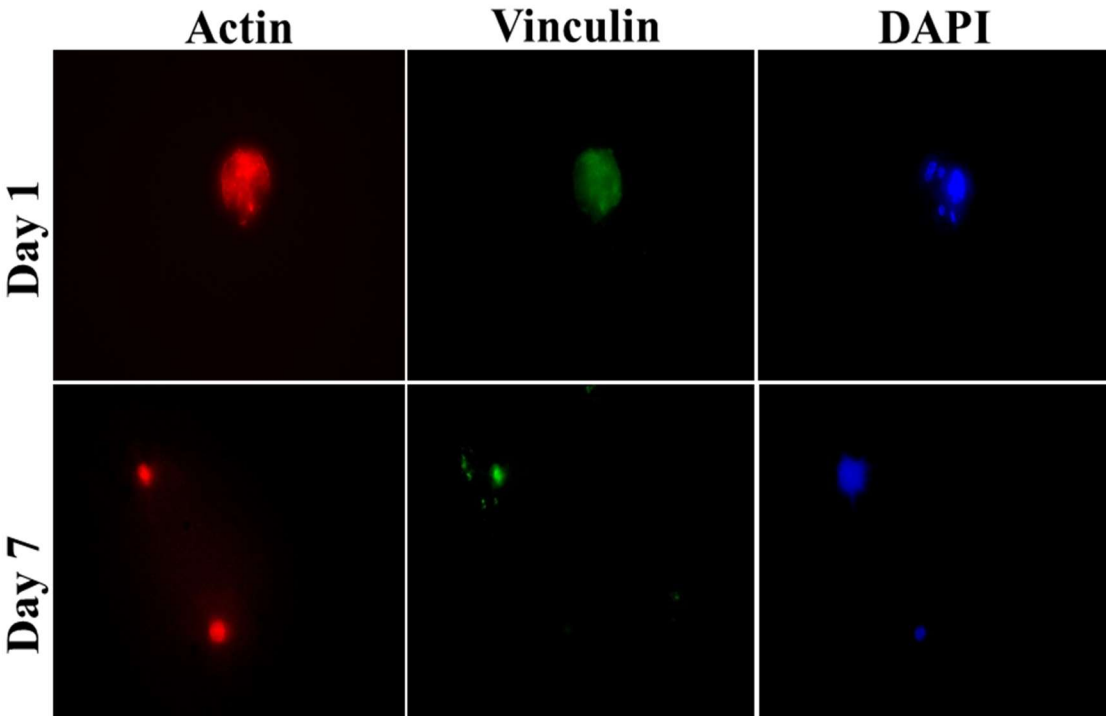


Fig S3: Actin- vinculin staining to identify focal adhesions (magnification 40X)

Accession	Description
Q5VCS6	Tudor domain-containing protein 5
Q01320	DNA topoisomerase 2-alpha
Q9ESN6	Tripartite motif-containing protein 2
P11369;Q8BHM9	LINE-1 retrotransposable element ORF2 protein
Q9Z1S3	RAS guanyl-releasing protein 1
P07724	Serum albumin
Q75N62	GTPase IMAP family member 8
Q8CG73	Protein fantom
Q921I1	Serotransferrin
P52480	Pyruvate kinase PKM
Q6PDI5;Q6ZPJ0;Q8CDZ2	Proteasome adapter and scaffold protein ECM29
Q9CPT0	Apoptosis facilitator Bcl-2-like protein 14
Q60949	TBC1 domain family member 1
Q61410	cGMP-dependent protein kinase 2
P10854;P10853;P70696;Q64475;Q64478;Q64524;Q64525;Q6ZWY9;Q8CGP0;Q8CGP1;Q8CGP2;Q9D2U9	Histone H2B type 1-M
Q70UZ7	von Willebrand factor A domain-containing protein 2
Q91W10	Zinc transporter ZIP8
Q99MX1	Ubiquitin carboxyl-terminal hydrolase 26
P68369;P68373;P05214	Tubulin alpha-1A chain
P05213	Tubulin alpha-1B chain
P16858	Glyceraldehyde-3-phosphate dehydrogenase
P70281	Synaptonemal complex protein 3
Q9D7P9	Serpin B12
Q7TSZ8	Nucleus accumbens-associated protein 1
Q9JJT9	Phosphorylated adapter RNA export protein
Q920Q2	DNA repair protein REV1
Q6ZQ06	Centrosomal protein of 162 kDa
Q99PN3;Q80ZQ5	Tripartite motif-containing protein 26
Q587J6	LINE-1 type transposase domain-containing protein 1
Q9D4H9;Q5SV66	PHD finger protein 14
P53569	CCAAT/enhancer-binding protein zeta
A2AHC3	Calmodulin-regulated spectrin-associated protein 1
P29699	Alpha-2-HS-glycoprotein
Q8BRH0	Protein O-mannosyl-transferase TMTC3
P35922	Synaptic functional regulator FMR1
Q9CZX0	Elongator complex protein 3
Q9R0E2	Procollagen-lysine_2-oxoglutarate 5-dioxygenase 1
P19426	Negative elongation factor E
Q8BFZ3	Beta-actin-like protein 2
Q1HKZ5	Mitogen-activated protein kinase kinase kinase 13
Q3URE1	Malonate--CoA ligase ACSF3_mitochondrial
O55028	[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase_ mitochondrial
Q6P9L6	Kinesin-like protein KIF15

Q9D8S4 Oligoribonuclease_ mitochondrial
Q9Z1L5 Voltage-dependent calcium channel subunit alpha-2/delta-3
Q99PT1 Rho GDP-dissociation inhibitor 1
Q69ZU6 Thrombospondin type-1 domain-containing protein 7A
P53349 Mitogen-activated protein kinase kinase kinase 1
Q6P542 ATP-binding cassette sub-family F member 1
Q60605 Myosin light polypeptide 6
P02104 Hemoglobin subunit epsilon-Y2
Q8BU03 Periodic tryptophan protein 2 homolog
Q8BGV7 BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 1
Q8VDS4 Regulation of nuclear pre-mRNA domain-containing protein 1A
Q6ZQK5 Arf-GAP with coiled-coil_ ANK repeat and PH domain-containing protein 2
P68134 Actin_ alpha skeletal muscle
P62737;P63268;P68033 Actin_ aortic smooth muscle
P56480 ATP synthase subunit beta_ mitochondrial
A0A140LI88 Ankyrin repeat domain-containing protein 31
P62806 Histone H4 OS=Mus musculus
Q91WD4 UPF0415 protein C7orf25 homolog
P02089;P02088 Hemoglobin subunit beta-2
C0HKE1;C0HKE2;C0HKE3;C0HKE4;C0HKE5;C0HKE6;C0HKE7;C0HKE8;C0HKE9;Q64523;Q6GSS7;Q8BFU2;Q8CGP5;Q8CGP6;Q8CGP7;Q8R1M2;P0C0S6;P
27661;Q3THW5;Q64522 Histone H2A type 1-B
Q3LAC4 Phosphatidylinositol 3_4_5-trisphosphate-dependent Rac exchanger 2 protein
Q91ZW3 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5
Q61703 Inter-alpha-trypsin inhibitor heavy chain H2
P35441 Thrombospondin-1
P01942;P06467 Hemoglobin subunit alpha
Q8CIB5 Fermitin family homolog 2
P40224 Stromal cell-derived factor 1
Q6PIX9 Uncharacterized protein C17orf80 homolog P0CG49;P0CG50;P62983;P62984 Polyubiquitin-B
P54071 Isocitrate dehydrogenase [NADP]_ mitochondrial
Q61043;O35166 Ninein
Q6ZQJ5 DNA replication ATP-dependent helicase/nuclease DNA2
P20029 Endoplasmic reticulum chaperone
Q8VCR7 Protein ABHD14B Q69Z26 Contactin-4 P51667;Q06806 Myosin regulatory light chain 2_
ventricular/cardiac muscle isoform
P31001 Desmin
A2AQP0 Myosin-7B
P58771;P21107 Tropomyosin alpha-1 chain Q6GQT1;B2RQE8;Q6ZQ82;Q8C0R0;Q8VI24;Q99JT2;Q99KH8 Alpha-2-macroglobulin-P
Q61292 Laminin subunit beta-2
Q8BJS8 Mdm2-binding protein
P63017 Heat shock cognate 71 kDa
Q9D071 MMS19 nucleotide excision repair protein
A2A863 Integrin beta-4
Q8CJ40;P10076 Rootletin
Q8BVD5 MAGUK p55 subfamily member 7
Q501J6 Probable ATP-dependent RNA helicase DDX17
P17156 Heat shock-related 70 kDa protein 2
Q9Z2Q5 39S ribosomal protein L40_ mitochondrial Q8VCM7 Fibrinogen gamma chain
Q7M6Z4 Kinesin-like protein KIF27
P20152 Vimentin
Q9JJ89 Coiled-coil domain-containing protein 86
Q9WVM8 Kynurenine/alpha-aminoadipate aminotransferase_ mitochondrial
P63260;P60710 Actin_ cytoplasmic
P01831 Thy-1 membrane glycoprotein B2RR83 3'-5' RNA helicase

Fig S4: Protein accession numbers and description analyzed in LC-MS

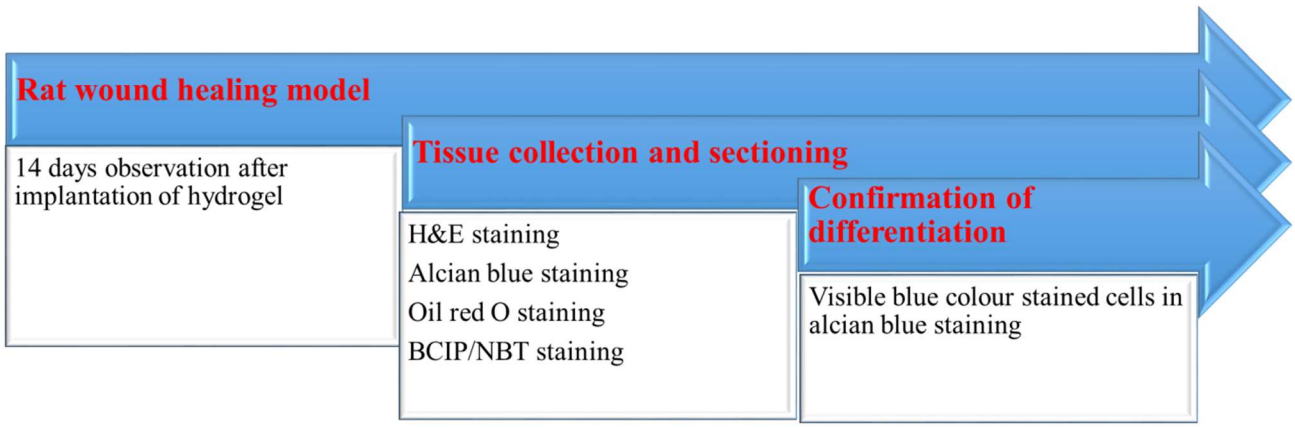


Fig S5: Illustration of *in vivo* differentiation studies

Pathways	P value
cellular component organization or biogenesis	2.06E-07
cellular component organization	3.79E-07
cellular component biogenesis	4.04E-06
cellular component assembly	4.90E-06
cellular response to stress	1.52E-05
female meiosis chromosome separation	2.98E-05
cell development	3.20E-05
hydrogen peroxide catabolic process	3.40E-05
response to stress	3.87E-05
cytoskeleton organization	1.22E-06
cellular component organization or biogenesis	1.64E-06
cellular component organization	5.91E-06
organelle organization	9.02E-06
positive regulation of cellular process	1.12E-05
movement of cell or subcellular component	1.34E-05
positive regulation of cell migration	2.52E-05
positive regulation of cell motility	3.16E-05
positive regulation of cellular component movement	3.81E-05
positive regulation of locomotion	4.31E-05
regulation of cell migration	4.55E-05

Fig S6: LC-MS data analysis on up-regulated and down-regulated pathways along with the p-value