Chapter 4

Engineering Cartilage Tissue by Pellet Coculture of Chondrocytes and Mesenchymal Stromal Cells

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Abstract

Coculture of chondrocytes and mesenchymal stromal cells (MSCs) in pellets has been shown to be beneficial in engineering cartilage tissue in vitro. In these cultures trophic effects of MSCs increase the proliferation and matrix deposition of chondrocytes. Thus, large cartilage constructs can be made with a relatively small number of chondrocytes. In this chapter, we describe the methods for making coculture pellets of MSCs and chondrocytes. We also provide detailed protocols for analyzing coculture pellets with cell tracking, proliferation assays, species specific polymerase chain reactions (PCR), short tandem repeats analysis, and histological examination.

Key words Chondrocytes, Mesenchymal stromal cells, Coculture, Trophic effects, Cartilage engineering, Matrix deposition

1 Introduction

Partial replacement of chondrocytes by alternative cell sources can reduce the number of chondrocytes needed to engineering cartilage constructs in vitro [1–3]. Hendriks et al., cocultured bovine primary chondrocytes with human expanded chondrocytes, human dermal fibroblasts, mouse embryonic stem cells, mouse-3T3 feeder cells, or human mesenchymal stromal cells (MSCs) in cell pellets [4]. Their data indicated that cartilage matrix deposition increased in coculture pellets. Replacement of 80 % of the chondrocytes with other cell types resulted in similar amounts of GAG production when compared to pure chondrocyte pellets. This beneficial effect on cartilage formation is most prominent in cocultures of chondrocytes with mesenchymal stromal cells [5]. In a more recent study, we used a xenogeneic coculture model of human MSCs and bovine chondrocytes to study the contribution of each cell type to cartilage matrix formation [6, 7]. Our data showed a significant decrease in MSCs in coculture pellets over time, resulting in an almost homogeneous cartilage tissue predominantly derived from

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the initially seeded chondrocytes. Our data showed that the beneficial effect of coculture is largely due to increased chondrocyte proliferation and matrix formation, while chondrogenic differentiation of MSCs only marginally contributed to cartilage formation. We also demonstrated that these observations present in coculture pellets of chondrocytes and MSCs are independent of donor variation and culture conditions [8]. Subsequent experiments indicated that increased secretion of fibroblast growth factor 1 (FGF1) in coculture of MSCs and chondrocytes is responsible for increased chondrocyte proliferation in pellet cocultures [9]. Thrombospondin-2 has also been reported to be secreted by MSCs to promote chondrogenic differentiation both in vitro and in vivo [10]. These reports are the first to show the trophic role of MSCs in stimulating chondrocyte proliferation and matrix production.

2 Materials

2.1 Cell Sources

1. Bovine primary chondrocytes (bPCs) are isolated from full-thickness cartilage knee biopsies of female calves that are approximately 6 months old. Cartilage is separated and digested to extract primary chondrocytes (*see* Subheading 3.1).
2. Human primary chondrocytes (hPCs) are obtained from full thickness cartilage dissected from knee biopsies of a patient undergoing total knee replacement (*see* Subheading 3.2).
3. Human MSCs (hMSCs) are isolated from bone marrow aspirates of healthy donors (*see* **Note 1**).

2.2 Media, Solutions, Chemicals, and Kits

1. Chondrocyte proliferation medium: DMEM supplemented with 10 % FBS, 1 × nonessential amino acids, 0.2 mM ascorbic acid 2-phosphate (AsAP), 0.4 mM proline, 100 U penicillin/ml and 100 μg/ml streptomycin.
2. Chondrogenic differentiation medium: DMEM supplemented with 40 μg/ml of proline, 50 μg/ml ITS-premix, 50 μg/ml of AsAP, 100 μg/ml of Sodium Pyruvate, 10 ng/ml of Transforming Growth Factor beta 3 (TGFβ3), 10^{-7} M of dexamethasone, 500 ng/ml of Bone Morphogenetic Protein 6 (BMP6), 100 U penicillin/ml and 100 μg/ml streptomycin.
3. MSC proliferation medium:α-MEM plus 10 % fetal bovine serum, 1 % L-glutamine, 0.2 mM ascorbic acid, 100 U/ml penicillin, 10 μg/ml streptomycin and 1 ng/ml basic Fibroblast Growth Factor (bFGF).
4. Proteinase K digestion buffer: 1 mg/ml proteinase K (Sigma) in Tris–EDTA buffer (pH7.6), 18.5 μg/ml iodoacetamide and 1 μg/ml pepstatin A. The proteinase K solution can be stored

in aliquots at −20 °C for several weeks. After one thaw, do not freeze again. Tris–EDTA buffer: Dissolve 6.055 g Tris and 0.372 g EDTA·2 H_2O in 1,000 ml of H_2O. Adjust pH to 7.6.

5. PBE buffer: 14.2 g/l Na_2HPO_4 and 3.72 g/l Na_2EDTA, pH 6.5.
6. GAG stock solution: 50 mg/ml, 17.5 mg of cysteine–HCl was dissolved in 10 ml of PBE buffer. Aliquoted and store in −20 °C freezer.
7. GAG working solution (200 μg/ml): Dilute GAG stock solution 1:250 in PBE buffer.
8. DMMB solution: add 9.5 ml of 0.1 M HCl solution to 90.5 ml of d_2H_2O plus 0.304 g of glycine and 0.237 g of NaCl; adjust to pH 3 before adding 1.6 mg of DMMB to the buffer. When stored in the dark at RT, the solution is stable for 3 months; filter to get rid of precipitates before use.
9. Organic fluorescent dye (CM-DiI), Click-iT® EdU Imaging Kit, and the CyQuant DNA Kit.
10. QIAamp DNA Mini Kit and RNeasy Mini Kit (Qiagen).
11. iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio-Rad).
12. PowerPlex 16 System (Promega).
13. Collagenase type II (Worthington).
14. Click-iT® EdU Imaging Kit (Invitrogen).
15. Round bottom ultra low attachment 96-well plate.
16. Cryomatrix (Shandon).
17. DMMB (1, 9-Dimethyl-Methylene Blue).

2.3 Equipment

1. BD pathway 435 confocal microscope (BD Biosciences).
2. ELISA reader.
3. MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad).

3 Methods

3.1 Isolation of Human Articular Chondrocytes

1. Human cartilage tissue were obtained from total knee or hip joint replacement.
2. Cartilage tissue is cut from underlying bone and connective tissue with scalpels and chopped into pieces of approximately 2×2 mm.
3. Digest cartilage pieces for 20–22 h in collagenase type II (0.15 %) in DMEM supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml).

3.2 Isolation of Human Bone Marrow Mesenchymal Stromal Cells

1. Collect bone marrow aspirates in sterile heparin tubes.
2. Pour aspirate into 50 ml Falcon tubes.
3. Remove red blood cells by incubating 100 μl aliquots of aspirate with 900 μl red blood cell lysing buffer for 5–10 min on ice or until transparent.
4. Count cell numbers with Trypan blue staining. Plate cells at 50,000/cm^2 in T75 in MSC proliferation medium plus 1 % heparin.

3.3 Cell Tracking of Cell Populations in Pellet Cocultures with Organic Fluorescent Dyes CM-Dil

1. Trypsinize bovine or human chondrocytes and resuspend in PBS at a concentration of 2×10^6 cells/ml.
2. Incubate the cells with the fluorescent dye CM-DiI (final concentration of 4 μM) at 37 °C for 5 min followed by incubation at 4 °C for 15 min.
3. Wash cells two times by suspending cells in PBS followed by collecting cells by centrifuging at $300 \times g$ for 3 min.

3.4 Coculture of bPCs and hMSCs in Pellets

1. Trypsinize hMSCs and suspend in chondrocyte proliferation medium at a concentration of 1×10^6 cells/ml. Resuspend labeled bPCs or hPCs from Subheading 3.1 at the same concentration as hMSCs in chondrocyte proliferation medium.
2. Mix hMSCs with bPCs or hPCs at ratios of 80/20 % and 50/50 %. Seed a total of 200,000 cells in one well of a round bottom ultra low attachment 96-well plate in chondrocyte proliferation medium.
3. Use mono-culture of hMSCs only or bPCs only or hPCs only as controls. Cell numbers per well are the same as in coculture pellets.
4. Make pellets by centrifugation of the plate at $500 \times g$ for 5 min.
5. Xenogeneic cocultures (bPCs and hMSCs), including corresponding controls, are cultured in chondrocyte proliferation medium at all times.
6. For allogenic cocultures (hPCs and hMSCs), including corresponding controls, medium is changed to chondrogenic differentiation medium (*see* Subheading 2.2) on the second day after seeding.

3.5 Examination of Cell Proliferation in Pellets by EdU Labeling and Staining

1. 2 or 3 days after making pellets, add EdU (5-ethynyl-2′-deoxyuridine, provided in Click-iT® EdU Imaging Kit) to the culture medium of pellets at a concentration of 10 μM.
2. Harvest samples for analysis, 24 h later by transferring pellets to eppendorf tubes.
3. Wash cell pellets with PBS and fix with 10 % formalin for 15 min.

4. Embed samples in cryomatrix, and cut 10 μM sections with a cryotome.
5. Permeabilize sections and stain for EdU with Click-iT® EdU Imaging Kit according to the manufacturer's protocol. In this kit, nuclei are counterstained with LP435 (Hoechst 33342, provided in Click-iT® EdU Imaging Kit).

3.6 Image Acquisition and Analysis by Fluorescent Microscopy

1. Make fluorescent images with a BD pathway 435 confocal microscope (*see* **Note 2**).
2. Capture three separate images for each pellet section, using BP536/40 (Alexa 488), BP593/40 (DiI), and LP435 (Hoechst 33342) and pseudo color green, red, and blue respectively.
3. Open blue image of one pellet section with ImageJ software [11].
4. Set threshold by click drop-down menu via Image→Adjust→Threshold (*see* **Note 3**).
5. Open particle analyzer via Analyze→analyze particles.
6. Set area restrictions: 100-infinite; choose Display results, Exclude on edges, Include holes; click OK to count $\text{NUMBER}_{\text{of total cell}}$ (*see* **Note 4**).
7. Open red image of the same pellet section; set threshold as described above (*see* **Note 5**).
8. Open image calculator via Process→Image calculator.
9. Select blue image in the box of Image 1; select red image in the box of Image 2; select "AND" in the box of Operation; then click OK to generate a new image named "result of blue."
10. Run "Analyze particles" on new image "result of blue" with the same setting as above to count $\text{NUMBER}_{\text{of red cell}}$.
11. Open green image of the same pellet; set threshold and area restriction (*see* **step 6**) to count $\text{NUMBER}_{\text{of green cell}}$.
12. Run "Image calculator" by selecting green image in Image 1 box and red image in Image 2 box, with AND in Operation box to generate new image named "result of green."
13. Run "Analyze particles" on new image "result of green" with same setting as above to count $\text{NUMBER}_{\text{of green plus red cell}}$.
14. Input all NUMBERs into an Excel spreadsheet and perform the following calculations: Rate of EdU positive Chondrocyte = $\text{NUMBER}_{\text{of green plus red cell}} \div \text{NUMBER}_{\text{of red cell}} \times 100\ \%$; Rate of EdU positive MSCs = $(\text{NUMBER}_{\text{of green cell}} - \text{NUMBER}_{\text{of green plus red cell}}) \div (\text{NUMBER}_{\text{of total cell}} - \text{NUMBER}_{\text{of red cell}}) \times 100\ \%$; Labeling efficiency = $\text{NUMBER}_{\text{of red cell}} \div \text{NUMBER}_{\text{of total cell}} \times 100\ \%$ (*see* **Note 6**).

Table 1
Series dilution of GAG standards

GAG amount	Blank	0.5 μg	1 μg	1.5 μg	2 μg	2.5 μg
GAG working solution (*see* **Note 9**)	0 μl	10 μl	20 μl	30 μl	40 μl	50 μl
PBE buffer (*see* **Note 10**)	100 μl	90 μl	80 μl	70 μl	60 μl	50 μl

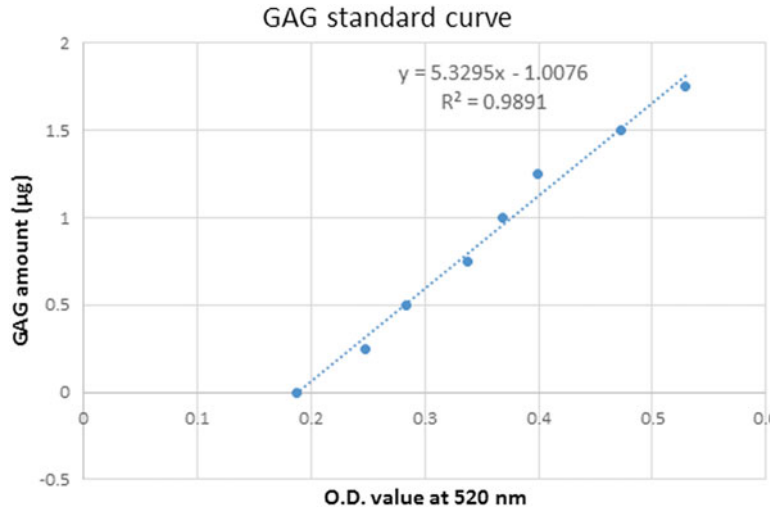

Fig. 1 An example of standard curve for GAG quantification. The blank (25 μl PBE, 5 μl 2.3 M NaCl and 150 μl DMMB solution) has an O.D. value of 0.18 ± 0.03

3.7 Quantitative GAG and DNA Assay

1. Perform glycosaminoglycan (GAG) and DNA assay at the end of coculture (i.e. 4 weeks).
2. Wash cell pellets ($n = 6$) with PBS and freeze pellets overnight at −80 °C.
3. Digest pellets in 500 μl of proteinase K digestion buffer (*see* **Note 7**) for more than 16 h at 56 °C.
4. To prepare a standard curve, make dilution series of cysteine–HCl, according to Table 1.
5. Add 5 μl of a 2.3 M NaCl solution and 25 μl of the samples or the standard in one well of a 96-well nontissue-culture-treated plate.
6. Add 150 μl of the DMMB (1, 9-Dimethyl-Methylene Blue) solution (*see* Subheading 2.2) and read the absorbance at 520 nm on an ELISA reader. Figure 1 gives an example of a standard curve (*see* Subheading 2.2).
7. Determine cell number by quantification of total DNA using a CyQuant DNA Kit, according to the manufacturer's instructions.

3.8 Cell Tracking with Species Specific PCR

1. Perform species-specific PCR to determine the ratio of MSCs and chondrocytes in xenogeneic coculture (hMSCs and bPCs) pellets at the end of culture (i.e. 4 weeks).
2. Isolate DNA samples of pellets with a QIAamp DNA Mini Kit according to the manufacturer's protocols.
3. Extract RNA samples of pellets with an RNeasy Mini Kit (*see* **Note 8**).
4. Reverse-transcribe one microgram of total RNA into cDNA using the iScript cDNA Synthesis kit.
5. Perform species-specific quantitative PCR (qPCR) on genomic DNA or cDNA samples by using the iQ SYBR Green Supermix.
6. Carry out PCR Reactions on MyiQ2 Two-Color Real-Time PCR Detection System under the following conditions: Denature cDNA for 5 min at 95 °C, follow with 45 cycles consisting of 15 s at 95 °C, 15 s at 60 °C and 30 s at 72 °C.
7. Generate a melting curve for each reaction to test primer dimer formation and nonspecific priming.
8. The primers for real-time PCR, either species specific or cross species-specific, are listed in Tables 2 and 3.
9. For each gene, standard curves are obtained by serial dilutions of cDNA (*see* **Note 9**). Figure 2 gives an example of standard curve for qPCR.
10. Use Bio-Rad iQ5 optical system software (version 2.0) to calculate copy numbers for each condition using the standard curve as reference.
11. Ratio of bovine or human cells in the xenogenic coculture pellets are defined as the proportion of human or bovine GAPDH copy numbers as percentage of the total copy numbers

Table 2
Forward (F) and reverse (R) primers used for quantitative PCR on genomic DNA

Gene name	Primer sequence	Product size	Gene bank No.
Cross-species GAPDH	F: 5′ GCATTGCCCTCAACGACCA 3′ R: 5′ CACCACCCTGTTGCTGTAGCC 3′	179 or 171[a]	NC_000012 and NC_007303
Human-specific GAPDH	F: 5′ TTCCACCCATGGCAAATTCC 3′ R: 5′ TTGCCTCCCCAAAGCACATT 3′	131	NC_000012
Bovine-specific GAPDH	F: 5′ AGCCGCATCCCTGAGACAAG 3′ R: 5′ CAGAGACCCGCTAGCGCAAT 3′	132	NC_007303

[a]Product size of human genomic GAPDH is 179, of bovine genomic GAPDH is 171

Table 3
Forward (F) and reverse (R) primers used for quantitative RT-PCR

Gene name	Primer sequence	Product size	Gene bank No.
Cross-species β-Actin	F: 5′ GCGCAAGTACTCCGTGTGGA 3′ R: 5′ AAGCATTTGCGGTGGACGAT 3′	123	NM_001101 and NM_173979
Cross-species GAPDH	F: 5′ AGCTCACTGGCATGGCCTTC 3′ R: 5′ CGCCTGCTTCACCACCTTCT 3′	116	NM_002046 and NM_001034034
Human-specific GAPDH	F: 5′ CGCTCTCTGCTCCTCCTGTT 3′ R: 5′CCATGGTGTCTGAGCGATGT 3′	82	NM_002046
Bovine-specific GAPDH	F: 5′ GCCAT CACTG CCACC CAGAA 3′ R: 5′ GCGGCAGGTCAGATCCACAA 3′	207	NM_001034034
Human-specific aggrecan	F: 5′ TTCCCATCGTGCCTTTCCA 3′ R: 5′ AACCAACGATTGCACTGCTCTT 3′	121	NM_013227
Bovine-specific aggrecan	F: 5′ CCAAGCTCTGGGGAGGTGTC 3′ R: 5′ GAGGGCTGCCCACTGAAGTC 3′	98	NM_173981
Human-specific collagen II	F: 5′ GGCGGGGAGAAGACGCAGAG 3′ R: 5′ CGCAGCGAAACGGCAGGA 3′	129	NM_001844
Bovine-specific collagen II	F: 5′ AGGTCTGACTGGCCCCATTG 3′ R: 5′ CTCGAGCACCAGCAGTTCCA 3′	101	NM_001001135
Human-specific collagen IX	F: 5′ GGCAGAAATGGCCGAGACG 3′ R: 5′ CCCTTTGTTAAATGCTCGCTGA 3′	150	NM_001851
Bovine-specific collagen IX	F: 5′GGACTCAACACGGGTCCACA 3′ R: 5′ ACAGGTCCAGCAGGGCTTTG 3′	102	XM_601325

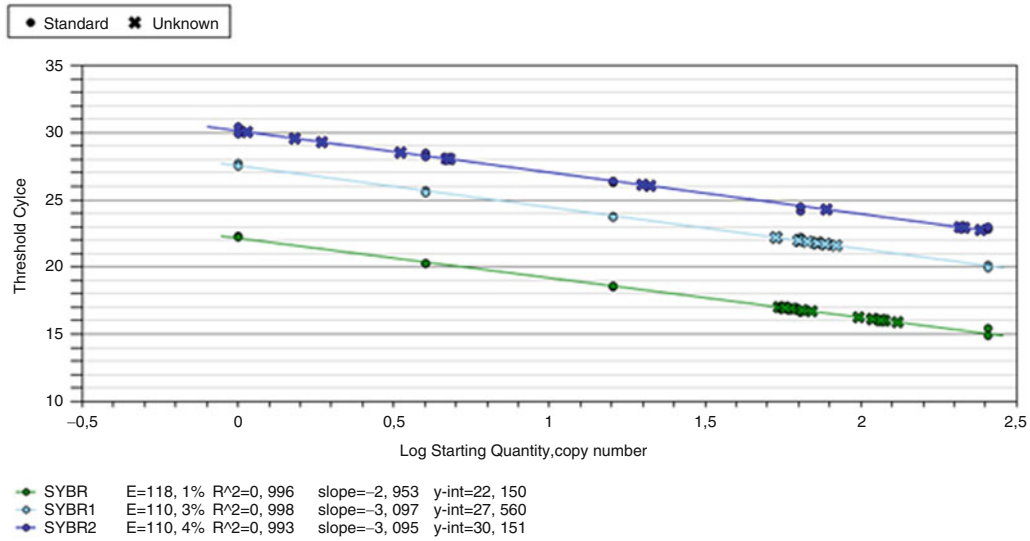

Fig. 2 An example of standard curve for qPCR. SYBR, SYBR1, and SYBR2 stand for three different primer sets

of both human and bovine genes determined by species specific PCR using genomic DNA as a template.

12. The relative mRNA expression level of bovine or human genes in xenogenic cocultures is determined by normalizing the values using cross species-specific GAPDH and β-actin primers.

3.9 Short Tandem Repeats (STR) Analysis

1. Perform STR analysis to determine the ratio of MSCs and chondrocytes in allogeneic cocultures (hMSCs and hPCs) pellets.
2. Extract genomic DNA samples from pellets ($n=6$) with the QIAamp DNA Mini Kit.
3. Amplify the 16 loci of the kit PowerPlex 16 System, type "sequence," and analyze all loci according to manufacturer's protocol.
4. Compare mono-cultures of hMSCs or hPCs to find informative alleles only present in either the hMSCs or the hPCs donor (*see* **Note 10**).
5. Make electropherograms of the informative loci.
6. As shown in Fig. 3, calculate the area under the peaks, which stand for the abundancy of the alleles.
7. The sum of the area under the peak for the two donor specific alleles represents a relative amount of DNA for this donor.
8. Calculate the relative DNA amount for both the hMSC and the hPC donor.
9. Calculate the ratio of hMSCs and hPCs in the pellet by dividing through the total amount of relative DNA present in the pellet.

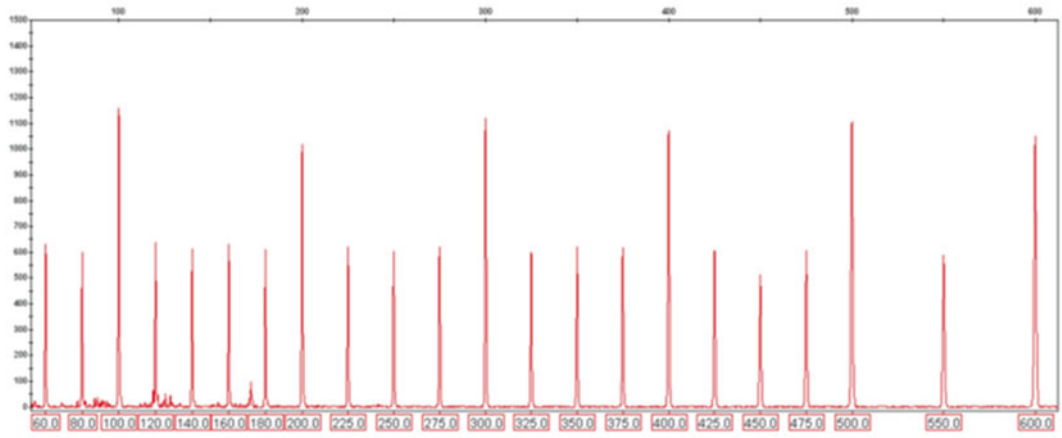

Fig. 3 An example of electropherogram of fragments after amplification. Adapted from the instructions of use of the "PowerPlex® 16 System" provided by Promega. Copyright to Promega

4 Notes

1. We define the "primary" cells (bPCs, hPCs, and hMSCs) in this manuscript as cells with low passage number (<2) without immortalization.
2. Using montage capture, images of high resolutions were obtained covering the entire section of a pellet. Choose the 20× objective. Use standard setting for the microscope and software.
3. Thresholds can usually be set by clicking "Dark background" option on the "Threshold window". If large artifacts appear, set threshold manually by adjusting the threshold bars so that the objects are red; click set and then ok.
4. By setting 100-infinite, any artifacts smaller than 100 pixel^2 (10×10 pixel) will be excluded. In images made with 20× objective, cell nuclei (either bovine or human) are larger than 100 pixel^2.
5. Setting the threshold for red image is tricky. Labeling efficiency is calculated to estimate the accuracy of threshold setting. Labeling efficiency should be similar to the ratio of chondrocytes used to establish the cocultures particularly in early time points (up to a few days maximum) after establishing the culture.
6. It is possible to automatically analyze all images by running customized plugins, which are written specifically for counting cells in different colors, using macro language of ImageJ. Basic knowledge about computer programing is required. Our plugin is available upon request.
7. Reading of absorbance at 520 nm gives variations. Always do triplicates for standards and samples.
8. Coculture pellets usually contain a lot of extracellular matrix, which makes it very difficult to extract RNA. After washing with PBS, pellets must be snap frozen with liquid nitrogen and smashed with pestle and mortar. Add lysis buffer to mortar to collet total RNA. To get 1 μg of RNA, at least three pellets (200,000 cells per pellet) are needed.
9. Take equal amount of cDNA from all samples in the same experiment to make a stock solution of cDNA templates. From the stock solution, make a series dilution: 1×, 4×, 16×, 64×, and 256× times. Run standards on the same plate as Unknown (samples to be tested), then make standards curves with Ct values in Bio-Rad iQ5 optical system software (version 2.0).
10. Theoretically, a random pair of human individuals has at least one locus (within the 16 loci tested in the kit), which is informative, except for identical twins. Normally, 2-3 loci are informative to distinguish the hMSC and the hPC donor at the DNA level.

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