

# Preconditioning of Human Adipose-derived Stromal/Stem Cells: Evaluation of Short-term Preincubation Regimens to Enhance their Regenerative Potential

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## Abstract

**Objective:** Stem cell-based therapy seems to be a promising option to support regeneration after organ failure and tissue injury. The transplantation of MSCs requires cells with a maximum regenerative capacity. Therefore, the research on new strategies to improve the release of regenerative factors of MSCs is urgently needed.

**Methods:** Human adipose-derived stromal/stem cells (ASC) were isolated from lipoaspirates, characterized, and cultured. Cells were either cultured under standard conditions or preconditioned by incubation in a hypoxic environment (0.5% O<sub>2</sub>) or in normoxia in the presence of recombinant human tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) or recombinant human epidermal growth factor (EGF) for 48 hours. First, seven selected regeneration promoting factors were evaluated by qPCR analysis. Afterwards the secretome of ASCs was estimated using a commercially available protein array for 507 proteins.

**Results:** PCR analysis showed a differential induction of ASCs' gene expression by the three pretreatments. Whereas ASCs in hypoxia showed a significant mRNA induction of VEGF, FGF-7, and IGF-II, the other pretreatments induced no significant change in VEGF expression. The gene expression of HB-EGF and M-CSF was significantly induced in hypoxia and by incubation with TNF $\alpha$ , but not EGF. Angiotensin-like 1 mRNA was not significantly induced by all three preconditioning regimens. Evaluation by the protein array revealed that from the 507 proteins investigated 21.9% were found to be more than five-fold increased after incubation in hypoxia (111 of 507 proteins). Preincubation with EGF resulted in an upregulation of 32.3% (164/507), whereas TNF $\alpha$  upregulated 28.8% of all proteins evaluated (146/507).

**Conclusion:** The findings indicate that all three preconditioning regimens induced a wide variety of proteins. However, short-term pretreatment with EGF induced the highest quantity of proteins, and, therefore, appears to be the best preconditioning regime for cell therapeutic approaches.

**Keywords:** Preconditioning; Pretreatment; Stem cells; Medium; Regeneration; Hypoxia; Mesenchymal stromal/stem cells

**Abbreviations:** ASCs: Adipose-derived Mesenchymal Stromal/Stem Cells; EGF: Epidermal Growth Factor; FGF-7: Fibroblast Growth Factor-7; HB-EGF: Heparin-Binding EGF-like Growth Factor; Hyp: Preconditioning in a Hypoxic Environment; IGF-II: Insulin-like Growth Factor-II; M-CSF: Macrophage Colony-Stimulating Factor; MIP-1: Macrophage Inflammatory Protein 1; MSCs: Mesenchymal Stromal/Stem Cells; qPCR: quantitative Real Time Polymerase Chain Reaction; TNF $\alpha$ : Tumor Necrosis Factor-alpha; VEGF: Vascular Endothelial Growth Factor

## Introduction

Stem cell-based therapy seems to be a promising option to support regeneration after organ failure and tissue injury. The organ-protective effects of mesenchymal stromal/stem cells (MSCs) and their conditioned medium (CM) have been investigated in the last decade, demonstrating that either transplanted cells or their CM promote regeneration after several organ- and tissue injuries. The transplantation of MSCs requires cells with a maximum regenerative capacity. Therefore, the development of new strategies to improve the release of regenerative factors of MSCs is urgently needed. *In vitro* pretreatment regimens able to optimize the regenerative capacity of stem cells should be in the focus of further studies aiming to enhance the regeneration process. During the last years, optimization of the beneficial effects of cell therapy has been investigated, seeking to

enhance survival, engraftment, and paracrine properties of MSCs [1]. In this case, various *in vitro* pretreatment strategies ("preconditioning") have been recently applied to enhance the regenerative capacity of MSCs [2-4]. MSC pretreatment may include exposure of cells to physiological stimuli such as cytokines, growth factors, biophysical stimuli, heat shock, pharmacological agents, cell-cell-contacts, or hypoxia [1-4]. Preconditioning by hypoxia has been shown to initiate the secretion of regenerative factors and to enhance cell survival [5,6]. Findings of others suggest that hypoxic preincubation of MSCs for two days induces metabolic changes that yield higher *in vivo* cell retention after transplantation [6]. Therefore, choosing appropriate pretreatment regimens may provide a simple but effective way of promoting survival

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and migration to the site of injury, enhancing regenerative properties, and boosting the regenerative capability of transplanted stem cells in cell therapy approaches [1].

In general, MSCs have been identified throughout the whole body as immature cells. Classically they were isolated from the bone-marrow, but also from nearly all adult tissues (e.g. adipose tissue) and solid organs (e.g. liver, kidney) [7-9]. MSCs represent a rare population (or populations) in the perivascular niche (or are derived from perivascular cells or pericytes [10]) within fully specialized tissues. MSCs release a number of regeneration promoting, pro-angiogenic, anti-inflammatory and immune-modulatory factors that improve regeneration in injured organs or tissue [11]. Although numerous studies using MSCs (or their CM) as potential therapeutic agents have been published, our understanding is incomplete regarding how this regenerative activity is governed by interactions with resident cells, growth factors and cytokines.

The present study investigates the potential of different preconditioning regimens to enhance the release of factors, and, therefore, the regenerative potential of human adipose-derived MSCs (ASCs). We firstly evaluated the effects of three different preconditioning regimens on seven selected regeneration promoting factors by quantitative real-time polymerase chain reaction (qPCR) analysis. Afterwards we identified the release of 507 proteins into the cell culture medium using a commercially available protein array.

## Methods

### Cell isolation and culture

Human adipose-derived adult mesenchymal stromal/stem cells (ASC) were isolated from lipoaspirates from seven female donors undergoing cosmetic liposuction in accordance to the local ethical committee. Aspirated tissue was digested at 37°C with 0.075% collagenase I (Biochrom, Berlin, Germany) under continuous agitation for 45 minutes. The stromal-vascular fraction was separated from the remaining fibrous material and the floating adipocytes by centrifugation at 300 g. The sedimented cells were washed with PBS and filtered through a 100 µm pore filter (Millipore, Schwalbach, Germany). Erythrocyte contamination was reduced by density gradient centrifugation with Bicoll (Biochrom, Berlin, Germany). Finally, cells were plated for initial cell culture, and cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> in humid air. Primary cell isolates and cultured cells were fully characterized, as described previously [12,13].

Dulbecco's modified Eagle's medium (DMEM; Sigma, Taufkirchen, Germany) was used with a physiologic glucose concentration (100 mg/dl) supplemented with 10% fetal calf serum (FCS; PAA, Cölbe, Germany) as the culture medium. The medium was replaced every three days. Subconfluent cells (85-90% confluency) were passaged by trypsinization. The 1<sup>st</sup> - 4<sup>th</sup> passage of ASC from 7 different female donors was used for the experiments. Cell morphology was examined by phase contrast microscopy. Expression of characteristic markers was proven by immunofluorescence staining for CD90 expression and flow cytometric analysis of CD73, CD90, and CD105 expression, as described previously [13,14]. Tri-lineage differentiation potential of cultured ASC was proven by specific media, as described previously [14,15].

### Preconditioning regimen and conditioned medium

Cells were either cultured under standard conditions (controls in

normoxia (21% O<sub>2</sub>) or preconditioned by incubation in a hypoxic environment (0.5% O<sub>2</sub>) or in normoxia supplemented with recombinant human tumor necrosis factor-α (TNFα Immunotools, Friesoythe, Germany No. 11343015) or recombinant human epidermal growth factor (EGF, Immunotools, Friesoythe, Germany No. 11343406). All treatments were performed for 48 h in serum-free low-glucose DMEM. For this purpose, cells were grown to subconfluency and washed twice with PBS. Then, TNFα or EGF was added to fresh serum-free DMEM in a final concentration of 10 ng/ml. Cells treated with hypoxia received fresh serum-free DMEM without supplements and were placed in an *in vivo* O<sub>2</sub> 400 (Baker and Baker Ruskinn, Sanford, USA) at 0.5 % oxygen. After 48 hours, the medium was removed, centrifuged at 1,000 g for 10 min and processed as described below or stored at -80°C for further processing. Furthermore, cell viability after preconditioning was determined by a photometric assay using 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT), as described previously [16]. In brief, subconfluent cells in 96-well plates were preconditioned for 48 hours as described above. Afterwards, XTT reagent was added to wells as described by the manufacturer (Applichem, Darmstadt, Germany) and incubated at 37°C. Absorbance was measured in a microplate reader at 490 nm vs. 650 nm.

### Quantitative Real Time PCR (qPCR)

Total cellular RNA was isolated immediately after preconditioning. RNA extraction was performed using the single-step RNA isolation from cultured cells by a modified protocol of Chomczynski and Sacchi [17]. After RNA extraction and synthesis of cDNA, quantitative PCR was carried out using a SYBR Green Rox Mix with the following conditions: 15 min at 95°C for enzyme activation and 35 cycles of 15 s at 95°C for denaturation, 30 s at 63°C for annealing, 30 s at 72°C for elongation, and 4°C for 5 min. Reactions were carried out in duplicate in 96 well plates. Quantification of the PCR fragment was carried out using the Eppendorf realplex<sup>2</sup> Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). Melting curve analysis was performed and the mean cycle threshold (Ct) values were used to calculate gene expression levels. Relative quantification was estimated by the  $\Delta\Delta C_t$  method [18] with normalization to β-actin. The relative mRNA expression of the target gene was calculated by using  $2^{-\Delta\Delta C_t}$ . PCR products were verified on an agarose gel electrophoresis. Primer pairs were synthesized by Invitrogen (Karlsruhe, Germany) and are listed in Table 1.

### Protein array

We used a commercially available protein array (Tebu-Bio, Offenbach, Germany, Human Antibody Array No. AAH-BLG-1-4) for the simultaneous detection of the relative expression of 507 human proteins in the supernatant. In brief, all supernatants were collected and centrifuged for 10 min at 1,000 g. Then, the protein content of the supernatants was determined by a routine assay using bicinchoninic acid (BCA). Supernatants were concentrated using a 3 kDa molecular weight cut-off Amicon Ultra-4 filter (No. UFC800324, Merck Millipore, Darmstadt, Deutschland) for 24 min at 3,060 g. The protein content of the supernatants was again determined by the BCA assay. Finally, the protein array was exactly processed according to the manufacturer's protocol. The readily prepared array was then sent to the manufacturer, which performed the measurement of the slides and normalization of the measured data (positive control normalization without background).

### Statistical analysis

The data were expressed as mean ± SD. The comparison between

Gene	Primer forward	Primer reverse	Product length	NCBI Reference
VEGF	CTGTCTAATGCCTGGAG	TATCGATCGTTCTGTATCAGT	268 bp	NM_001025366
HB-EGF	AAGAGGGACCCATGTCTTCG	GATTTTCCACTGGGAGGCTCA	148 bp	NM_001945
IGF-II	GATTAATTACACGCTTTCTGT	CTGTTGTATCAAGGATAGAGG	165 bp	NM_000612
Angiopoietin-like 1	GTTATTTCAGAAAAGAACAGACG	TTTTTACTCTCCAGTCTTCTAAT	175 bp	NM_004673
MIP-1 $\alpha$	TCAGACTTCAGAAGGACAC	TAGTCAGCTATGAAATTCTGTG	243 bp	NM_002983
FGF-7	CCCTGAGCGACACACAAGAA	TTCCACCCCTTTGATTGCCA	199 bp	NM_002009
M-CSF	GCAGCTGCAGGAACCTCTT	TGGTCACCACATCTTGGCTG	227 bp	NM_000757
$\beta$ -Actin	ACTGGAACGGTGAAGGTGAC	AGAGAAGTGGGGTGGCTTTT	169 bp	NM_001101

Table 1: Primers used for PCR.

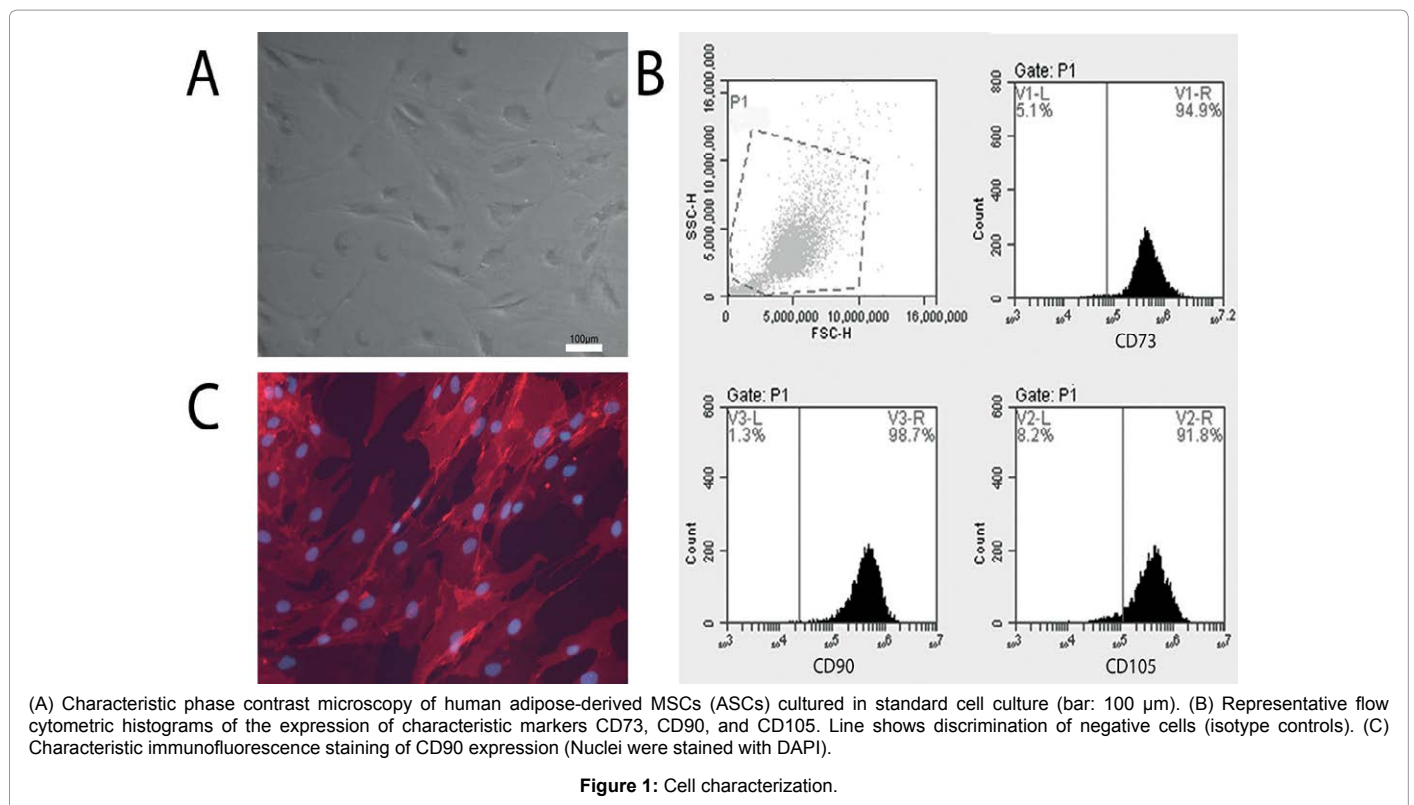


Figure 1: Cell characterization.

groups was performed by one-way analysis of variance (ANOVA) and Bonferroni post-hoc test using Prism 5 software (GraphPad). p values < 0.05 were considered significant.

## Results

### Characterization of human adipose-derived mesenchymal stromal/stem cells (ASCs)

Cell morphology was evaluated by phase contrast microscopy. The cells displayed a spindle-shaped fibroblastoid morphology in culture (Figure 1A). Expression of characteristic markers for mesenchymal stromal/stem cells (CD73, CD90, CD105) was shown by flow cytometric analysis (Figure 1B). A comprehensive characterization of ASCs has been shown earlier [13]. In addition, expression of CD90 was shown by immunofluorescence staining (Figure 1C).

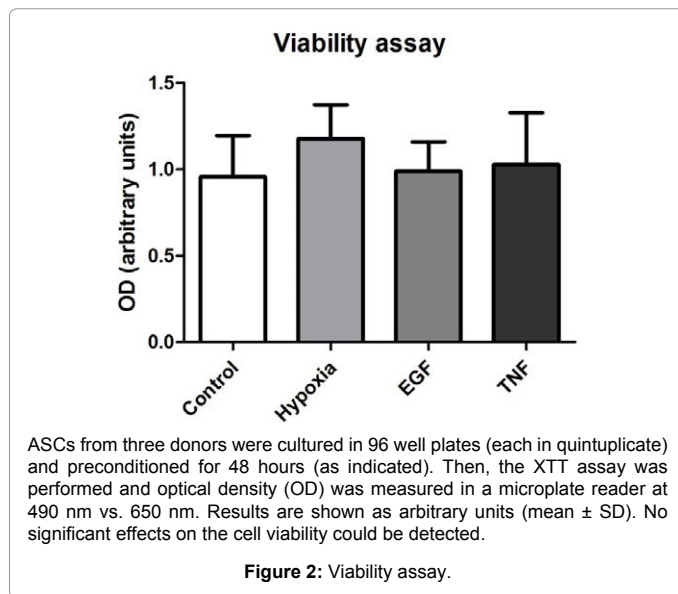
### Effect of preconditioning regimens on cell viability

Cell viability after the preconditioning regimens was determined by XTT assay. The XTT assay is a colorimetric assay used to determine cell viability as a function of cell number based on metabolic activity of

the cultured cells. We detected no significant differences in cell viability after the pretreatments compared to the control (Figure 2).

### Evaluation of preconditioning by qPCR

We investigated the potency of three different preconditioning regimens on the gene expression of ASCs. Therefore, we analysed the mRNA of seven selected potential regeneration promoting factors (VEGF, FGF-7, HB-EGF, IGF-II, Angiopoietin-like 1, MIP-1 (CCL3), M-CSF) by qPCR analysis (Figure 3). The qPCR results highlight the differential induction of ASCs' gene expression by those three pretreatments. Whereas the Hyp treated ASCs showed a significant VEGF mRNA induction, the other preincubation regimens induced no significant changes in the VEGF mRNA expression. Also FGF-7 and IGF-II mRNA were only induced by the Hyp. Angiopoietin-like 1 mRNA was not significantly induced by all three preconditioning regimens. The gene expression of the other two factors (HB-EGF, M-CSF) was significantly induced by incubation with TNF $\alpha$  or by a Hyp. Interestingly, none of the evaluated mRNAs was induced after incubation with EGF at the chosen endpoint (48 h). Nevertheless, in regard of the results of the protein array it should be mentioned that



none of the seven factors was *de novo* induced by the preconditioning methods. All factors were constitutively expressed by ASCs – as proven by the Ct value and the melting curve analysis of the qPCR data (data not shown).

### Evaluation of preconditioning by protein array

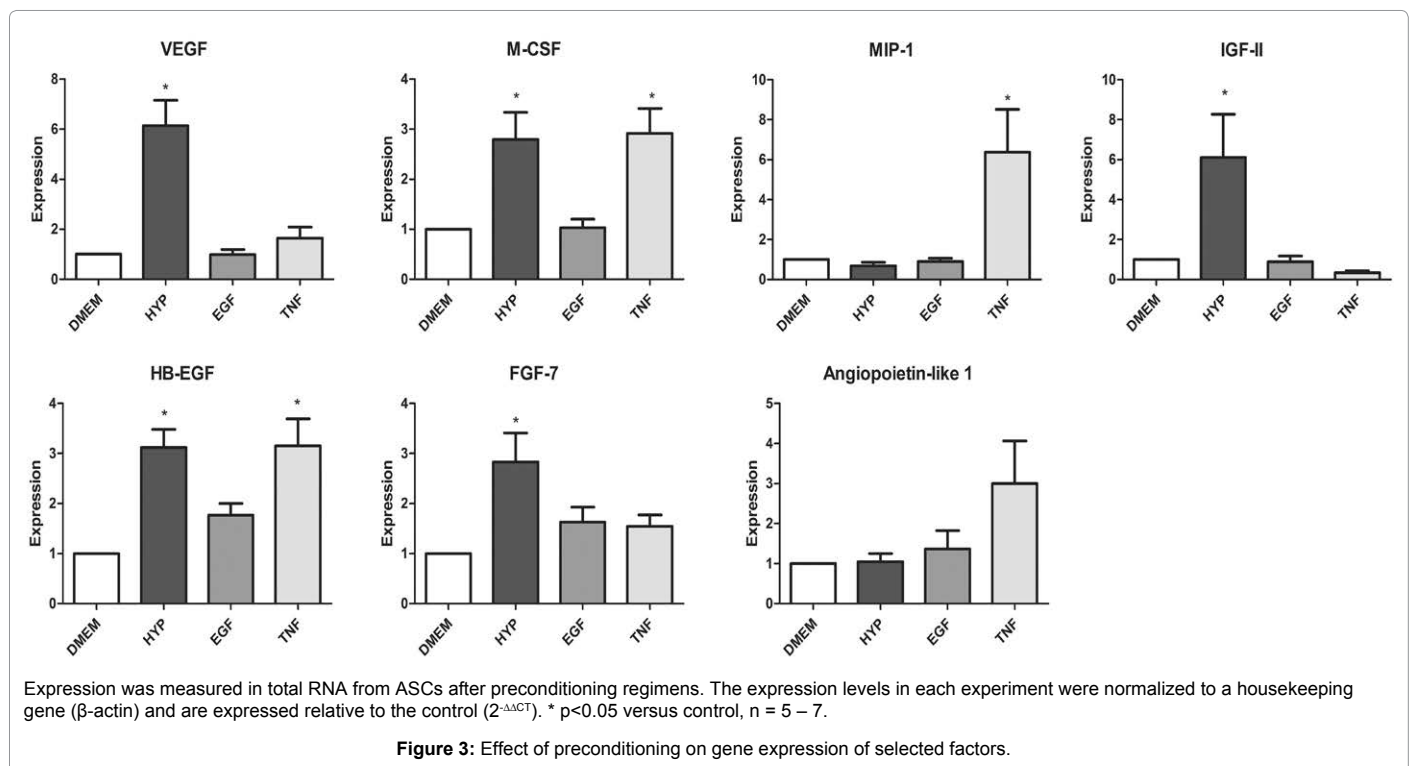
Based on the hypothesis that factors released from ASCs represent a key mechanism to enhance organ and tissue regeneration after injury, we evaluated whether the preconditioning regimens increase the release of proteins into the culture supernatant. Therefore, we used a commercially available protein array for the simultaneous detection of 507 human proteins in the supernatant of the preconditioned ASCs

in comparison to ASCs cultured under standard conditions (control). The data from this array were evaluated, whereas only values (arbitrary units) which increased more than five-fold compared to the control were regarded as induced (Figure 4). Furthermore, we did not use stimulation values < 500. From the 507 proteins in the assay, 21.9 % were found to be more than five-fold increased after incubation in a Hyp (111 of 507 proteins) (Figure 4). Preincubation with EGF resulted in an upregulation of 32.3% (164/507), whereas TNF $\alpha$  upregulated 28.8% of all proteins evaluated (146/507) (Figure 4). Complete results from all 507 proteins and the internal controls are shown in a supplemental online table (Table S1).

Compared to the seven selected regeneration promoting factors evaluated by PCR analysis, the results of the protein array are not completely consistent. Interestingly, the amount of protein release of all seven factors was induced by each pretreatment regimen, whereas qPCR analysis revealed a differential induction of the mRNA expression. For example the amount of Angiotensin-like 1 protein in the cell supernatant exhibits a more than five-fold induction by each preconditioning regimen, whereas no significant induction of the mRNA expression could be detected. On the other hand, HB-EGF protein was significantly enhanced in the supernatant by all treatments similarly to the mRNA expression (Hyp and TNF $\alpha$  significant, EGF by trend). It should also be mentioned that similar to the mRNA none of the proteins was *de novo* induced by the preconditioning methods. All seven proteins were constitutively expressed in non-pretreated ASCs, as proven by the protein array (Supplemental Table S1).

### Discussion

Adult adipose-derived stromal/stem cells are multipotent cells with strong paracrine activities based on the release of various regeneration promoting factors [19]. Therefore, ASCs are promising cells for regenerative medicine and cell therapy. In this regard, studies have demonstrated that transplantation of MSCs exerts beneficial effects,



	Control	Hypox	EGF	TNF		Control	Hypox	EGF	TNF
Positive Control	41276	41276	41276	41276	Negative control	6	40	9	43
6CKine	482	3718	4299	3584	IL-2	542	1799	3320	2584
Activin B	324	1151	3398	2685	IL-4	99	671	1	585
Activin C	514	2472	5244	3317	IL-4 R	1801	5864	19027	22947
Activin RIA / ALK-2	352	1752	3705	2191	IL-6	416	1850	3732	3954
Activin RIB / ALK4	385	1474	2784	1796	IL-7	266	1224	1778	1213
Activin RIIA	836	3132	6713	6329	IL-7 R alpha	201	1238	753	1124
Adiponectin / Acrp30	232	1271	1336	1220	IL-8	298	1120	2234	2194
Angiogenin	67	3215	1130	913	IL-10	813	3801	7164	6067
Angiopoietin-1	202	1368	1701	2977	IL-12 p40	14	584	1	518
Angiopoietin-2	888	3277	4816	2163	IL-13	635	3554	4782	3497
Angiopoietin-like 1	2397	13734	21442	19523	IL-15	394	1491	2775	2795
Angiopoietin-like 2	2067	8850	13632	5703	IL-16	158	595	867	636
Angiopoietin-like Factor	203	1143	2244	967	IL-17	79	51	592	442
Angiotensin	654	4005	6493	3744	IL-17B	366	1513	3262	4006
APJ	104	1	1	3089	IL-17F	915	7295	19377	19062
APRH	813	5484	10396	8303	IL-18 R alpha / IL-1 R5	245	1376	1327	2151
BD-1 / CDB0	111	756	733	501	IL-19	1045	11442	9664	7905
beta NGF	158	984	811	887	IL-21	198	999	579	1740
BIK	416	368	469	3973	IL-29	970	9210	19923	19409
BLC / BCA-1 / CXCL13	287	1435	1544	1256	Inhibin A	473	1071	3438	1486
BMP-2	468	1852	4036	3210	Insulin R	1543	8321	17858	10299
BMP-3	519	2687	4519	3645	Insulysin / IDE	115	739	878	1296
BMP-8	108	561	1016	439	IP-10	204	917	1071	39133
BMP-15	94	579	932	522	I-TAC / CXCL11	970	7922	24815	20349
CCL14 / HCC-1 / HCC-3	507	2490	5175	3519	Kremen-2	459	2721	5656	2922
CCL28 / VIC	365	5441	5642	4524	Latent TGF-beta bp1	154	1061	1904	1512
CCR1	300	960	2111	1952	Lefty - A	209	1226	6665	8726
CCR2	326	1610	3164	2490	LFA-1 alpha	403	760	2219	1087
CCR3	282	1192	1901	1506	LIGHT / TNF-SF14	207	1368	1623	1458
CCR4	270	1275	1723	1616	Lipocalin-1	1306	7654	31737	40591
CCR5	161	1091	1512	1178	Lipocalin 2	16	431	162	2804
CCR6	102	484	912	712	Lymphotactin / XCL1	147	499	1203	1172
CCR7	2032	8928	30402	24069	Lymphotoxin beta R / TNFRSF3	142	896	770	832
CCR8	658	2475	4626	3019	MAC-1	713	2436	4761	1846
CCR9	1555	7053	17241	17885	MCP-1	456	74	1	2280
CD30 Ligand / TNFSF8	469	2831	3258	2420	MCP-2	437	2185	3448	6054
CD40 Ligand / TNFSF5 / CD154	287	1751	309	1654	MCP-3	190	2014	2927	3405
CD 163	1144	6571	8570	7641	MCP-4 / CCL13	108	790	1522	1216
Cerberus 1	199	544	1257	754	M-CSF	1581	7113	24788	23804
Chem R2C	192	559	1435	993	MDC	288	1644	3616	2799
Chordin-Like 1	558	7390	9860	6687	MFG-E8	1194	12217	11842	16170
CLC	103	290	408	686	MIF	281	1782	1556	1220
CNTF	1111	4990	6725	1475	MIG	759	3439	6847	5812
CTACK / CCL27	379	2186	1862	1677	MIP-1a	699	4683	9726	11091
CTGF / CCN2	225	1430	3711	2292	MIP-1b	273	857	1214	46272
CV-2 / Crossveinless-2	393	3951	6835	15274	MIP-1d	111	705	497	340
CXCL14 / BRAK	1558	9972	24385	19444	MIP-2	502	4294	6422	64204
CXCR1 / IL-8 RA	1732	7981	15021	12076	MIP-3 alpha	191	1601	1727	2555
CXCR2 / IL-8 RB	1451	7244	13855	9177	MMP-1	141	838	928	3779
CXCR3	213	1126	2361	644	MMP-10	286	1507	1809	1598
CXCR5 / BLR-1	119	579	1295	245	MMP-12	141	768	436	541
D6	201	565	801	1397	MMP-13	218	1242	929	1119
DcR3 / TNFRSF6B	619	3929	6191	4675	MMP-20	1701	5279	5056	17199
Decorin	1583	21478	22621	24093	Mus	565	4748	8916	6073
Dkk-1	404	7698	20716	23285	NOV / ECN3	131	954	316	119
DR3 / TNFRSF25	396	2325	4477	3310	NrCAM	1513	10122	22846	23550
Endocan	197	519	601	1686	NT-4	149	874	1093	755
Endoglin / CD105	460	3470	4760	3939	Orexin B	247	1135	3455	2856
EN-RAGE	184	1127	1713	1272	OSM	707	7236	8759	6574
Eotaxin / CCL11	160	771	1768	2563	Osteocalcin / GPMB	328	1992	3169	2276
ErbB2	39	455	535	98	Osteopontin / TNFRSF11B	107	2084	4361	5079
Erythropoietin	1978	15472	34803	28081	PD-ECCG	269	1543	2563	1145
Fas / TNFRSF6	251	816	1118	1292	PDGF-AA	245	819	3217	5281
FGF-BP	433	1608	2690	1977	PDGF-C	100	560	653	1144
FGF-R3	382	2706	5217	3169	Pentraxin3 / TSG-14	2238	7253	13890	8639
FGF-R4	270	704	1546	879	PLUNC	146	1084	281	848
FGF-7 / KGF	801	3984	11315	11625	Pref-1	122	990	667	1035
FGF-8	105	590	457	734	Progranulin	10823	36512	52103	53037
FGF-9	534	3926	8933	8240	RAGE	100	672	678	550
FGF-13 1B	390	1661	2253	1825	RANTES	9	87	1	39449
FGF-16	331	2180	1317	1029	ROBO4	800	5107	11175	8205
FGF-18	293	1062	2117	1579	SDF-1 / CXCL12	134	1022	733	609
FGF-23	115	725	841	770	sgp130	320	1748	2677	2378
FLRG	273	1225	1893	1	SIGIRR	1117	7354	21485	28168
Follistatin	1625	11107	28157	34699	Siglec-9	1454	8153	15643	21226
Follistatin-like 1	587	2797	6237	13152	Smad 4	4578	19610	33277	17206
Frizzled-5	117	586	1255	1155	Smad 5	81	564	603	528
Frizzled-6	106	338	647	624	Smad 7	217	1195	1160	980
Galectin-3	504	2924	2313	2669	Soggy-1	167	910	567	1319
GDF-15	650	5625	11245	8676	Sonic Hedgehog (Shh N-terminal)	267	1326	2333	692
Glut1	124	713	1436	957	Spinesin	408	1464	2295	1457
Glut2	1030	7594	19595	14965	TAC1 / TNFRSF13B	224	581	132	2442
Glut5	1453	12400	25649	25399	Thrombopoietin (TPO)	605	1235	1528	3429
Granzyme A	525	3230	4593	2755	Thrombospondin-2	716	6250	10915	11641
GREMLIN	454	2470	3183	2609	TIMP-1	556	6143	16118	14449
HB-EGF	1478	8769	18545	21210	TIMP-2	5139	360853	261595	263598
HCC-4 / CCL16	106	254	649	103	TIMP-3	2586	17304	30732	16892
HGFR	335	1329	3709	2332	TLR2	23	558	1	458
I-309	107	629	997	4798	TMEFF1 / Tomoregulin-1	2887	14500	33180	27680
ICAM-1	112	784	383	850	TNF-alpha	607	3133	5015	6436
IGFBP-1	325	2813	6201	3235	TNF-beta	522	4162	7325	5371
IGFBP-3	517	10139	18863	11196	TRADD	390	2569	4841	6329
IGFBP-4	73	341	341	527	TRAIL R1 / DR4 / TNFRSF10A	306	1684	1483	1013
IGFBP-6	112	510	947	444	TSG-6	1	1	181	3293
IGF-II	423	2922	4867	5902	uPA	289	2622	14363	17005
IL-1 alpha	383	1833	3126	2367	VCAM-1 (CD106)	111	621	157	74
IL-1 beta	156	926	928	457	VE-Cadherin	372	2398	2479	1530
IL-1 F6 / FIL1 epsilon	199	1363	699	1019	VEGF	1615	44916	41489	31710
IL-1 R4 / ST2	93	408	664	462	VEGF-C	215	2903	4814	6812
IL-1 sRII	79	392	950	437	WIF-1	335	1833	3367	1603

>10 versus control

>5 versus control

Cells were either cultured under standard conditions (Ctrl) or preconditioned by incubation in a hypoxic environment (Hyp; 0.5% O<sub>2</sub>), in the presence of EGF (10 ng/ml) or TNFα (10 ng/ml) for 48 h. Hereafter, expression of 507 proteins was measured in the cell supernatant by a commercially available protein array. Heatmap displays proteins enhanced at least >5-fold versus control and arbitrary unit of stimulation >500.

Figure 4: Heat map of differentially expressed proteins after preconditioning.

e.g. in acute kidney injury [20] or cardiovascular diseases [2]. Herein, paracrine factors secreted by transplanted MSCs have been reported to be largely responsible for neovascularization and tissue regeneration [21]. Their ability to express and release these factors has been analysed in many *in vitro* studies, including various *in vitro* pretreatments [5,6,9]. Cell therapy heavily relies on the strong capability of the transplanted cells to deliver regeneration promoting factors to promote angiogenesis, wound repair, and tissue regeneration. It is also well known that pretreated MSCs hold an improvement for organ engraftment and for enhanced *in vivo* cell retention after transplantation compared to untreated cells.

Several strategies have been explored how to enhance the secretion of paracrine factors and, therefore, the regenerative potential of MSCs (or their CM) before cell transplantation, including gene modification [22], preincubation with drugs [23] or low-dose lipopolysaccharide [24], growth factors [3,4], cytokines [21], cell-cell-interactions [25], or culture in hypoxia [5,6]. Nevertheless, the secretome of ASCs after different preconditioning regimens has yet not been investigated in a comprehensive manner. Thus, further studies *in vitro* and *in vivo* are required to fully unfold ASCs' paracrine secretome and to optimize the cells as an effective tool for cell therapeutical approaches. Advances in high-throughput technologies, protein microarrays, and bioinformatics have already facilitated analysis of the secretome and will continue to aid in identification of soluble factors released by stem cells under different conditions [9].

In this study we focussed on the comparison of different preconditioning regimens on cultures of ASCs. First, we analysed gene expression levels of seven factors treated with three different preconditioning regimens. Hereafter, we then completed the analysis with a commercially available human protein array. Our investigation illustrates that the release of factors into the supernatant can effectively be enhanced by all three regimens, although we achieved an individual pattern of secreted growth factors and cytokines (Figure 4 and Table S1). Interestingly, we could show that the short-term preconditioning with EGF yielded the strongest release of proteins, whereas all three regimens induced an increase of many factors. In contrast, mRNA expression of the seven investigated genes remained relatively unchanged via preconditioning with EGF in comparison the control.

Several recently published studies have shown that preconditioning by hypoxia strongly enhances the regenerative potential of MSCs [4,26,27]. Preconditioning by hypoxia was described to stimulate the secretion of growth factors, cytokines and other proteins and the release of microvesicles with mRNA/miRNA from MSCs (and ASCs) [28]. It was shown that hypoxic preconditioning is likewise able to enhance the angiogenic potential of human ASCs [23] and to improve cell survival in both *in vitro* and *in vivo* studies [5,6]. The positive effect of hypoxic preconditioned transplanted human ASCs has also been shown in a rat model of ischemic acute kidney injury [26]. In this study, vascularization, apoptosis, histological injury and levels of serum creatinine and blood urea nitrogen were significantly improved in the preconditioned transplanted group compared with the control groups [26].

In addition to hypoxia, several other factors can stimulate ASCs in culture. To date, however, methods for *in vitro* pretreatment or preconditioning, eventually by a combination of factors, have not been fully optimized to improve ASC- or their conditioned medium-based therapies. Indeed, also the growth factor EGF and the proinflammatory mediator TNF $\alpha$  have been shown to enhance paracrine and autocrine functions of MSCs [29-32]. EGF was shown to facilitate *in vitro*

expansion of MSCs without altering the multipotency of the cells [31,32].

Understanding the mechanisms by which ASCs secrete growth factors and cytokines could be an important step to further enhance their regeneration promoting effects [33]. Whereas EGF and TNF $\alpha$  bind to specific receptors, the effects of hypoxia are mainly mediated through a reduced hydroxylation of hypoxia-inducible transcription factors (HIF) resulting in stabilization of the factors and translocation to the nucleus [27]. Nevertheless, hypoxic incubation has also been shown to induce phosphorylation of surface receptors and, therefore, induce several intracellular signaling pathways [27]. On the other hand, also the downstream signaling mechanisms following EGF or TNF $\alpha$  receptor binding initiate several signal transduction cascades (e.g. ERK, p38, and JNK MAP Kinases, IP $_3$ , AKT) resulting in proliferation, transcription and protein synthesis. Due to the involvement of these various signaling pathways in all of the three pretreatment regimens further studies are needed to determine the mechanisms ultimately responsible for ASCs enhanced secretion of proteins.

Data from Tamama et al. also suggest that the molecular machinery underlying MSCs' strong paracrine capability lies downstream of EGF receptor (EGFR) signaling. The expression of a functional EGFR and the signal transduction of ASCs have been shown by previous data from our group [34]. Whereas the influence of EGF on MSCs proliferation, multipotency, and cell motility and migration are well described [31,32,34], no comprehensive study investigating the effect of EGF on the release of factors has been published. The effect of EGF has only been shown to enhance the release of factors like VEGF, hepatocyte growth factor, HB-EGF, and interleukin-6 and -11 [35]. These data strongly suggest that EGF can be used for *in vitro* MSC expansion and for the enhancement of ASCs' paracrine capability.

TNF $\alpha$ -pretreated ASCs have been shown to release various proteins, including cytokines, extracellular matrix, proteases, and protease inhibitors [24]. For example, treatment with TNF $\alpha$  has been reported to stimulate VEGF secretion in ASCs up to 1.5 fold [30]. Lee and coworkers identified 118 enhanced secreted proteins (mainly cytokines, chemokines, and proteases) by liquid chromatography coupled with tandem mass spectrometry in ASC-conditioned media upon TNF $\alpha$  pretreatment [24]. In addition, conditioned medium derived from TNF $\alpha$  pretreated ASCs has been shown to accelerate wound healing and angiogenesis *in vivo* [29].

The present study clearly showed that ASCs' release of soluble factors can be largely enhanced by different preconditioning regimens. Nevertheless, results from qPCR analysis were not congruent to results of the protein array, maybe due to the chosen endpoint analysis after 48 hours for both assays. It is possible that mRNA is already degraded at this time point and additional experiments at an earlier time point are needed.

In conclusion, all three different preconditioning regimens induced a wide variety of proteins, yet short-term pretreatment with EGF induced the highest quantity of proteins, and, therefore, appears to be the best preconditioning regime for cell therapeutic approaches.

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