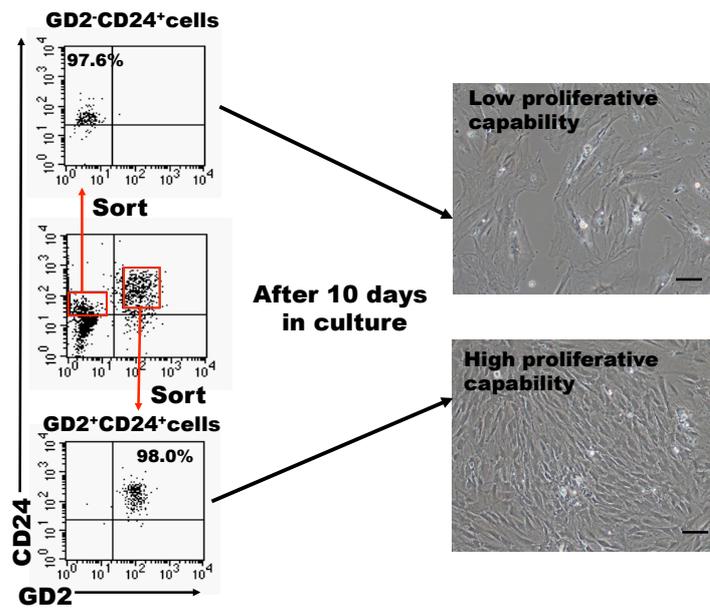


Supporting Online Material for
**Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the
intervertebral disc**

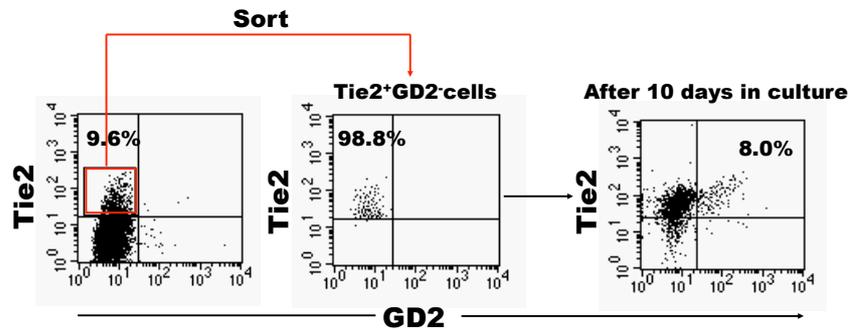
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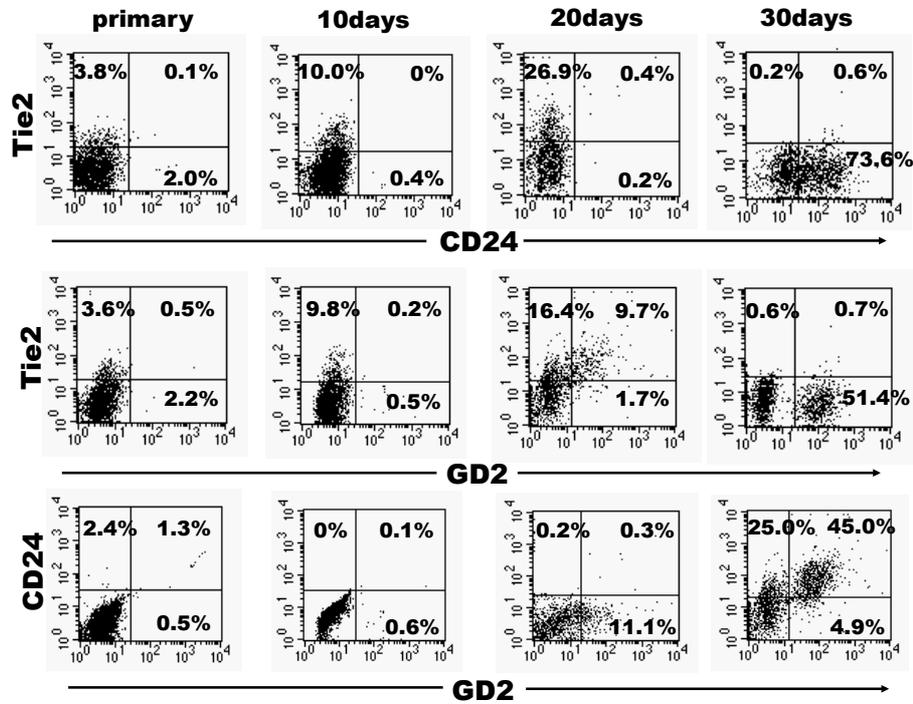
Supplementary Figure S1

GD2 positive cells sorted from mouse nucleus pulposus (mNP) showed high proliferative capability. Cells were sorted from 20-day-cultured mNP. Sorted GD2⁺CD24⁺ cells were superior to GD2⁻CD24⁺ cells in proliferative capacity at 10 days in additional liquid culture. Scale bars are 50 μ m.



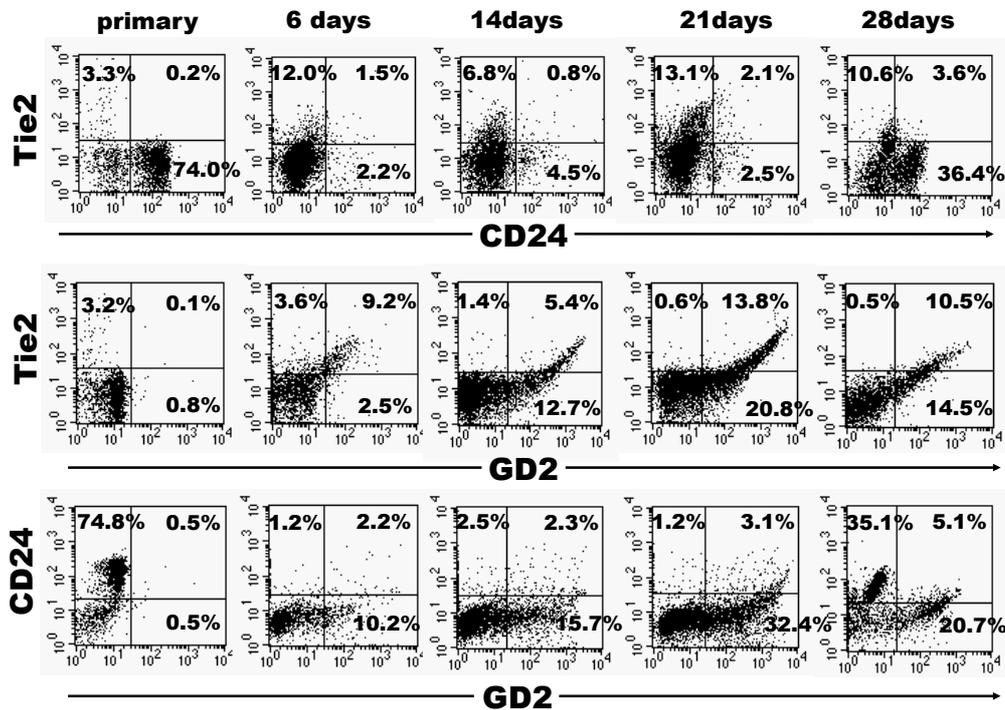
Supplementary Figure S2

Generation of GD2⁺ cells from sorted mouse Tie2⁺GD2⁻ NP cells. Tie2⁺GD2⁻ NP cells were sorted from 10-day-cultured mNP cells and passaged in liquid medium for 10 days. Generation of Tie2⁺GD2⁺ was confirmed.



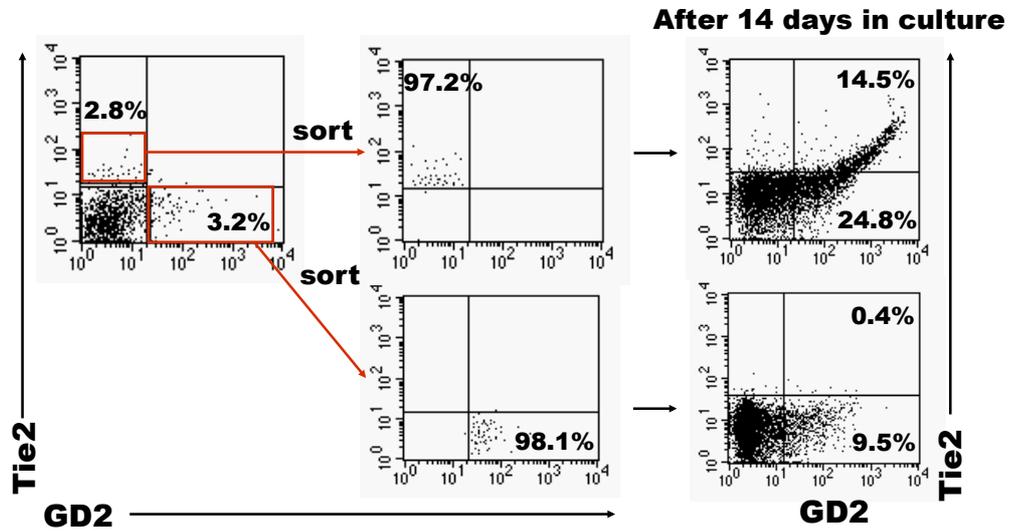
Supplementary Figure S3

Expression of Tie2, GD2 and CD24 in mNP cells during liquid culture. A population positive for tyrosine kinase receptor Tie2 was found (primary and 10 days) and Tie2⁺GD2⁻ changed into Tie2⁺GD2⁺ population in process of culture expansion. Tie2⁺GD2⁺ cells increased and then disappeared by day 30, while GD2⁺CD24⁺ or GD2⁻CD24⁺ cells increased. Typical expression patterns of three experiments are shown.



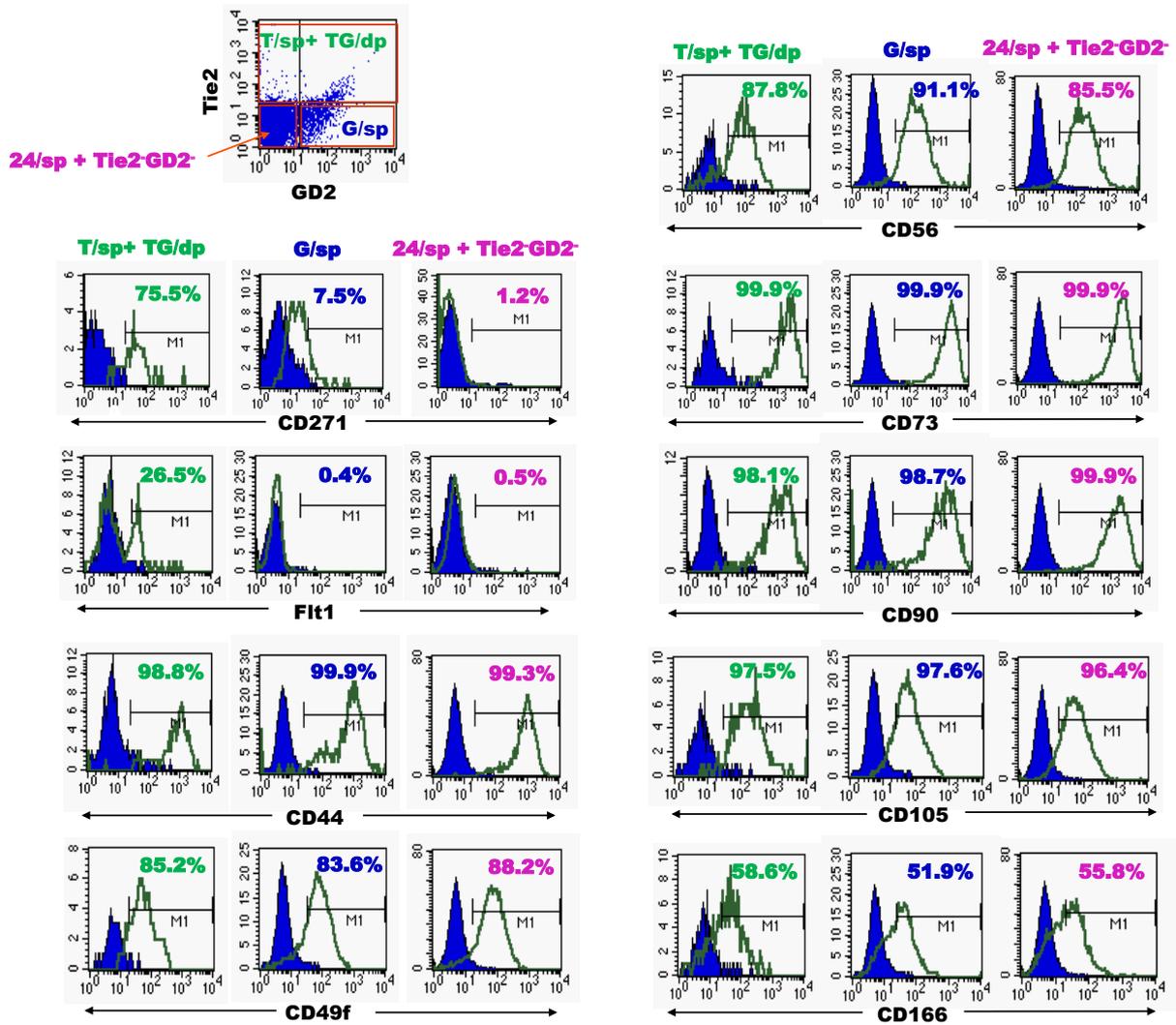
Supplementary Figure S4

Expression of Tie2, GD2 and CD24 during liquid culture of human NP (hNP) cells. In human NP cell culture a small number of Tie2⁺GD2⁻CD24⁻ cells (Tie2 Single Positive cells: T/sp) were detected in the beginning but hardly increased (middle panels) whereas Tie2⁺GD2⁺CD24⁻ cells (Tie2 and GD2 Double Positive cells: TG/dp) and Tie2⁻GD2⁺CD24⁻ cells (GD2 Single Positive cells: G/sp) increased. Finally at the 28th day of culture, 35% of NP cells were recognized as Tie2⁻GD2⁻CD24⁺ cells (CD24 Single Positive cells: 24/sp) aside from 24/sp that used to make up to 74% of primary cells had disappeared by day 6 suggesting their poor proliferation capability. As a whole, a hierarchy that originates from T/sp and followed by in the order of TG/dp, G/sp and 24/sp was observed. Typical expression patterns of five experiments are shown.



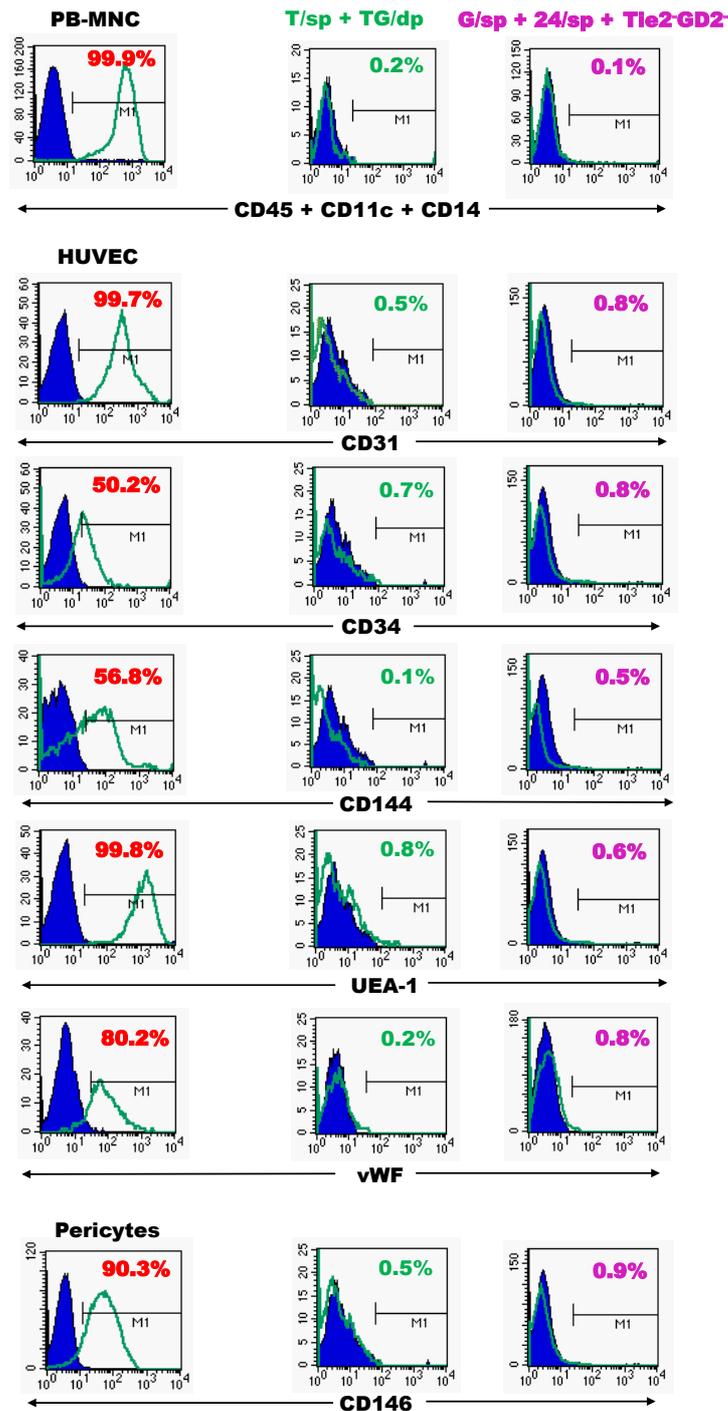
Supplementary Figure S5

Both TG/dp and G/sp generated from T/sp whereas G/sp did not generate T/sp or TG/dp. T/sp and G/sp were purified from hNP cells. Each cell population was cultured in liquid culture condition and analyzed for changes in expression of Tie2 and GD2. After 14 days in culture, T/sp cells generated TG/dp and G/sp however, G/sp cells did not generate T/sp or TG/dp. Typical expression patterns of five experiments are shown.



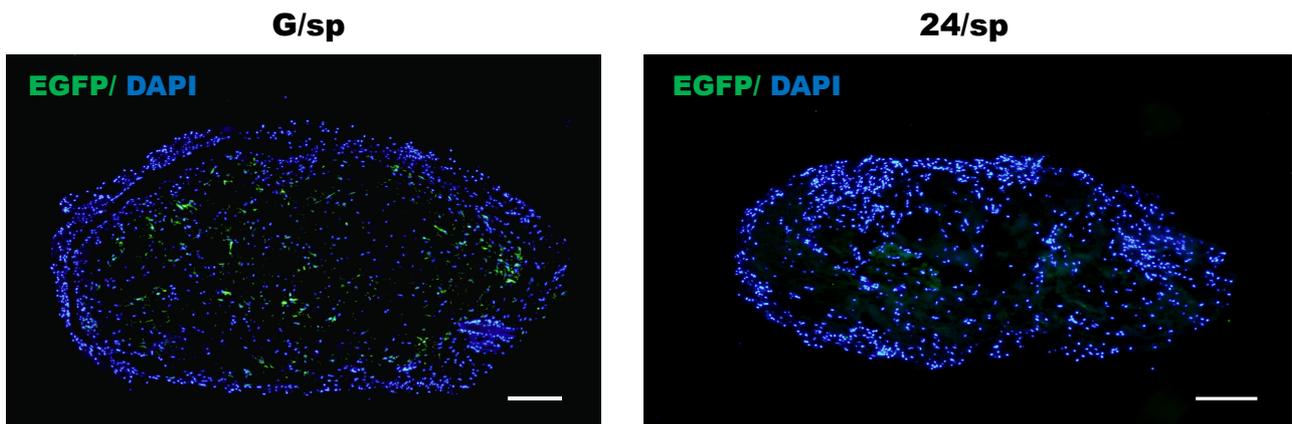
Supplementary Figure S6

Expression of other surface markers on hNP cells. The expression of other cell surface markers specific for bone marrow stromal cells were tested. hNP cells that had been cultured for seven days were stained with Tie2, GD2 and CD24 antibodies and then stained with antibodies for other surface markers. T/sp + TG/dp showed distinction from the downstream two populations G/sp and 24/sp in Flt1 and CD271. However CD44, CD49f, CD56, CD73, CD90, CD105, and CD166 were positively expressed universally. Typical expression patterns of five experiments are shown.



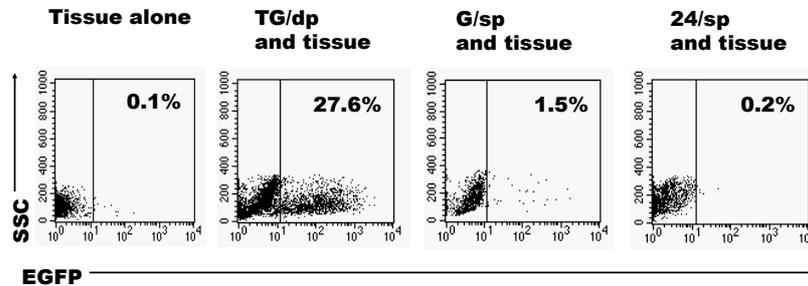
Supplementary Figure S7

Expression of macrophage, endothelial cell and pericyte markers on hNP cells. Primary hNP cells were stained with Tie2, GD2 and CD24 antibodies and then stained with antibodies for CD11c, CD14, CD31, CD34, CD45, CD144, CD146 and Ulex europaeus Lectin 1(UEA-1) as cell surface markers. Cytoplasmic Von Willebrand Factor (vWF) was also stained after membrane permeabilization. Peripheral blood mononuclear cells (PB-MNC: from normal human volunteer donor), normal human umbilical vein endothelial cells (HUVEC: Lonza) and pericytes (from human placenta : PromoCell) were used as positive control cells. Primary hNP cells did not express these cell surface markers and vWF. Typical expression patterns of three experiments are shown.



Supplementary Figure S8

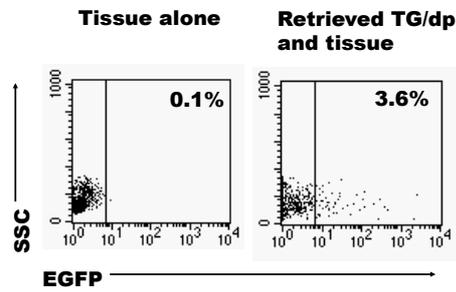
Evaluation of self-renewal capability *in vivo*. 1×10^5 of EGFP-labeled G/sp (left) 24/sp (right) transplanted subcutaneously in NOD/SCID mice with lethally irradiated (15Gy) allogeneic hNP tissue as scaffold. Very few (left) or almost no (right) EGFP labeled cells were detected. Scale Bars are 200 μm .



Supplementary Figure S9

FACS analysis of EGFP⁺ cells retrieved from the primary transplants.

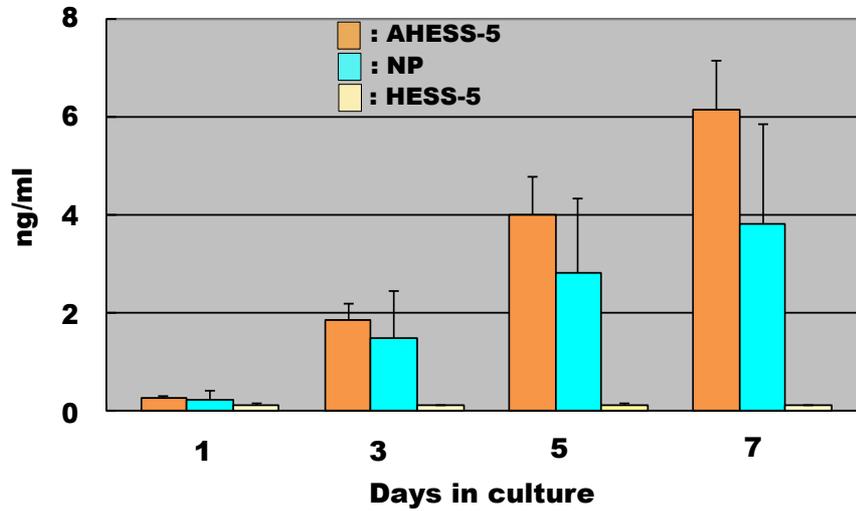
Detection of EGFP⁺ cells in the digestively retrieved cells from each transplant is shown. Tissue alone means that lethally irradiated (15Gy) allogeneic hNP tissue was transplanted without cells. Labeled cells were detected in the retrieved cells from the TG/dp transplants in every five recipients whereas it was hardly detected from G/sp or from 24/sp transplants. Typical result of five experiments are shown.



Supplementary Figure S10

FACS analysis of EGFP⁺ cells retrieved from the secondary transplants.

Detection of EGFP⁺ cells in the digestively retrieved cells from each transplant is shown. Tissue alone means that lethally irradiated (15Gy) allogeneic hNP tissue was transplanted without cells. Labeled cells were detected in the retrieved cells from the TG/dp transplants of every five recipients. Typical result of five experiments are shown.



Supplementary Figure S11

Human Ang-1 productions from AHES-5 (murine stromal cells overexpressing human Ang-1)²², primary hNP cells and murine stromal cell line HESS-5 to cell culture supernatant were determined using an Ang-1 ELISA kit (R&D System). Cell culture supernatant of confluent AHES-5, primary NP cells and HESS-5 were measured at 1, 3, 5, and 7 days in each culture period ($n=5$).

Mouse No.	Chon.	Adipo.	Osteo.	Multipotent
1	-	+	+	**
2	-	+	+	**
3	+	+	+	***
4	-	+	+	**
5	+	+	+	***
Ratio	2/5	5/5	5/5	Tri-2/5
				Bi-5/5

Supplementary Table S1

Result of mesenchymal lineage differentiation assay *in vitro* in cells retrieved from TG/dp transplants.

Case	Age	Gender	Disc grade	pathology	Operation
1	18	M	II	LDH	Hemiotomy
2	19	F	I	Burst Fx	ASF
3	19	M	II	LDH	ASF
4	20	F	II	LDH	Hemiotomy
5	21	M	II	Burst Fx	ASF
6	21	M	II	LDH	Hemiotomy
7	21	M	II	LDH	Hemiotomy
8	21	M	II	LDH	Hemiotomy
9	26	M	II	Burst Fx	ASF
10	28	M	II	LDH	PLIF
11	28	F	II	Burst Fx	ASF
12	31	M	II	Spondylosis	PLIF
13	31	F	III	LDH	PLIF
14	35	M	III	LDH	Hemiotomy
15	37	M	IV	LDH	Hemiotomy
16	38	M	III	Burst Fx	ASF
17	38	M	III	LDH	PLIF
18	39	M	III	LDH	Hemiotomy
19	47	M	III	Spondylosis	PLIF
20	48	M	IV	Spondylosis	PLIF
21	52	F	III	Burst Fx	ASF
22	60	F	IV	LDH	PLIF
23	69	M	IV	LDH	PLIF

Supplementary Table S2

Clinical data of IVD donors

Supplementary Methods

Cell isolation and culture. Human NP (hNP) tissues were cut into small pieces and digested with TrypLE Express (Gibco) for 1 hour followed by 0.25 mg/ml Collagense-P (Roche) for 2 hours at 37°C. The digested cells were washed twice with α -MEM (Gibco), and primarily seeded at 3,000 to 5,000 cells/cm². Mouse NP (mNP) tissues were also digested with the same enzymes for 30 minutes. hNP or mNP cells were cultured in α -MEM (Gibco) supplemented with 10% FBS (Cell Culture Bioscience) at 37°C, 5% CO₂ and 5% O₂.

FACS analysis and purification of cells. hNP cells were stained with PE-conjugated mouse specific monoclonal antibody (mAb) to human CD11c (BD Biosciences; S-HCL-3), CD14 (Coulter Immunotech; RMO52), CD31 (Coulter Immunotech; 1F11), CD34 (Coulter Immunotech; 581), CD44 (BD Biosciences; 515), CD45 (Coulter Immunotech; J.33), CD49f (BD Biosciences; GoH3), CD56 (Beckman Coulter; N901), CD73 (BD Biosciences; AD2), CD90 (BD Biosciences; 5E10), CD105 (Beckman Coulter; 1G2), CD144 (Coulter Immunotech; TEA1/31), CD166 (BD Biosciences; 3A6), CD271 (Miltenyi; ME20.4-1.H4) VEGF-R1 (Flt1) (R&D system; 49560), and FITC conjugated anti-human CD146 (Serotec; OJ79C) mAb. FITC-conjugated Ulex Europaeus Agglutinin 1 Lectin (UEA-1) (Biogenesis) was also used. For FACS analysis of Cytoplasmic Von Willebrand Factor (vWF) staining, cells were permeabilized by IntraPrep (Beckman Coulter) using mouse anti-human vWF mAb (DAKO; F8/81). Staining procedure was performed according to the manufacture's procedure. After washing, cells were analyzed using an FACS Calibur flow cytometer (BD Biosciences). For purification of cells, hNP cells were incubated with anti-human disialoganglioside GD2 (GD2) (BD Pharmingen; 14; G2a) mAb for 30 minutes and FITC-conjugated anti-mouse Ig goat (BD Biosciences) or Alexa Fluor 633 (invitrogen) goat anti-mouse IgG secondary antibody for 30 minutes at 4°C. After washing, cells were stained with PE- or allophycocyanin-conjugated anti-human Tie2 (R&D Systems, clone 83715) mAb and PE-conjugated or biotinylated anti-human CD24 (BD Biosciences; clone ML5) mAb. Streptavidin-allophycocyanin-Cy7 (BD Pharmingen) was also used as needed. mNP cells also stained with anti-human GD2 mAb, anti-mouse Tie2 biotinylated goat polyclonal antibody (pAb) (R&D Systems) and PE-conjugated anti-human CD24 (BD Biosciences; clone ML5) mAb. In the cases where biotinylated primary antibodies were used cells were washed and then incubated with allophycocyanin-conjugated streptavidin (BD Biosciences). Stained hNP or mNP cells sorted using a FACS Vantage cell sorter (BD Biosciences).

Preparation for staining the NP-CFU-F colonies formed in methylcellulose were detached with 2mM EDTA for 10min at room temperature while the NP-CFU-S colonies were collected by pipetting softly and then the samples were washed with PBS. Approximately 5×10^4 cells were suspended in 0.5 ml PBS and centrifuged at cytopins for 3min at 700 r.p.m. Then the cells attached the slides were fixed with 4% paraformaldehyde in PB for 15min and stained. For FACS analysis of Cytoplasmic ECM staining, NP-CFU-F and NP-CFU-S were dispersed using TrypLE Express for 30 min and were permeabilized by IntraPrep.

Co-culture study. Primary hNP cells were harvested following four days of monolayer culture in α -MEM (Gibco) supplemented with 10% FBS. Six-well culture plates and inserts (BD Biosciences) containing a polyethylene terephthalate track-etched membrane with 0.4 μ m pores at the bottom, were used for co-culture²². 1.0×10^4 hNP cells were seeded onto each culture insert, which had a confluent monolayer of AHES-5 on the back side of the culture insert membrane with direct cell-to-cell contact. AHES-5 is engineered from a hematopoietic-supportive mouse BM stromal cell line HES-5²², to produce human angiopoietin-1. Soluble human Ang-1 protein (500ng/ml) was also used instead of AHES-5. The blocking assay of Ang-1 was performed using 10 μ g/ml goat anti-human Tie-2 blocking pAb or normal goat Ig (both from R&D Systems) as controls. After seven days in culture, NP cells were removed by TrypLE Express and applied for FACS analysis and colony assay.

Immunohistochemistry. The following primary antibodies were used: anti-human Tie2/TEK mAb (Upstate Millipore, Ab33), anti-mouse Tie2/TEK biotinylated goat pAb (R&D systems), anti-human disialoganglioside GD2 mAb, (BD Biosciences, 14.G2a), anti-collagen type II mAb (Seikagaku Biobusiness Corporation, II-4C11), anti-human aggrecan mAb (Chemicon, Millipore, MAB2015), anti-mouse aggrecan rabbit pAb (Chemicon, Millipore, AB1031), anti-human angiopoietin-1 rabbit anti sera (Acris Antibodies GmbH), anti-human and mouse osteocalcin rabbit anti sera (Biomedical Technologies Inc.). Alexa Fluor-488-conjugated goat anti mouse IgG, Alexa Fluor-594-conjugated goat anti-mouse IgG, Alexa Fluor-488-conjugated goat anti rabbit IgG, Alexa Fluor-594-conjugated goat anti rabbit IgG, (Invitrogen) were used as secondary antibodies with co-staining for nuclei using DAPI (Vector Laboratories). were visualized by (Invitrogen).

Alexa Fluor-488-conjugated phalloidin was for F-actin fibers and Alexa Fluor-488-conjugated rabbit anti-green fluorescent protein (EGFP) pAb (Invitrogen) was used for amplification of the fluorescence from the EGFP labeled transplanted cells. In addition, incubation with anti-rabbit Envision (Dako) or biotinylated goat anti-mouse antibody (Cortex Biochem Inc. CA USA) followed by peroxidase-conjugated streptavidin (Dako) and DAB staining with hematoxylin counter-staining were used. For negative controls, isotype-matched mouse or rabbit IgG was used. Decalcification solution A, Triton-X100 (WAKO) and TrypLE Express (Gibco) were used if necessary.

Multi-potent differentiation. Purified hNP cells were assessed their multi-differentiation potential toward osteogenesis, adipogenesis and chondrogenesis as described previously³. Osteogenic differentiation was confirmed by staining for alkaline phosphatase, von Kossa, Masson's trichrome and staining for osteocalcin. For adipogenesis, Oil-red O staining was used and for chondrogenesis, safranin O, toluidine blue, staining for aggrecan and type II collagen were used.

Real-Time RT-PCR for the Angiopoietin-1 mRNA Cells were homogenized in lysis buffer and total RNA was prepared using the RNeasy-Microscale RNA isolation kit (Ambion, Applied Biosystems). mRNA was reverse transcribed using High Capacity RNA-to cDNA Kit (Applied Biosystems). cDNA was amplified using TaqMan Gene Expression Assay (Taqman probe ID:Hs00919202_m1, Product detail can be obtained from the URL http://bioinfo.invitrogen.com/genome-database/details/gene-expression/Hs00919202_m1, Applied Biosystems) including gene-specific primers and TaqMan probes (Gene symbol: ANGPT1, Ref. Sequence: NM_001146). Quantification of mRNA expression was performed according to the comparative CT method (ABI PRISM User Bulletin 2, Applied Biosystems) with 18S ribosomal RNA as the endogenous control. Gene expression levels of sorted cell fractions were normalized to the level of Tie2⁻/GD2⁻/CD24⁻ cell fraction. For hNP sorting, Tie2⁺GD2⁻CD24⁻ (Tie2 Single Positive: T/sp) cells were from primary hNP cells; Tie2⁺GD2⁺CD24⁻ (Tie2 and GD2 Double Positive: TG/dp) cells and Tie2⁻GD2⁺CD24⁻ (GD2 Single Positive: G/sp) cells were from 7 to 14 days-cultured NP cells; Tie2⁻GD2⁻CD24⁺ (CD24 Single Positive: 24/sp) cells were from 30 days-cultured NP cells.

Multi-potent differentiation in vivo by transplantation of purified human NP cells into injured tail IVDs and bone defect in mice. Under surgical microscope and general anesthesia, the injury to the tail disc of mature NOD/SCID mouse (12Wks, female, n=6) was performed prior to cell injections by a single aspiration of the NP using a 27 gauge needle.

EGFP-labeled and sorted hNP cells (5.0×10^5) were suspended in 50 μ L of PBS using an insulin syringe with a 27 gauge needle (Terumo). About 10 μ L of the cell suspension was injected into injured tail IVDs in mice. Twelve weeks after transplantation, the mice were sacrificed under ether anesthesia and the tail IVDs were excised and examined for immunohistochemistry. In the case of osteogenic differentiation, left femur of mature NOD/SCID mouse (12Wks, female, n=6) was exposed under surgical microscope and general anesthesia. With a surgical bone rongeur forceps luer, 5mm bone defect was created at the middle of femur. An internal fixation was achieved by using an 18 gauge needle as an intramedurally nail. Hydroxyapatite granules loaded with purified TG/dp or 24/sp clones (5.0×10^5) were implanted in the defect and the wound closed. Eight weeks after, the healed femur was harvested, intramedurally nail was removed and examined for histology and immunohistochemistry.

Bioluminescence imaging (BLI). The mice were anesthetized with inhaled isoflurane and placed into the BLI system (Xenogen) and images were acquired using the CCD camera. Range of interest was set at coccygeal intervertebral disc. Filter to detect GFP was used and negative control mice without cell transplantation were used for calibration of autofluorescence. Data analysis was performed using the Living ImageTM Software (Caliper Life Sciences). Images were analyzed every week in all groups up to 12 weeks.