Electronic Supplementary Material (ESI) for Integrative Biology.

Supplementary Data

Table S1 ***Primer sequences***

Gene	Forward Primer	Reverse Primer
B2M	GACTTGTCTTTCAGCAAGGA	ACAAAGTCACATGGTTCACA
ALP	ACAAGCACTCCCACTTCATC	TTCAGCTCGTACTGCATGTC
Sox9	TGGGCAAGCTCTGGAGACTTC	ATCCGGGTGGTCCTTCTTGTG
Col-1	GTCACCCACCGACCAAGAAACC	AAGTCCAGGCTGTCCAGGGATG
Col-2	CGTCCAGATGACCTTCCTACG	TGAGCAGGGCCTTCTTGAG
ACAN	AGGCAGCGTGATCCTTACC	GGCCTCTCCAGTCTCATTCTC
ALCAM	ACGATGAGGCAGACGAGATAAGT	CAGCAAGGAGGAGACCAACAA
BMP2	GCTAGACCTGTATCGCAGGC	TTTTCCCACTCGTTTCTGGT

Table S2 ***Overview of the complete data-set.*** The legend of the table is given in the lower right panel. Per donor, per substrate type and per time point the applied culture media and the analysis are indicated. Whenever 'C' is noted in the table both differentiation and dedifferentiation were conducted. In those particular cases the dedifferentiation phase equals the indicated time point minus 14 days of differentiation.

		Donor 1																Donor 2									
		TCPS				FDM						ES						TCPS				FDM					
	time point	d1	d3	d7	d14	d3	d7	d14	d17	d21	d28	d3	d7	d14	d17	d21	d28	d1	d3	d7	d14	d3	d7	d14	d17	d21	d28
Gene expression	ALP	BM	A	A	A	A	A	A	C	A	C	A	A	A	C	A	C	BM	A	A	A	A	A	A	C	A	C
Gene expression	Sox9	BM	A	A	A	A	A	A	C	A	C	A	A	A	C	A	C	BM	A	A	A	A	A	A	C	A	C
Gene expression	Col-1	BM	A	A	A	A	A	A	C	A	C	A	A	A	C	A	C	BM	A	A	A	A	A	A	C	A	C
Gene expression	Col-2	BM	A	A	A	A	A	A	C	A	C	A	A	A	C	A	C	BM	A	A	A	A	A	A	C	A	C
Gene expression	ACAN	BM	A	A	A	A	A	A		A	A							BM	A	A	A	A	A	A	C	A	C
Gene expression	BMP2	BM	BM	BM	BM	A	A	A		A	A																
Gene expression	ALCAM	BM	BM	BM	BM	A	A	A		A	A							BM	A	A	A	A	A	A	C	A	C
Protein	DNA	BM		BM		A	A	A	C	A	C	A	A	A	C	A	C					A	A	A	C	A	C
Protein	ALP	BM		BM		A	A	A	C	A	C	A	A	A	C	A	C					A	A	A	C	A	C
Protein	GAG	BM		BM		A	A	A	C	A	C	A	A	A	C	A	C						A	A	C	A	C

		Donor 3						Donor 4									
		ES						TCPS				FDM					
	time point	d3	d7	d14	d17	d21	d28	d1	d3	d7	d14	d3	d7	d14	d17	d21	d28
Gene expression	ALP	A	A	A	C	A	C		A	A	A			A	C		C
Gene expression	Sox9	A	A	A	C	A	C		A	A	A			A	C		C
Gene expression	Col-1	A	A	A	C	A	C		A	A	A			A	C		C
Gene expression	Col-2	A	A	A	C	A	C		A	A	A			A	C		C
Gene expression	ACAN	A	A	A	C	A	C		A	A	A			A	C		C
Gene expression	BMP2																
Gene expression	ALCAM	A	A	A	C	A	C		A	A	A			A	C		C
Protein	DNA	A	A	A	C	A	C							A	C		C
Protein	ALP	A	A	A	C	A	C							A	C		C
Protein	GAG	A	A	A	C	A	C							A	C		C

BM= Only basic medium
A= BM, CM, OM
C= BM, CM, OM, CB, OB

Table S3 ***Results of protein expression levels.*** Measured values before normalization to the basal level at first time point for the indicated substrate. **Note:** ALP activity assay results should always be considered as arbitrary numbers showing relative differences between samples within the same assay. Therefore, no direct comparison between ALP activity after culture in 2D and 3D can be made.

Substrate:	ALP/DNA	GAG/DNA	DNA
2D TCPS day1	1 ± 0.2	3 ± 1.31	1.36 ± 0.23
3D FDM day 3	0.7 ± 0.1	3.66 ± 1.35	2.23 ± 0.39
3D ES day 3	2.0 ± 1.2	6.24 ± 1.43	2.50 ± 0.37

Supplementary Fig. S1 Soluble-factor-induced differentiation of pre-selected hMSCs in 2D as monolayer (BM and OM) or pellet culture (CM) and in 3D on FDM-scaffolds was assessed for ACAN, ALCAM and BMP2 mRNA expression levels. In pellet culture no activation of ACAN was found upon culture in CM. On FDM-scaffolds, culture in CM resulted in a significant up-regulation of ACAN after 7 and 28 days of culture. BMP2 showed a significant down-regulation in 2D after 3, 7 and 14 days in BM and a significant up-regulation in 3D after 7, 14 and 21 days in BM. ALCAM was significantly down-regulated in all conditions for nearly all time-points. (n=5, except for ACAN in CM day 28 n=4, # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 2D day 1 as control).

Supplementary Fig. S2 Soluble factor induced differentiation of hMSCs from donor 2 in 2D on TCPS. ALP, Sox9 and ALCAM expression levels were significantly down-regulated in all three types of media. Collagen type-1 and collagen type-2 expression followed similar trends in which initially a small up-regulation in CM and a down-regulation at nearly all time-points in BM and OM was found. ACAN expression was in some samples in CM too low to be detected. Some samples showed too low expression levels of ACAN and ALCAM expression to be detected within a reliable range of CT-values and were therefore not presented. (n=5, except for ACAN in CM day 3 (n=1), day 7 (n=0), day 14 (n=2) and ALCAM in CM day 14 (n=1) and OM day 14 (not expressed) # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 2D day 1 as control).

Supplementary Fig. S3 Soluble factor induced differentiation of hMSCs from donor 4 in 2D on TCPS. ALP expression levels were initially significantly down-regulated in CM. Sox-9 was significantly down-regulated after 7 days in BM and after 3 and 7 days in OM. Collagen type-1 and collagen type-2 expression followed similar trends in which there was initially a small down-regulation in CM and an up-regulation at day 3 in OM. ACAN expression showed a significant up-regulation in CM after 14 days of culture. (n=5, # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 2D day 1 as control).

Supplementary Fig. S4 Soluble factor induced differentiation of hMSCs from donor 2 on 3D FDM-scaffolds. No profound response on OM was found in the mRNA expression of the markers for osteogenesis ALP and collagen type-1. Sox9 expression was initially increased in CM, and significantly increased in BM and OM. This increase was lost after 7 days of culture. After 28 days of culture a small down-regulation was found compared to the expression levels after 1 day of culture in BM on TCPS. ACAN expression levels did not correlate to chondrogenic induction in CM. ALCAM expression levels showed a two-fold down-regulation after 7 days of culture, yet the expression levels

remained stable up to 28 days of culture. (n=3-8, # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 2D day 1 as control).

Supplementary Fig. S5 Soluble factor induced differentiation of hMSCs from donor 3 on 3D ES- scaffolds. ALP and Sox9 expression levels were both significantly down-regulated in BM, CM and OM at nearly all time-points. Collagen type-1 and collagen type-2 expression showed similar trends and fluctuate between a 3-fold down and 5 fold up-regulation for Collagen-1 and a 2-fold down- and up-regulation for collagen-2. ACAN expression levels decreased over time for all conditions. ALCAM showed to be significantly up-regulated within 3 days of 3D culture, yet the relative mRNA expression levels were slightly reduced over time. (n=5, # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 2D day 0 as control).

Supplementary Fig. S6 DNA quantification of samples analyzed for ALP activity and GAG expression (Figure 4). It can be observed that in all conditions the amount of DNA remains in a comparable range between 0.5 and 2.2µg per FDM scaffold and 1.9 and 4.5µg per ES-scaffold. (n=5, no statistics since data is further processed paired with ALP and GAG protein expression analysis).

Supplementary Fig. S7 Protein expression levels after soluble factor induced differentiation of hMSCs from donors 2 and 3 in 3D on FDM- and ES-scaffolds, respectively. In both scaffold-systems an increase in ALP activity was found after culture in OM. In ES-scaffolds ALP activity was also increased after 14 and 28 days in CM. The GAG production on FDM-scaffolds only showed an increase after 7 days of culture in CM. In BM and OM no changes in GAG production were observed. The GAG production per µg of DNA on ES-scaffolds was lower than on FDM-scaffolds due to the higher amounts of DNA detected. In CM an increase in GAG-production was found after 7 days of culture and remained stable up to day 28. Surprisingly, also in BM and OM a significant increase in GAG-production was found after 14 and 21 days of culture respectively. (n=4, # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 2D day 3 as control for ALP donor 2, ALP donor 3 and GAG donor 3; Dunnett's posttest with BM 2D day 7 as control for GAG donor 2).

Supplementary Fig. S8 DNA quantification of samples analyzed for ALP activity and GAG expression (Supplementary Fig. 7). It can be observed that in all conditions the amount of DNA remains in a comparable range between 0.1 and 0.5µg per FDM scaffold for donor 2 and between 2.2 and 4µg per ES-scaffold for donor 3. (n=5, no statistics since data is further processed paired with ALP and GAG protein expression analysis)

Supplementary Fig. S9 The mRNA expression levels of donor 2 hMSCs on FDM-scaffolds after 3 and 14 days of de-differentiation subsequent to 14 days of differentiation were compared to the expression levels resulting from 14 days of differentiation. ALP showed a significant up-regulation after 14 days in CM. This increased expression level was lost not only after soluble factor removal (CB) but also when culture in CM was continued. For all other markers no significant differences were found at day 14 between BM, CM and OM indicating that the presence of soluble factors did not result in differentiation of the hMSCs within 14 days. Therefore, no conclusions with respect to de-differentiation could be drawn for this donor on FDM-scaffolds. (n=3-8, # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 3D day 14 as control)

Supplementary Fig. S10 The mRNA expression levels of donor 3 hMSCs on ES-scaffolds after 3 and 14 days of de-differentiation subsequent to 14 days of differentiation were compared to the expression levels resulting from 14 days of differentiation. ALP expression levels were significantly decreased for hMSCs in OM compared to hMSCs in BM after 14 days of culture and remained to decrease over time in OM. When OM after 14 days of culture was replaced with BM (OB) it was observed that the expression levels were restored to the same levels as for hMSCs cultured in BM for 28 days. For Sox-9 and ACAN expression, the same behavior was found when CM was replaced with BM after 14 days of differentiation. Collagen type-1, collagen type-2 and ALCAM did not show profound changes between BM, CM and OM after 14 days of differentiation and the expression levels for CM versus CB and OM versus OB remained within the same range. (n=4-5 except for day 14 OM (n=2) and day 14 + 3 BM (n=3), # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 3D day 14 as control)

Supplementary Fig. S11 The mRNA expression levels of donor 4 hMSCs on FDM-scaffolds after 3 and 14 days of de-differentiation subsequent to 14 days of differentiation were compared to the expression levels resulting from 14 days of differentiation. ALP expression was up-regulated for hMSCs in OM, after 3 days of de-differentiation the expression levels in OM remained stable whereas in OB the expression levels where down-regulated within the same range as hMSCs cultured in BM for 17 days. ALP expression levels for CM, OM, CB and OB were too low to be detected, which was also the case for the levels of Sox9, ACAN and ALCAM. Sox-9 expression levels were slightly down-regulated in OM after 14 days of culture. This decrease progressed after another 3 days of differentiation while at that time-point hMSCs in OB remained comparable to the levels in OM at day 14. Collagen type-1 and collagen type-2 expression showed differences between hMSCs cultured in CM or in OM. After soluble factor removal these

differences were retained. For ACAN and ALCAM no profound changes in expression levels were observed between hMSCs cultured in BM, CM and OM. Therefore the maintenance of expression levels after soluble factor removal for these markers was not considered relevant. (n=3-5, except for day 14 CM, day 14 + 3 CB and OB, day 14 + 14 CM (n=2) and Day 28 CB (n=1), # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 3D day 14 as control)

Supplementary Fig. S12 Protein expression after 3 or 14 days of de-differentiation subsequent to 14 days of differentiation in CM or OM. The ALP activity was found to be significantly increased for hMSCs cultured in OM in two out of three hMSCs populations. After 3 days of de-differentiation donor 2 hMSCs still showed an increase in ALP activity. Yet, after 14 days of de-differentiation the ALP activity in OM showed to be significantly higher than in OB. Also for hMSCs from donor 3 on ES-scaffolds, the ALP activity in OM was significantly higher than in OB after 14 days of de-differentiation. For donor 4 the ALP activity was not significantly increased in OM after 14, 17 or 28 days of differentiation compared to BM at the same time-point. Only after 3 and 14 days of de-differentiation a significant increase in ALP activity was found for hMSCs in OB and CB, respectively, compared to BM day 14. When comparing the ALP activity of hMSCs in BM, CM, OM, CB and OB after 14 days of de-differentiation, however, no differences were observed. The GAG expression levels did not change significantly for two donors. Only donor 4 on FDM-scaffolds showed an increased GAG production after hMSCs were cultured in CM. These elevated production levels were lost both when culture in CM was continued as well as for hMSCs that were de-differentiated in CB. (n=4, # $p<0.05$, @ $p<0.01$, * $p<0.001$, Dunnett's posttest with BM 3D day 14 as control)