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A luciferase-based quick potency assay to predict chondrogenic differentiation¹

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Abstract

Chondrogenic differentiation of adipose derived stem cells (ASC) is challenging but highly promising for cartilage repair. Large donor variability of chondrogenic differentiation potential raises the risk for transplantation of cells with reduced efficacy and a low chondrogenic potential. Therefore quick potency assays are required in order to control the potency of the isolated cells before cell transplantation. Current in vitro methods to analyze the differentiation capacity are time consuming and thus, a novel enhancer and tissue-specific promoter combination was employed for the detection of chondrogenic differentiation of ASC in a novel quick potency bioassay. Human primary ASC were co-transfected with the *Metridia* luciferase based collagen type II reporter gene pCMVE_ACDCII-MetLuc together with a *Renilla* control plasmid and analyzed for their chondrogenic potential. On day 3 after chondrogenic induction, the luciferase activity was induced in all tested donors under three dimensional (3D) culture conditions and in a second approach also under 2D culture conditions. With our newly developed quick potency bioassay we can determine chondrogenic potential already after 3 days of chondrogenic induction and under 2D culture conditions. This will enhance the efficiency of testing cell functionality, which should allow in the future to predict the suitability of cells derived from individual patients for cell therapies, in a very short time and at low costs.

Introduction

Cartilage repair strategies after traumatic injuries range from standard autologous cartilage transplantation (mosaicplasty) or marrow-stimulating techniques (microfracture) up to novel cell based strategies. For the matrix associated chondrocyte transplantation (MACT)¹, biomaterials are used in combination with expanded human autologous chondrocytes (hAC), a method which originates from a scaffold free procedure, the autologous chondrocyte transplantation (ACT)². One of the biggest drawbacks of cartilage/chondrocyte based methods is that healthy autologous cartilage needs to be harvested from the patient. This creates an additional defect which enhances the risk for developing osteoarthritis. In order to obtain enough cells for treatment it is further necessary to expand chondrocytes *in vitro* which is accompanied by a dedifferentiation process, leading to fibroblast-like, collagen type I expressing cells³. Instead of hAC, mesenchymal stem cells (MSC) may overcome drawbacks accompanied with cartilage harvest and cell expansion. Bone marrow-derived MSC (BMSC) represent the most intensively investigated MSC type in cartilage tissue engineering. They can be isolated from bone marrow aspirates, expanded in monolayer and induced for chondrogenic differentiation^{4, 5}. Zuk *et al.* found that adipose-derived stem cells (ASC) also show ability to form cartilage-like matrix, as indicated by Alcian blue and collagen type II staining⁶. In contrast to bone marrow, adipose tissue can be harvested in large amounts by simple surgical procedure. However, a large donor variability for the chondrogenic differentiation potential has been reported^{7, 8}, which raises the risk for transplantation of low-potent cells. Therefore, a quick potency assay is required to predict cell potency before transplantation or long-term storage. For assessing the chondrogenic potential collagen type II might be regarded as the most specific and unique marker for hyaline cartilage synthesis. Unfortunately, collagen type II expression starts very

late: a significant increase can be detected by quantitative reverse transcription-(qRT) PCR after 2-3 weeks of differentiation *in vitro*⁷ which impedes quick decisions of cells' quality. This late but specific marker needs to be monitored in an early phase to anticipate cellular alteration and modification upon differentiation. Therefore we designed and developed a luciferase-based reporter-vector together with a quick potency assay to monitor the capability of cells synthesizing collagen type II upon chondrogenic induction. Luciferase based bioassays represent a useful alternative to conventional analytical methods due to their simple and extremely sensitive properties. The assay employed in this work is based on measuring bioluminescence derived from the reaction of *Metridia* luciferase under control of a tissue-specific collagen type II promoter coupled to a signal amplified viral enhancer (CMV)⁹. With our luciferase reporter system we have established a proof of principle concept for the prediction of the chondrogenic differentiation potential within 3 days.

Methods

ASC isolation and cultivation

The collection of human tissue was approved by the local ethical board with patient's consent. Subcutaneous human adipose tissue was obtained during routine outpatient liposuction procedures under local tumescence anaesthesia. Isolation of ASC was performed according to Wolbank et al.¹⁰. After isolation, cells were cultured in endothelial growth medium (EGM-2; Lonza, Austria) at 37°C, 5% CO₂, and 95% air humidity to a subconfluent state before passaging. ASC were seeded at a density of $2 \times 10^3 / \text{cm}^2$ and media was changed every 3-4 days. ASC from passage 1 were used

for flow cytometry, histological analysis and reporter experiments. For qRT-PCR experiments cells were expanded up to passage 2-4.

ASC immunophenotype

ASC from passage 1 were characterized by flow cytometry analysis using the following antibodies: CD73-PE (BD), CD90-PE (BD), CD105-FITC (Abcam), CD14-FITC (Immunotools), CD34-PE (Immunotools), CD45-FITC (BD), HLA-ABC-PE (BD) and HLA-DR-FITC (BD). For staining, 2×10^5 cells in 50 μ L PBS with 1% FCS were incubated with 5 μ L primary labeled antibodies at room temperature for 15 min in the dark. Cells were washed with 1.5 ml Cell Wash™ (BD) and centrifuged for 5 min at 400 g. The supernatant was discarded and the cell pellet resuspended in 300 μ L 1 x Cell Fix™ (BD; diluted 1:10 with aqua dest) and analyzed on a FACSCanto (BD).

Chondrogenic 3D pellet culture

For chondrogenic differentiation and 3D micromass pellet cultures 3×10^5 ASC were centrifuged in chondrogenic differentiation media (hMSC Chondro BulletKit (Lonza) containing 10ng/ml BMP-6 (R&D Systems, Austria) and 10ng/ml TGF- β 3 (Lonza)) in 1.5 mL polypropylene screw cap micro tubes (Sarstedt, Austria). The tubes were placed in an incubator at 37°C, 5% CO₂, and 95% humidity with slightly open cap for gas exchange. After 2 days the pellets were transferred to 96-well U-bottom plates. Media was changed every 2-3 days.

Histological analysis

The 3D micromass pellet cultures incubated over 35 days in chondrogenic differentiation media were measured once a week for their cross section area. Micromass pellets were fixed in 4% phosphate-buffered formalin overnight for histological analysis. The next day the pellets were washed in 1x PBS and dehydrated in increasing concentrations of alcohol. After rinsing the pellets in xylol and infiltration with paraffin, deparaffinized sections were stained with Alcian blue for 30 min and counterstained for 2 min with Mayers haematoxylin. For immunohistochemical staining, sections were treated with pepsin for 10 min at 37°C (AP-9007 RTU, Thermo Scientific, Austria). Endogenous peroxidase was quenched with freshly prepared 3% H₂O₂ for 10 min at room temperature, followed by normal horse serum 2.5% (Vector RTU) to block unspecific binding. Sections were incubated 1 hour with monoclonal anti- collagen type II (MS-306 P0 Thermo Scientific) at 1:100. After washing with TBS, sections were incubated with the secondary antibody (anti mouse DAKO EnVision+ System HRP labelled Polymer, Dako, Austria) for 30 min and rinsed in TBS again. Bindings were visualized using Nova Red (SK4800 Vector Labs, Austria) for 6 min. Counterstaining was performed with Mayers haematoxylin for 2 min. For quantification of the collagen type II immunohistological staining intensity ImageJ 1.47v (National Institutes of Health, USA) was used.

Quantitative RT-PCR

Samples for qRT-PCR were taken after 3 days and at the end of differentiation cultures (day 35). Three to five micromass pellets per donor were pooled in 1 ml TriReagent (Sigma) and incubated for 20 min at room temperature. Extraction was facilitated by repeated pipetting of the pellets. Total RNA isolation was performed according to the TriReagent protocol (Sigma) and RNA content and RNA integrity of

the samples was assessed by an Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Chips Kit (no. 5065 – 4476, Agilent Technologies, Germany). Isolated RNA was transcribed to cDNA according to the High Capacity cDNA Archive Kit protocol (Applied Biosystems, Austria). Quantification of specific cDNAs was conducted in triplicate, using a LightCycler W 480 (Roche, Germany) and Taqman gene expression assay (Applied Biosystems) for Col2A1 (collagen type II, Hs01064869_m1) and Sox9 (Hs00165814_m1). The PCR was programmed as follows: initial denaturation at 95°C for 10 min, followed by 95°C for 10 s, 60°C for 45 s, cycled 50 times. Cooling to 40°C was held for 30 s. Slope speed was 20°C/s. Standard curves were prepared for quantification and expression values were normalized to the housekeeping gene, hypoxanthine–guanine phosphoribosyl transferase (HPRT). The efficiency-corrected quantification was performed automatically, using LightCycler 480 Relative Quantification Software (Roche).

Reporter plasmid

All cloning procedures were carried out using a standard Taq hot-start polymerase (Peqlab Biotechnologie GesmbH, Austria) and the *E.coli* cloning strain TOP10. The human collagen II alpha 1 promoter (Col2A1 promoter and 5' mRNA UTR from -478 to +179 from the transcriptional start site, see NC_000012.12) was cloned from human genomic DNA isolated from whole blood using the cloning primers hCollIPs (5'- CTGTGGGCTCCTCCCTGTCCCCACTCC-3') and hCollIPas(*NotI*) (5'- GAGGCGGCCGCGCTCACCGCGGGGCCTGGCTGAGC-3') (Microsynth AG, Switzerland) and the product was ligated into the vector pCR2.1 by TA cloning for sequencing (data not shown) and subsequent cloning. The promoter was transferred to the in-house designed plasmid pCMVE-EYFPHis containing the CMV-enhancer

(from -524 to -120 from the transcriptional start site, according to ⁹) and an EYFPHis reporter gene by PCR amplification using the primers hC2PHIII_f. (5'-GAG AAG CTT CTG TGG GCT CCT CCC TGT CC-3') and hC2PHIII_r (5'-GAG AAG CTT GCT CAC CGC GGG GCC TGG-3') (Microsynth AG), transfer of the PCR product to pCR2.1 and subsequent cloning by *HindIII* (Fermentas/FisherScientific, Austria) digestion of insert and destination vector and ligation into pCMVE_ACDCII using standard procedures. The EYFPHis reporter gene was exchanged by a *Metridia* secreted luciferase by cloning the *Metridia* luciferase open reading frame 3' of the CMV enhancer and the collagen II promoter in pCMVE_ACDCII using *AgeI* (Fermentas/FisherScientific) and *NotI* (Fermentas/FisherScientific) restriction sites. All vectors and intermediate products were verified by control digests (data not shown) and the *Metridia* luciferase containing end product pCMVE_ACDCII-MetLuc (Col2A, Fig. 1) was verified by sequencing (data not shown).

Luciferase assay

Pellets were co-transfected with *Metridia* luciferase based reporter genes pCMVE_ACDCII-MetLuc together with the *Renilla* control plasmid using XtremeGENE HP DNA Transfection Reagent (Roche) and 0.4 µg DNA in a 3:1 reagent/DNA ratio after 2 days of pellet formation. For chondrogenic 2D luciferase assay 3×10^4 ASC per well were seeded in a white 96 well plate (Fisher Scientific, Austria) in EGM-2 media (9.4×10^4 / cm²). Sixteen hours later media was replaced by differentiation media. ASC were co-transfected with *Metridia* luciferase based reporter vector pCMVE_ACDCII-MetLuc together with *Renilla* control plasmid using the same concentrations like for 3D pellet transfection. *Metridia* luciferase activity was measured in the supernatant samples (50 µl) with Ready-To-Glow™ Secreted

Luciferase Reporter System (Clontech, Austria) according to the manufacturer's protocol on day 1, day 2 and day 3. For the evaluation of this bioassay and as internal control to normalize the transfection we co-transfected the cells with a *Renilla* reporter gene under control of an ubiquitous promoter. Prior to measurement of *Renilla* luciferase activity, ASC were lysed with 1x *Renilla* Luciferase Assay Lysis Buffer (Promega, Austria) for 15 minutes, pellets with 5x *Renilla* Luciferase Assay Lysis Buffer for 15 minutes at 1400 rpm. Then *Renilla* luciferase activity was measured using *Renilla* Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were recorded with an Infinite→M200 Multimode Microplate Reader (Tecan). Data were normalized for transfection efficiency to *Renilla* luciferase activity. Pellet cultures and adherent cells were transfected using green fluorescent protein (GFP) to evaluate transfection efficiency. GFP positive pellets and cells were microscopically analyzed (Axiovert 200, Zeiss, Germany).

Statistical Analysis

Data are presented as mean \pm standard deviation and statistical analysis was performed using PRISM6 (GraphPad, San Diego, CA, USA). P values of < 0.05 were considered to be significant acquired by parametric 2-way analysis of variance (ANOVA)-Bonferroni post hoc or parametric two-tailed t-test assuming normal distribution according to Kolmogorov-Smirnov normality test.

Results

Expression of specific ASC surface marker

ASC displayed strong expression of mesenchymal stem cell marker CD73 (99.68% \pm 0.35), CD90 (98.70% \pm 1.46) and CD105 (97.95% \pm 1.62) and almost no expression of the lymphatic marker CD14 (1.64% \pm 0.87), the endothelial marker CD34 (3.90% \pm 7.69) and the hematopoietic marker CD45 (1.23% \pm 0.52) (Suppl. Table 1), which verified the ASC character as described in Bourin et al. 2013¹¹. The histocompatibility antigen molecule HLA-ABC (93.74% \pm 6.17) was expressed at a very high level whereas only a small portion of ASC expressed the histocompatibility antigen class II HLA-DR (1.71% \pm 0.76).

Definition of donor potential by qRT-PCR, histology and pellet diameter area

Micromass pellets from 9 different donors were analyzed after 35 days of chondrogenic differentiation for their capability to express collagen type II at mRNA and protein level by means of qRT-PCR and immunohistological staining. Further, sulphated glycosaminoglycans (sGAGs) were stained with Alcian blue to analyze the potential of individual donors to synthesize proteoglycans. Donors 3, 4, 5, 8 and 9 showed Col2A1 expression levels in the range of 70.8-354.8 (relative mRNA expression normalized to HPRT), whereas donors 1, 2, 6 and 7 demonstrated significantly lower values in the range of 0.0066 – 3.4 (Fig. 2a,b). Immunohistological stainings showed that donors with low Col2A1 mRNA expression have also low potential to produce collagen type II and sGAGs on protein level, while donors with high Col2A1 expression on mRNA level showed intense staining for Alcian blue and collagen type II (Fig. 3a). Based on the results of qRT-PCR and histological evaluations we defined donors with high and low differentiation potential, as “good donors” and “bad donors”. The pellet diameter area of each good and bad donor was evaluated over time in culture (day 7, 14, 21, 28 and 35) (Fig. 3b). These results

corroborate the immunohistological stainings for Alcian blue and collagen type II. Donor 2 is in the range of the good donors regarding pellet diameter area but is still negative for Alcian blue and collagen type II staining.. According to the analyses, the threshold for Col2A1 expression on mRNA level was set to 10 and together with the histological and immunohistological data we identified 5 good donors (3, 4, 5, 8, 9) and 4 bad donors (1, 2, 6, 7).

Activation of Col2A luciferase reporter under 3D culture conditions

To visualize transfection efficiency of 3D micromass pellets a GFP plasmid was used (Suppl. Fig. 1a). The weak fluorescence of un-transfected pellets is probably due to the known autofluorescence of cartilage-like matrix ¹². For the analysis of Col2A reporter activation micromass pellets of the 9 different donors were transfected with a plasmid containing *Metridia* luciferase under control of the Col2A promoter together with a plasmid containing *Renilla* luciferase under control of an ubiquitary promoter. After 3 days of differentiation the activation of the Col2A luciferase reporter was analyzed and normalized to *Renilla* luciferase. While there were remarkable differences in the activation of the Col2A reporter (Fig. 4a), all donors showed similar activation of *Renilla* luciferase (Fig. 4b), indicating similar transfection efficiency and promoter activation. These differences in the Col2A reporter activation also occurred after normalization to *Renilla* activation (Fig. 4c). The data were compared to the results of qRT-PCR and histological stainings. Good donors exhibited relative luciferase units (RLU) in the range of 5.0×10^4 - 8.9×10^4 of Col2A activation corresponding to relative values of 112-335 after normalization to *Renilla*, while bad donors were in the range of 5.5×10^3 - 3.4×10^4 and 20.1-95.7 after normalization (Fig. 4a,c). Sequential analysis of Col2A reporter activation over the first 3 days of induction showed an increase for both groups, but significantly higher Col2A

activation for good donors on day3 (Fig. 4d). Even after normalization of Col2A activation to *Renilla* activation the induction of Col2A reporter was significantly stronger in good donors (199 ± 83) compared to bad donors (51 ± 34) (Fig. 4e).

Col2A1 and Sox9 mRNA expression under 3D culture conditions

As we have observed a luciferase reporter signal only after 3 days of differentiation we performed qRT-PCR for Col2A1 and Sox9. After 3 days Col2A1 expression was not detectable for any donor except minimal levels for donor 2 (Col2A1 mRNA expression of 0.0154) (data not shown). Early marker Sox9 expression after 3 days of differentiation showed also a very low but detectable signal in all donors (Suppl. Fig. 2a). However no difference between bad and good donors could be discriminated (Suppl. Fig. 2b).

Activation of Col2A luciferase reporter under 2D culture conditions

In order to simplify the assay we changed from the standard chondrogenic 3D culture conditions to 2D culture conditions, which reduces time and required cell numbers. Transfection efficiency was analyzed by using a GFP plasmid (Suppl. Fig. 1b) corresponding to $45.3\% \pm 11.8$ of transfected cells as determined by nucleocounting. ASC were seeded and induced in 2D culture and co-transfected with Col2A reporter plasmid together with *Renilla* luciferase control plasmid. There was a clear difference in the activation of Col2A reporter between good (6.2×10^5 - 1.1×10^6 RLU) and bad donors (2.4×10^5 - 7.0×10^5 RLU) found on day 3 of culture (Fig. 5a), while *Renilla* activation was similar in all tested donors (Fig. 5b). The differences in Col2A reporter activation were still present after normalization of *Metridia* to *Renilla* activation (19.9-

31.3 vs. 7.3-18.3) (Fig. 5c), which demonstrates that the potential for chondrogenic differentiation is reflected in Col2A promoter activity at early timepoints even under 2D conditions. Sequential analysis of Col2A reporter activation over the first 3 days showed an increase for both groups, but again a significantly higher Col2A activation for good versus bad donors on day 3 (Fig. 5d). Moreover, activation of Col2A reporter was still higher in good donors (25.6 ± 4.3) compared to bad donors (14.2 ± 4.8) after normalization to *Renilla* activation (Fig. 5e).

Relation of Col2A luciferase reporter activation to Col2A1 mRNA expression, collagen type II immunostaining or pellet diameter area

For a more clear relation of the 3D micromass pellet data, our day 3 quick potency assay was plotted against qRT-PCR, immunostainings and pellet diameter area of day 35 3D cultures (Fig. 6). By comparing values of Col2A luciferase reporter activation to Col2A1 mRNA expression (Fig. 6a) as well as to collagen type II immunostaining intensity (Fig. 6b) the determined good donors 3, 4, 5, 8, 9 and bad donors 1, 2, 6, 7 could clearly be identified. Regarding the relation between Col2A luciferase reporter activation and the pellet diameter area (Fig. 6c) there is no clear cut-off line to identify donor quality, which may however partly be due to the low correlation of pellet size and the quality of chondrogenic differentiation^{13, 14}.

Discussion

In the current study we developed a quick potency assay for fast determination of chondrogenic differentiation status of ASC. ASC can be induced for collagen type II expression after extensive passaging¹⁵, but also directly after isolation from adipose tissue¹⁶. The expression of collagen type II is mainly regulated via the TGF- β pathway^{17, 18}; in a previous work we could demonstrate that TGF- β and BMP-6 induce collagen type II expression in ASC, in a 3D culture system¹⁹. Also other pathways such as MAPK- and Wnt/ β -catenin signalling have impact on collagen type II expression but have not been investigated in detail as the TGF- β pathway²¹⁻²⁵. To predict chondrogenic potential of ASC from different donors, Kang *et al.* measured activation of TGF- β receptor expression. The chondrogenic potential was increased with a bicistronic vector system containing TGF- β receptors²⁰. Duryagina *et al.* developed a new reporter system based on Gaussia luciferase reporter to monitor HSC-supportive proteins in human MSC²¹. The advantages of assessing reporter genes with bioluminescent assays are the high sensitivity and the non-destructive and rapid application compared to protein and RNA analysis²². Based on a previous study, where we designed a reporter construct for detection of osteogenic differentiation⁹, we employed in this study a novel reporter for chondrogenic differentiation. Cells from 4 individuals demonstrated a value for Col2A1 relative mRNA expression lower than 5 which confirmed the weak Alcian blue and absent immunohistological staining for collagen type II. These donors were regarded as bad donors. The remaining 5 donors expressed Col2A1 in the range of 70-350 and showed intense Alcian blue and collagen type II expression on protein level. These donors were regarded as good donors. The pellet diameter area of each good and bad donor corroborate these results, except donor 2 which is in the range of the good donors but still negative for Alcian blue and collagen type II staining. This is slightly different to a study of Hennig *et al.*⁷, who investigated the chondrogenic

differentiation potential of 9 donors treated with TGF- β and BMP-6 resulting in 7 of 9 donors positive for collagen type II immunohistochemistry and all investigated samples positive for Col2A1 gene expression. They also found that low potency donors lack the expression of TGF- β receptor I which could be partly overcome by co-induction with TGF- β and BMP-6 ⁷. We have identified that 20-50% are low potency donors (with low or no collagen type II expression) also in presence of BMP-6, there is need for further analysis to find out differences between good and bad donors.

Our determined good and bad donors were evaluated with our reporter gene assay using the identical differentiation condition and compared to collagen type II expression on mRNA and protein level on day 35. To our knowledge we were the first who showed transfection of chondrogenic micromass pellet cultures. The results of the quick potency assay measured for *Metridia* luciferase in the supernatant showed an increase of Col2A promoter activation for all donors from 1- 3 days with significant higher values of the good donors on day 3. Even the values normalized for *Renilla* luciferase activity were significantly higher for the good donors. Notably, the Col2A reporter activation normalized against *Renilla* demonstrated higher values in each of the good donors compared to each single bad donor. These results corroborate the analyses of the histological stainings and qRT-PCR after 35 days with 5 good donors and 4 bad donors. At single donor level, Col2A luciferase reporter activation measured on day 3 was comparable to the readout from day 35 pellet culture Col2A1 mRNA levels and immunostaining for collagen type II and Alcian blue. We could hence identify the 4 donors with the weakest potential for chondrogenesis resulting in no deposition of collagen 2 in the pellets after 5 weeks. This result based on Col2A promoter-activity could be achieved although Col2A is a late marker and Col2A1 mRNA levels on day 3 were indeed negative. mRNA levels of the early marker Sox9

could be detected in all 9 donors already on day 3. However expression levels were extremely low and did not allow identification of the previously determined donors obtained by Col2A1 mRNA level and immunostainings on day 35. Hence, Sox9 expression on day 3 was not predictive for chondrogenic potency at an early time point. Although 3D micromass pellet culture is the prerequisite for induction of chondrogenic differentiation we simplified this assay and changed to 2D culture. Surprisingly, also under 2D culture conditions Col2A reporter activity increased over 3 days for all donors with a significant difference on day 3. The change from 3D to 2D contributed towards further improvement by saving time (2 days less, since no pellet formation was necessary) and cell number (1/10) while maintaining the functionality of the assay. Moreover, under 2D conditions no autofluorescence was visible which interferes with the luciferase reporter signal. Considering absolute *Metridia* (Fig. 4a, Fig. 5a) and *Renilla* (Fig. 4b, Fig. 5b) values, obviously 2D culture conditions give higher signals compared to 3D culture conditions. This might be due to inferior transfection or cell lysis efficiency in 3D micromass pellets compared to monolayer cultures. However, *Metridia* values normalized against *Renilla* are higher under 3D (Fig. 4c,e) than under 2D (Fig. 5c,e) culture conditions, which confirms the fact that ASC cultured in micromass pellets are more committed to the chondrogenic lineage compared to 2D culture. The results demonstrate that a determination between good and bad donors is possible under both 3D and 2D conditions. That implies the functionality and quality of the assay is maintained combined with the advantages of a quick potency assay.

In this study we have established a proof of principle concept for the analysis of donor quality in terms of chondrogenic differentiation potential. Based on this study the identification of donor material with poor chondrogenic differentiation potential should allow to exclude these cells at early timepoints. Before an implementation as

a valuable cGMP (current good manufacturing practice) quality control could be established in the future, further careful evaluation of the cut-off point with a high number of donors would be required.

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Author Disclosure Statement

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of this paper.

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Reference List

1. Marlovits, S., Zeller, P., Singer, P., Resinger, C., and Vecsei, V. Cartilage repair: generations of autologous chondrocyte transplantation. *European journal of radiology* **57**, 24, 2006.
2. Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *The New England journal of medicine* **331**, 889 1994.
3. Schlegel, W., Albrecht, C., Eckl, P., Freudenthaler, H., Berger, A., Vecsei, V., and Marlovits, S. Dedifferentiation of human articular chondrocytes is associated with alterations in expression patterns of GDF-5 and its receptors. *Journal of cellular and molecular medicine* **13**, 3398, 2009.
4. Johnstone, B., Hering, T., Caplan, A., Goldberg, V., and Yoo, J. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Experimental cell research* **238**, 265 1998.
5. Mackay, A., Beck, S., Murphy, J., Barry, F., Chichester, C., and Pittenger, M. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue engineering* **4**, 415 1998.
6. Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., and Hedrick, M.H. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue engineering* **7**, 211, 2001.
7. Hennig, T., Lorenz, H., Thiel, A., Goetzke, K., Dickhut, A., Geiger, F., and Richter, W. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. *Journal of cellular physiology* **211**, 682, 2007.
8. Hildner, F., Eder, M.J., Hofer, K., Aberl, J., Redl, H., van Griensven, M., Gabriel, C., and Peterbauer-Scherb, A. Human platelet lysate successfully promotes proliferation and subsequent chondrogenic differentiation of adipose-derived stem cells: a comparison with articular chondrocytes. *Journal of tissue engineering and regenerative medicine* 2013.
9. Feichtinger, G.A., Morton, T.J., Zimmermann, A., Dopler, D., Banerjee, A., Redl, H., and van Griensven, M. Enhanced reporter gene assay for the detection of osteogenic differentiation. *Tissue engineering Part C, Methods* **17**, 401, 2011.
10. Wolbank, S., Peterbauer, A., Fahrner, M., Hennerbichler, S., van Griensven, M., Stadler, G., Redl, H., and Gabriel, C. Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: a comparison with human mesenchymal stem cells from adipose tissue. *Tissue engineering* **13**, 1173, 2007.
11. Bourin, P., Bunnell, B.A., Casteilla, L., Dominici, M., Katz, A.J., March, K.L., Redl, H., Rubin, J.P., Yoshimura, K., and Gimble, J.M. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* **15**, 641, 2013.
12. Hagiwara, Y., Hattori, K., Aoki, T., Ohgushi, H., and Ito, H. Autofluorescence assessment of extracellular matrices of a cartilage-like tissue construct using a fluorescent image analyser. *Journal of tissue engineering and regenerative medicine* **5**, 163, 2011.
13. Payne, K.A., Didiano, D.M., and Chu, C.R. Donor sex and age influence the chondrogenic potential of human femoral bone marrow stem cells. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* **18**, 705, 2010.
14. Shirasawa, S., Sekiya, I., Sakaguchi, Y., Yagishita, K., Ichinose, S., and Muneta, T. In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. *Journal of cellular biochemistry* **97**, 84, 2006.
15. Estes, B.T., Wu, A.W., Storms, R.W., and Guilak, F. Extended passaging, but not aldehyde dehydrogenase activity, increases the chondrogenic potential of human adipose-derived adult stem cells. *Journal of cellular physiology* **209**, 987, 2006.
16. Jurgens, W.J., van Dijk, A., Doulabi, B.Z., Niessen, F.B., Ritt, M.J., van Milligen, F.J., and Helder, M.N. Freshly isolated stromal cells from the infrapatellar fat pad are suitable for a one-step surgical procedure to regenerate cartilage tissue. *Cytotherapy* **11**, 1052, 2009.

17. Moustakas, A., Souchelnytskyi, S., and Heldin, C.H. Smad regulation in TGF-beta signal transduction. *Journal of cell science* **114**, 4359, 2001.
18. Heldin, C., Miyazono, K., and ten Dijke, P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465 1997.
19. Hildner, F., Peterbauer, A., Wolbank, S., Nurnberger, S., Marlovits, S., Redl, H., van Griensven, M., and Gabriel, C. FGF-2 abolishes the chondrogenic effect of combined BMP-6 and TGF-beta in human adipose derived stem cells. *Journal of biomedical materials research Part A* **94**, 978, 2010.
20. Kang, S.W., Do, H.J., Han, I.B., Shin, D.A., Kim, H.O., Kim, J.H., and Lee, S.H. Increase of chondrogenic potentials in adipose-derived stromal cells by co-delivery of type I and type II TGFbeta receptors encoding bicistronic vector system. *Journal of controlled release : official journal of the Controlled Release Society* **160**, 577, 2012.
21. Duryagina, R., Anastassiadis, K., Maitz, M.F., Gramm, S., Schneide, S., Wobus, M., Thieme, S., Brenner, S., Werner, C., and Bornhauser, M. Cellular Reporter Systems for High-Throughput Screening of Interactions Between Bioactive Matrices and Human Mesenchymal Stromal Cells. *Tissue engineering Part C, Methods* 2014.
22. Fan, F., and Wood, K.V. Bioluminescent assays for high-throughput screening. *Assay and drug development technologies* **5**, 127, 2007.

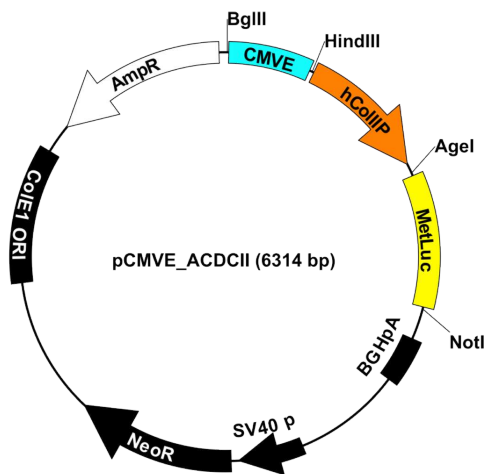


Figure 1

Fig. 1. *Metridia* luciferase based reporter vector pCMVE_ACDClI-MetLuc. This construct utilizes an artificial, chimeric cis-acting regulatory sequence through combination of a pan-active cytomegalovirus enhancer cloned directly upstream of the human COL2A promoter which drives the expression of the reporter gene.

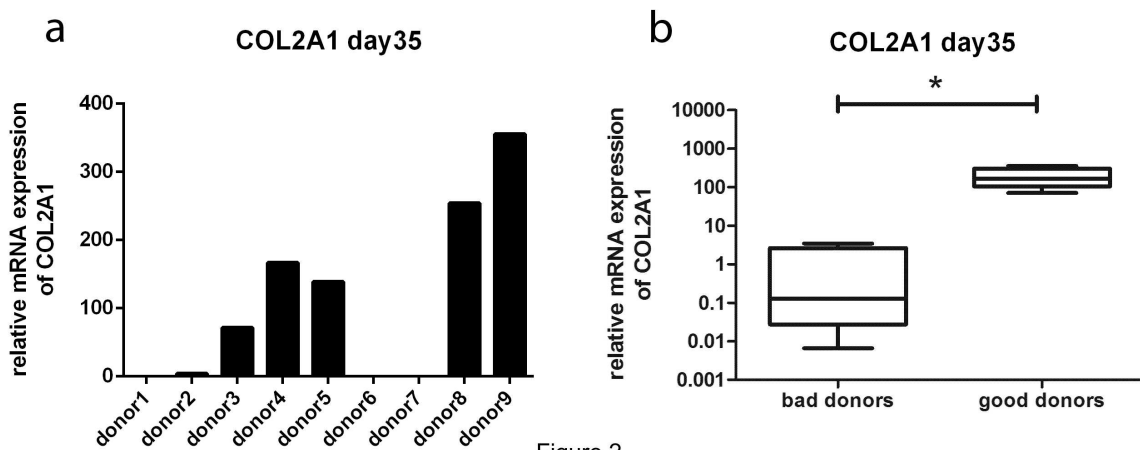


Figure 2

Fig. 2. COL2A1 mRNA expression. On day 35 of micromass pellet cultures 3 – 5 pellets were pooled from each donor. COL2A1 mRNA expression demonstrates variations between the 9 tested donors (a). These donors were separated into 5 good donors (3, 4, 5, 8, 9) and 4 bad donors (1, 2, 6, 7) with a significant difference (b). *p < 0.05.

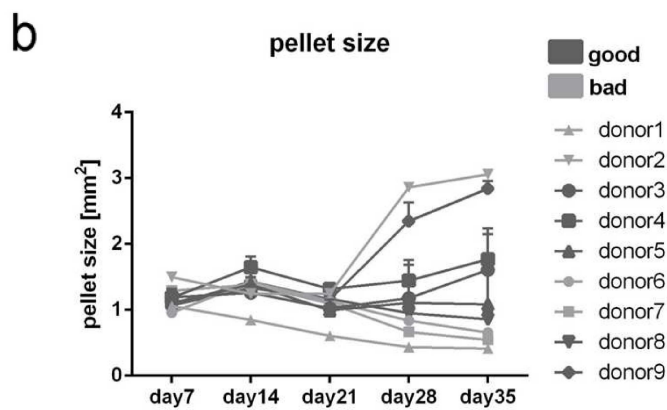
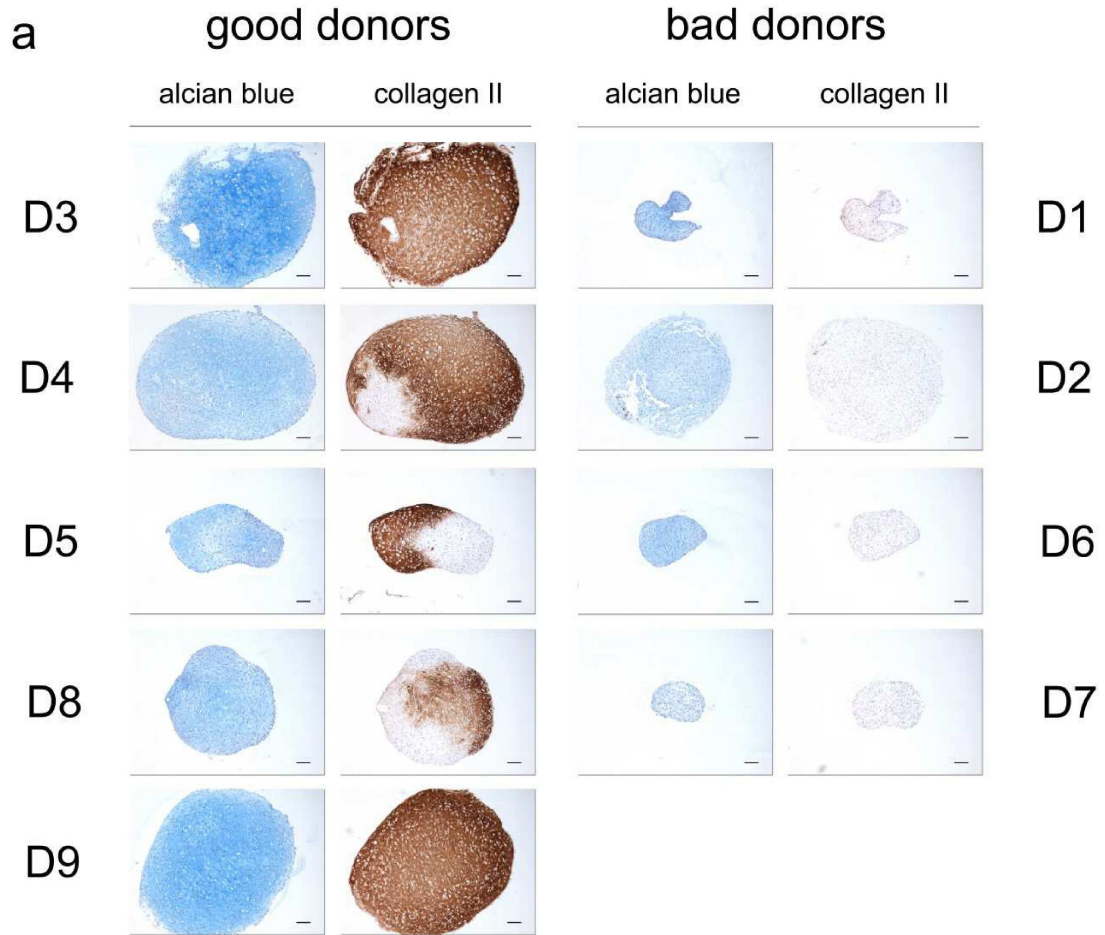


Figure 3

Fig. 3. Alcian blue and collagen type II staining (a). The immunohistological evaluation demonstrates weak or absent Alcian blue and collagen type II staining for bad donors (D1, D2, D6, D7) but intense staining for good donors (D3, D4, D5, D8, D9). Bar = 100 μ m. Pellet size of 3D chondrogenic micromass pellets measured in differentiation media over time (day 7, 14, 21, 28 and 35) (b). The pellet diameter

area of each good and bad donor corroborates the immunohistological stainings for Alcian blue and collagen type II. Apart from Donor 2, which is in the range of the good donors but still negative for Alcian blue and collagen type II staining.

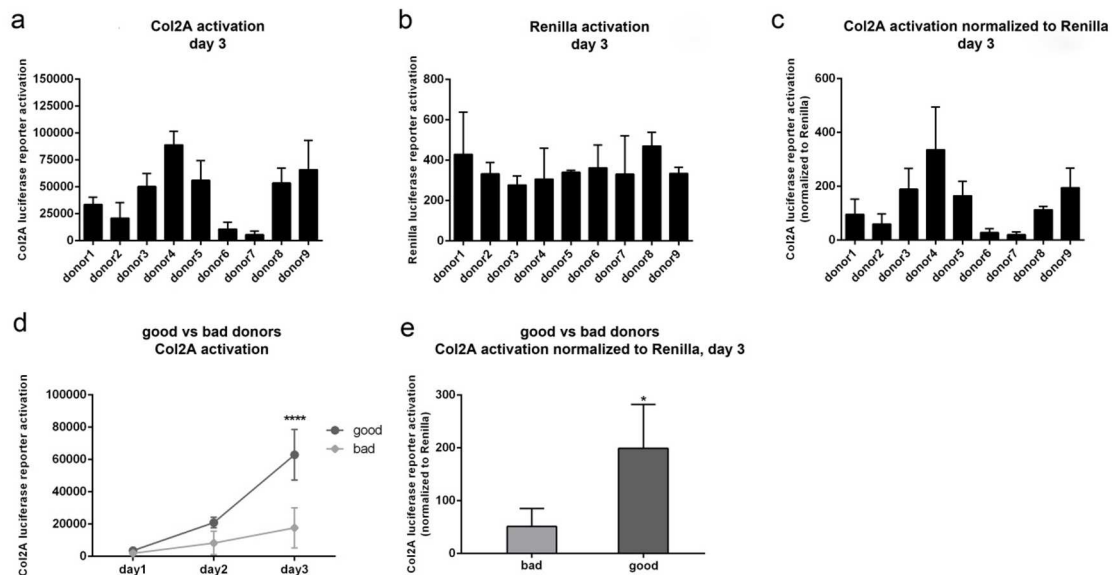


Figure 4

Fig. 4. Activation of Col2A luciferase reporter under 3D culture conditions. Micromass pellets were co-transfected with *Metridia* luciferase based reporter gene pCMVE_ACDCII-MetLuc together with *Renilla* control plasmid. Pellets were incubated in chondrogenic differentiation media with TGF- β 3 and BMP-6. Col2A *Metridia* luciferase activity was measured in the supernatant on day 1, day 2 and day 3. For measurement of *Renilla* luciferase activity pellets were lysed on day3. Activation of *Metridia* and *Renilla* luciferase reporter and normalization of *Metridia* luciferase to *Renilla* luciferase on day3 for each single donor (a-c). There was a clear difference in the activation of Col2A reporter (a) while *Renilla* activation was similar in all tested donors (b). These differences were still present after normalization of *Metridia* to *Renilla* activation (c). Donors were divided in good and bad donors as confirmed with qRT-PCR and regarding their specific reporter activation. Analysis of

Col2A reporter activation over 3 days showed an increase for both groups, but significant higher Col2A activation for good donors (d). Even after normalization of Col2A activation to *Renilla* activation the induction of Col2A reporter was much stronger in good donors compared to bad donors (e). Data are shown as mean \pm SD. Asterisks indicate significant difference. Units of Col2A luciferase reporter activation are shown in Relative Light Units (RLU). * $p < 0.05$; **** $p < 0.0001$

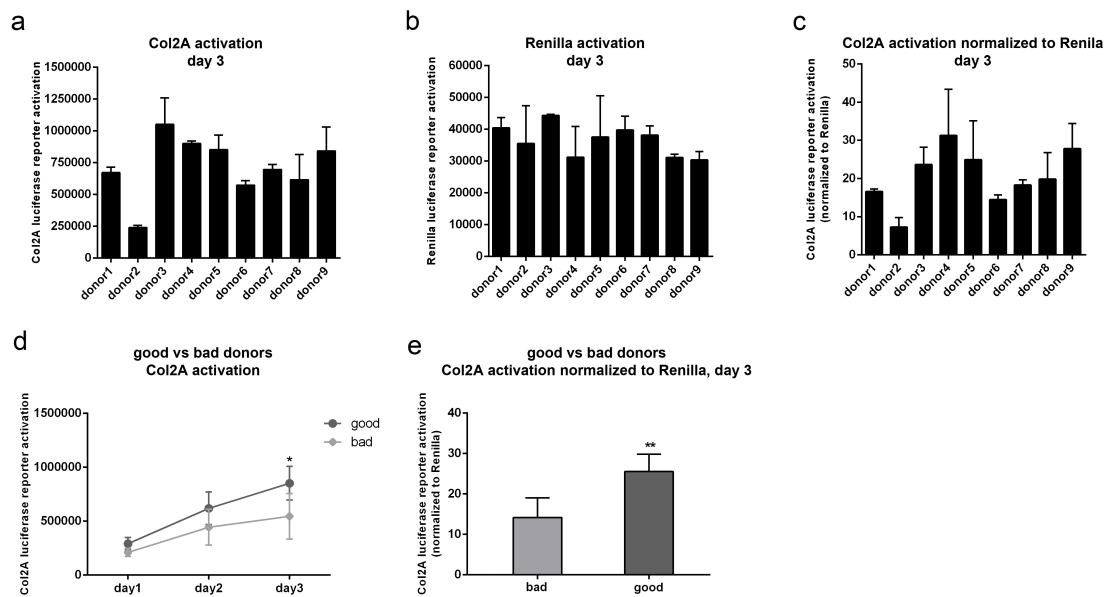


Figure 5

Fig. 5. Activation of Col2A luciferase reporter under 2D culture conditions. ASCs were co-transfected with *Metridia* luciferase based reporter gene pCMVE_ACDCII-MetLuc together with *Renilla* control plasmid and incubated for 3 days with chondrogenic differentiation media with TGF- β 3 and BMP-6. On day 1, day 2 and day 3 Col2A *Metridia* luciferase activity was measured in the supernatant. On day 3 cells were lysed for measurement of *Renilla* luciferase activity. Activation of *Metridia* and *Renilla* luciferase reporter and normalization of *Metridia* luciferase to *Renilla* luciferase on day3 for each single donor (a-c). There was a clear difference in the activation of Col2A reporter between the donors (a) while *Renilla* activation was similar in all tested donors (b). These differences were still present after

normalization of *Metridia* to *Renilla* activation (c). Donors were divided in good and bad donors as confirmed with qRT-PCR and regarding their specific reporter activation. Analysis of Col2A reporter activation over 3 days showed an increase for both groups, but significant higher Col2A activation for good donors (d). Even after normalization of Col2A activation to *Renilla* activation the induction of Col2A reporter was higher in good donors compared to bad donors (e). Data are shown as mean \pm SD. Asterisks indicate significant difference. Units of Col2A luciferase reporter activation are shown in Relative Light Units (RLU). * $p < 0.05$; ** $p < 0.01$

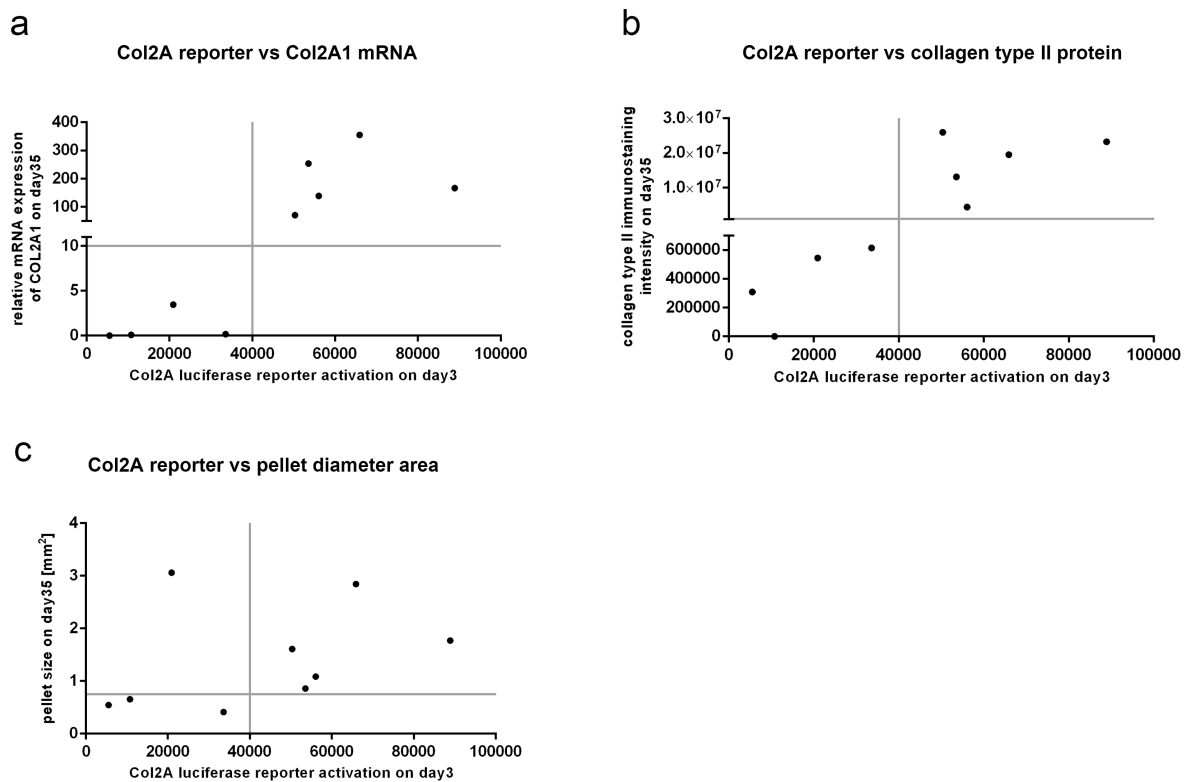


Figure 6

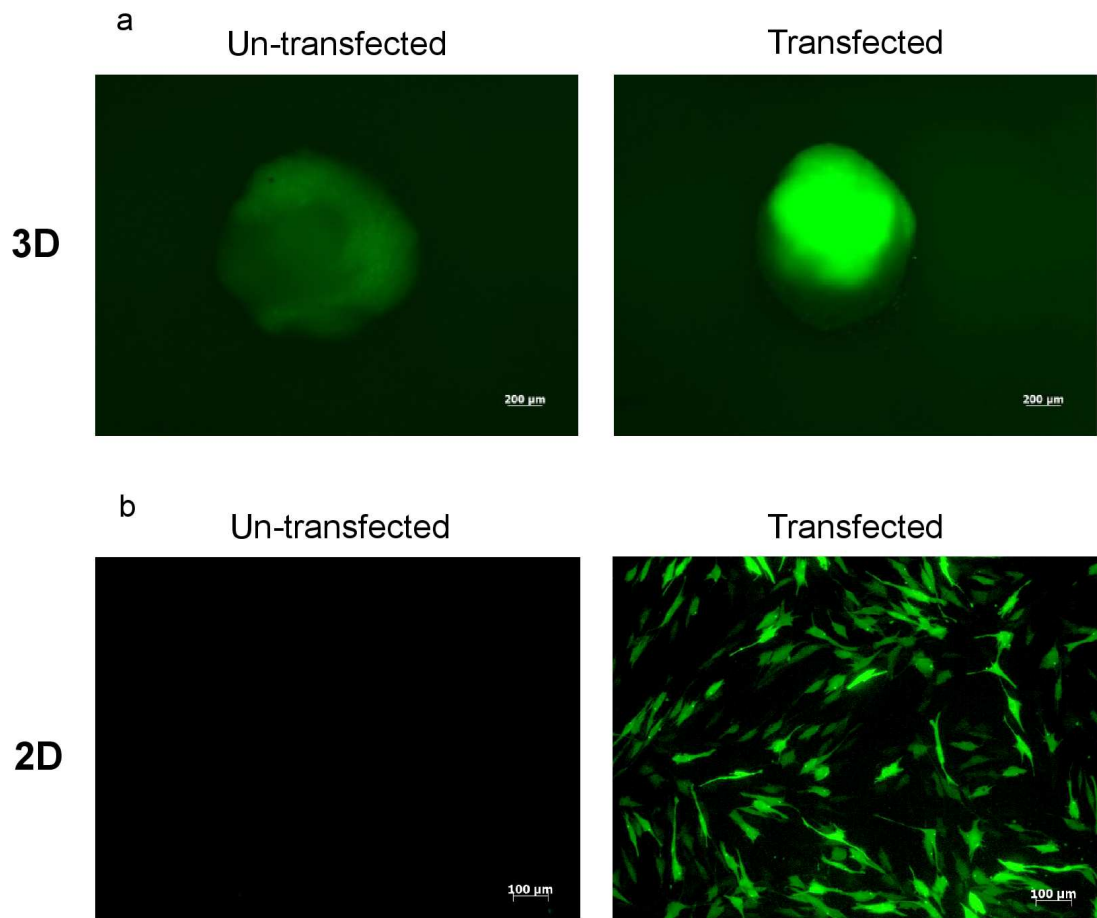
Fig. 6. Relation of Col2A luciferase reporter activation to Col2A1 mRNA expression, collagen type II immunostaining and pellet diameter area. Data of single donors are displayed with luciferase reporter activation on the x-axis and mRNA expression (a), immunostaining intensity (b) and the pellet diameter area (c) on the respective y-axis. A horizontal and vertical line indicate the cut-off point discriminating the good donors

3, 4, 5, 8, 9 and bad donors 1, 2, 6, 7 characterized by Col2A1 mRNA expression level (a) and collagen II immunostaining intensity (b) based on Col2A luciferase reporter activation. In contrast plotting pellet diameter area against the luciferase reporter activation (c) showed no clear cut-off line to discriminate good and bad donors.

Marker	Mean \pm SD
CD73	99.68% \pm 0.35
CD90	98.70% \pm 1.46
CD105	97.95% \pm 1.62
HLA-ABC	93.74% \pm 6.17
HLA-DR	1.71% \pm 0.76
CD14	1.64% \pm 0.87
CD34	3.90% \pm 7.69
CD45	1.23% \pm 0.52

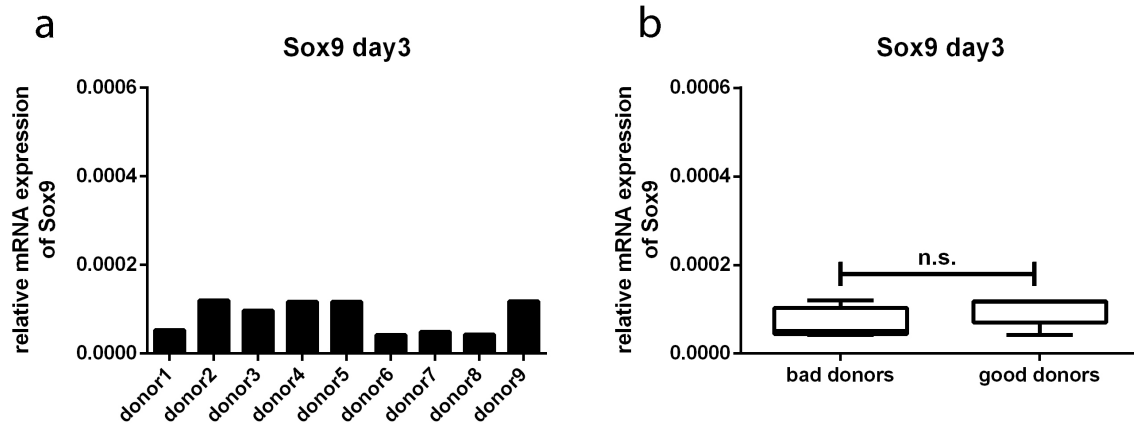
Table 1

Supplemental Table 1. Immunophenotype of ASC at passage 1. ASC displayed strong expression of mesenchymal stem cell marker CD73, CD90 and CD105 and almost no expression of CD14 (lymphatic), CD34 (endothelial) and CD45 (hematopoietic) which verified the ASC character. The histocompatibility antigen molecule HLA-ABC was expressed at a very high level whereas only a small portion of ASC expressed the histocompatibility antigen class II HLA-DR.



Supplemental Figure 1

Supplemental Fig. 1. GFP transfection of micromass pellet cultures. Un-transfected (left) and transfected (right) micromass pellets. Although the conventional autofluorescence is visible in un-transfected pellets, a clear positive signal for GFP is visible in transfected pellets (a) as well as 2D monolayer cell culture (b). Bar = 200 μ m (a), 100 μ m (b).



Supplemental Figure 2

Supplemental Fig. 2. SOX9 mRNA expression. On day 3 of micromass pellet cultures SOX9 demonstrates a very low expression in all single donors (a). There was no difference obvious when dividing the defined good donors (3, 4, 5, 8, 9) and bad donors (1, 2, 6, 7) (b).