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ARTICLE

Expression of Paracrine Effectors in Human Adipose-Derived Mesenchymal Stem Cells Treated With Plasma From Brown Bears (*Ursus arctos*)

Maria Berg von Linde[1,*], Karin Johansson[2], Robert Kruse[3,4], Gisela Helenius[2], Ninos Samano[5], Örjan Friberg[6], Anne Mette Frøbert[7] and Ole Fröbert[1]

Adipose-derived mesenchymal stem cells (ADSCs) are promising candidates for novel cell therapeutic applications. Hibernating brown bears sustain tissue integrity and function via unknown mechanisms, which might be plasma borne. We hypothesized that plasma from hibernating bears may increase the expression of favorable factors from human ADSCs. In an experimental study, ADSCs from patients with ischemic heart disease were treated with interventional media containing plasma from hibernating and active bears, respectively, and with control medium. Extracted RNA from the ADSCs was sequenced using next generation sequencing. Statistical analyses of differentially expressed genes were performed using fold change analysis, pathway analysis, and gene ontology. As a result, we found that genes associated with inflammation, such as *IGF1*, *PGF*, *IL11*, and *TGFA*, were downregulated by > 10-fold in ADSCs treated with winter plasma compared with control. Genes important for cardiovascular development, *ADM*, *ANGPTL4*, and *APOL3*, were upregulated in ADSCs when treated with winter plasma compared with summer plasma. ADSCs treated with bear plasma, regardless if it was from hibernating or active bears, showed downregulation of *IGF1*, *PGF*, *IL11, INHBA*, *IER3*, and *HMOX1* compared with control, suggesting reduced cell growth and differentiation. This can be summarized in the conclusion that plasma from hibernating bears suppresses inflammatory genes and activates genes associated with cardiovascular development in human ADSCs. Identifying the involved regulator(s) holds therapeutic potential.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Adipose-derived mesenchymal stem cells (ADSCs) are promising candidates for novel cell therapeutic applications. Previous cell culturing strategies with intention to improve the therapeutic potential of ADSCs hold promise by increasing proliferation and delaying senescence, but have not entered therapeutic use.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ Plasma from hibernating bears may enhance the potency of human ADSCs to secrete paracrine effectors with potential relevance for human disease.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ Plasma from hibernating bears could suppress inflammatory genes and activate genes associated with cardiovascular development in human ADSCs.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ Identifying the involved regulator(s) that suppresses inflammatory genes and activates genes associated with cardiovascular development in ADSCs holds therapeutic potential.

Adipose-derived mesenchymal stem cells (ADSCs) are promising candidates for autologous and allogenous regenerative therapy for organ failure and tissue injury. ADSCs are capable of differentiating into several cell types[1] and also secrete paracrine effectors, which provide injured tissues with a regenerative microenvironment.[2] However, ADSCs decline in regenerative capacities while aging[3] and, therefore, improving the therapeutic potential of ADSCs is warranted. Previous cell culturing strategies with intention to augment the therapeutic capability of stem cells,

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including dietary restriction,[4] prolonged hypoxia,[5] or adding the growth factors IGF-1 and FGF-2 combined,[6] hold promise by increasing proliferation, improving differentiation, or delaying senescence. However, none of the methods have entered therapeutic use.

In order to optimize culture medium supporting efficient stem cell growth, alternative strategies could be explored. Brown bears (*Ursus arctos)* have an evolutional advantage allowing them to withstand extreme conditions without organ damage. During 6 months of hibernation, bears avoid heart failure,[7] atherosclerosis,[8] diabetes,[9] osteoporosis,[10] significant loss of lean body mass,[11] and more, despite several "risk factors", such as immobilization, extreme bradycardia of < 15 beats/min, obesity, and high cholesterol levels during hibernation (summarized in ref. 12). Cardiac protection partly appears to stem from altered heart-related gene expression during hibernation,[13] which could be induced or complemented by blood-borne regulators. Bone formation markers decrease and bone resorption markers increase during hibernation.[10] ADSCs recovered from hibernating bears demonstrate spontaneously formed bone-like nodules and chondrogenic differentiation, possibly primed by protective circulating factors from bone degeneration during hibernation.[14]

We hypothesized that plasma from hibernating bears may enhance the potency of human ADSCs to secrete paracrine effectors with potential relevance for human disease.

METHODS

Isolation and cultivation of ADSCs

Human ADSCs were derived from subcutaneous adipose tissue obtained from patients, 4 men and 1 woman aged 70–88 years, undergoing coronary artery bypass surgery at Örebro University Hospital.

Adipose tissue was collected from the sternotomy and washed with phosphate-buffered saline (PBS). ADSCs were dissociated from the adipose tissue by incubating with 1:1 v/v collagenase (Collagenase NB4 standard grade; SERVA Electrophoresis, GmbH, Heidelberg, Germany) in Hank's Balanced Salt Solution (Gibco, Paisley, UK) for 45 minutes at 37°C, followed by centrifugation at 400 g for 10 minutes at 21°C. The cell pellet was resuspended in complete cell culture medium (Alpha-MEM (Life Technologies, Paisley, UK) with 10% fetal bovine serum (FBS; Australian Origin, Lonza BioWhittaker TM, Verviers, Belgium) and 1% penicillin-streptomycin (GE Healthcare Life Science, Wien, Austria)). The cells were then filtered through a 100 µm cell strainer, before centrifuging at 400 g for 10 minutes at 21°C, and the pellet resuspended in fresh culture medium. The procedure was repeated using a 60 µm cell strainer prior to seeding cells for primary culture.

The cells were seeded in T75 flasks and incubated at 37°C in 5% CO_2. After 1–2 days, the attached cells were washed with PBS and fresh culture medium was added and thereafter changed every 3–4 days during expansion until the cells were 80% confluent. The cells were then detached from the culture flasks using TrypLE Select (Gibco), and after 2 passages, aliquots of 1×10^6 cells/vial were cryopreserved.

Proliferation and identification of ADSCs

Approximately 3–5 mL cell pellets were obtained from 10–30 mL adipose tissue after tissue dissociation. Following the cell isolation, cells adhered to the culture flasks and displayed the characteristic shape of ADSCs and achieved confluency in 1–2 weeks. Furthermore, mesenchymal stem cell phenotype with capacity for tri-linage development was confirmed by culturing obtained cells in StemPro differentiation media (Gibco) and subsequent staining for adipogenesis (lipids) with Oil red O, chondrogenesis (proteoglycans) with Alcian blue and osteogenesis (alkaline phosphatase activity) with Alizarin Red.

Collection of bear plasma

Blood from free-ranging bears living in Dalarna and Gävleborg counties in Sweden was collected in lithium-heparin tubes in collaboration with the Scandinavian Brown Bear Research Project (http://bearproject.info/). Blood samples were collected from two active brown bears in summer 2010 (both males, aged 2 and 3 years, respectively) and from two different hibernating bears in the winter of 2011 (1 male and 1 female, aged 2 and 3 years, respectively) as described by Evans *et al*.[15]

Plasma was thawed from –70°C in a 37°C water bath and pooled directly before usage, and 0.1% heparin (Vianex S.A., Attiki, Greece) was added to avoid clotting.[16]

Experimental design

Cryopreserved human ADSCs from each of the 5 patients were thawed and cultured in 6-well plates (Sarstedt, Nümbrecht, Germany) during 5 days until 80% confluency. Prior to stimulation, ADSCs were starved for serum for 48 hours with the aim of minimizing the residual effects from FBS in the cell culture media. Confirmation of reversible cell cycle arrest in G0/G1 was achieved by flow cytometric analysis using a monoclonal antibody for Ki67 concomitant DAPI-staining. Recovery and proliferation of cells after serum starvation was monitored by staining with Alamar-blue. The cells were washed with PBS prior to the addition of intervention medium.

The ADSCs were treated with three different intervention media in two replicates per patient:

1. Alpha-MEM with 10% pooled plasma from hibernating bears, 0.1% heparin.
2. Alpha-MEM with 10% pooled plasma from active bears, 0.1% heparin.
3. Alpha-MEM with 10% FBS, 0.1% heparin.

The ADSCs were incubated as previously for 20 hours. ADSCs treated with intervention or control media did not show any differences regarding cell shape, adhesion, or other morphological changes as observed by standard light microscopy.

RNA preparation

The two replicates of ADSCs were pooled when harvested for total RNA extraction 20 hours after plasma-treatment. Total RNA was extracted using the RNeasy Plus Kit

(Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Total RNA quantity and purity was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and quality was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The samples had 260/280 ratios above 1.37, 260/230 ratios above 0.26, and RIN 10. The extracted RNA was stored at –70°C until shipment on dry ice.

RNA sequencing

Next generation sequencing was performed at Uppsala Genome Centre (Uppsala University, Uppsala, Sweden). The sequencing libraries were prepared using the Ion AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific), targeting 20,802 genes representing > 95% of human RefSeq genes. Adaptor ligated amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN) and amplified by polymerase chain reaction for 5 cycles. Size selection and purification were conducted using 0.5X Agencourt AMPure XP beads (Beckman Coulter). The amplicons were quantified using the Fragment Analyzer instrument (Advanced Analytical Technologies, Santa Clara, CA) with the DNF-474 High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical Technologies).

Up to eight barcoded libraries were then pooled, followed by emulsion polymerase chain reaction on the Ion Chef System using the Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific). The multiplexed libraries were loaded on Ion PI version 3 chips and sequenced on the Ion Proton System using the Ion PI Hi-Q Sequencing 200 Kit (200 bp read length; Thermo Fisher Scientific).

Single-end reads of 4.3–9.9 × 10^6 were obtained per sample. Generated data were adaptor and quality trimmed using standard settings of *ampliSeqRNA* plug-in within the Torrent Suite software (Thermo Fisher Scientific). Mapping was performed against hg19_ampliSeq_transcriptome_21k_v1 genomic reference by *tmap* plug-in of Torrent Suite, using the manufacturer's standard settings.

Statistical gene expression analysis

Normalization, fold change analysis, pathway analysis and gene ontology (GO) were conducted with GeneSpring GX Software version 14.0 (Agilent Technologies, Palo Alto, CA).

Quantile and 75th percentile normalizations were in parallel performed with baseline adjustment. Statistical significance was determined by analysis of variance with Tukey *post hoc* test and Benjamini–Hochberg false discovery rate correction. The overall outcome was similar with nearly all of the genes showing overlapping differential expression between both normalization methods. The outcome of 75th percentile normalization was selected for further analyses. Upregulated and downregulated genes with a P value < 0.05 were defined as statistically significant and a minimum fold change of 2-fold was considered biologically significant.

Differentially expressed genes were analyzed for GO enrichment and pathways enrichment. Significance for GO term enrichment and pathway analysis were set at $P < 0.05$.

Ethics

Ethical approval for collecting adipose tissue for experimental stem cell research from patients undergoing coronary artery bypass surgery was obtained from the regional ethical committee in Uppsala, Sweden (Dnr 2012/139).

All captures were approved by the Swedish Ethical Committee on Animal Research (C212/9) and the Swedish Environmental Protection Agency.

RESULTS

Differentially expressed genes of plasma-treated ADSCs

A total of 212 genes were statistically ($P < 0.05$) and biologically (fold change ≥ 2) differentially expressed between ADSCs treated with plasma from hibernating bears compared with the control (Winter vs. Control), and of these, 87 genes were upregulated and 125 genes were downregulated. In the comparison between ADSCs treated with plasma from active bears and control (Summer vs. Control), a total of 149 genes were differentially expressed of which 50 genes were upregulated and 99 genes were downregulated. In the group where winter plasma-treated ADSCs was compared with summer plasma-treated ADSCs (Winter vs. Summer), 63 genes were differentially expressed. From these, 28 genes were upregulated and 35 genes downregulated. The expressed gene variations in the different groups (Winter vs. Control, Summer vs. Control, and Winter vs. Summer) are presented in a Venn diagram (**Figure 1**). Between the groups Winter vs. Control and Summer vs.

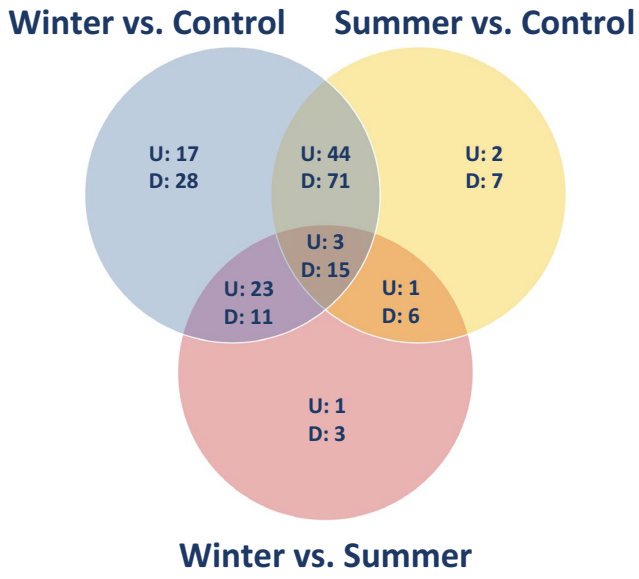

Figure 1 Venn diagram of differential expression and overlap between groups. Venn diagram of upregulated (U) and downregulated (D) genes within the groups Winter vs. Control (blue circle), Summer vs. Control (yellow circle), and Winter vs. Summer (red circle). The overlapping regions are representing shared genes with differential expression between groups.

Control, 47 upregulated genes and 86 downregulated genes were shared. The overlapping region between the groups Winter vs. Control and Winter vs. Summer showed that 26 upregulated genes and 26 downregulated genes were shared. Three upregulated genes and 15 downregulated genes were shared among all three groups. Fold changes and *P* values for all of the differentially expressed genes within each category are stated in **Supplementary Table S1**, **Table S2**, and **Table S3**.

Gene ontology analysis

In order to study the logical function of differentially expressed entities of ADSCs treated with plasma from hibernating and active bears compared with controls, GO analysis was performed. In the group Winter vs. Control, 325 GO terms showed significant enrichment. From all of these, enriched GO terms specifically relevant for adaptive changes found in hibernating bears were sorted out. Thereby, the following categories were identified: cell growth to which 29 GO terms were related, cell differentiation with 18 related terms, hypoxia with 3 terms, inflammation with 4 terms, senescence with 5 terms, the cardiovascular system with 15 terms, bone remodeling with 13 terms, muscle metabolism with 7 terms, and adipogenesis with 1 term. When analyzing Summer vs. Control, 229 GO terms were found significantly enriched. From the GO terms relevant for adaptive changes found in hibernating bears, 21 GO terms were related to cell growth, 12 related to cell differentiation, 5 to hypoxia, 5 to inflammation, 5 to senescence, 12 to the cardiovascular system, 8 to bone remodeling, and 3 to muscle metabolism. The 10 most significantly enriched GO terms within these sections are presented in **Table 1** (Winter vs. Control) and in **Table 2** (Summer vs. Control).

Pathway analysis

For pathway analysis, 121 biological pathways showed significant variations in the category Winter vs. Control. From these, 22 pathways were related to cell growth, 15 related to cell differentiation, 2 to hypoxia, 4 to inflammation, 4 to senescence, 10 to the cardiovascular system, 6 to bone remodeling, 4 to muscle metabolism, and 3 to adipogenesis. In the category Summer vs. Control, 87 pathways were significantly changed, of which 13 pathways were related to cell growth, 12 related to cell differentiation, 2 to hypoxia, 4 to inflammation, 4 to senescence, 5 to the cardiovascular system, 5 to bone remodeling, 5 to muscle metabolism, and 2 to adipogenesis. The top 10 most significant pathways within these categories are stated in **Table 3** (Winter vs. Control) and **Table 4** (Summer vs. Control).

Gene entities involved in the over-represented pathways

A curated selection of significant differentially expressed genes in the categories Winter vs. Control and Summer vs. Control demonstrative for over-represented GO terms and pathways with relevance for adaptive changes in hibernating bears is presented in **Table 5**. Additionally, a selection of genes from the category Winter vs. Summer were assessed.

DISCUSSION

Main findings

As seen in the Venn diagram (**Figure 1**), the highest number of differentially expressed genes was found in the overlapping region between the categories Winter vs. Control and Summer vs. Control. In addition, most of the modulated pathways and GO terms showed a distinct pattern of similarities between these categories. This demonstrates an effect of bear plasma for treatment of ADSCs regardless if it is collected from hibernating or active bears. Overall, a higher count of significantly upregulated or downregulated genes and mainly with a greater fold change were seen in the category Winter vs. Control compared with Summer vs. Control. These findings point to a greater response by ADSCs treated with plasma from hibernating bears compared with active bears. The expression of factors from ADSCs as a response from adding summer plasma is initially relevant to implicate the differences between FBS and bear plasma in general, whereas winter plasma-treated cultures illustrate the more biologically interesting findings with relevance to human disease. As the result was evaluated, focus was added on gene variations related to cell growth and differentiation, hypoxia, inflammation, senescence,

Table 1 Top 10 over-represented GO categories: Winter vs. Control

GO ID	GO accession	GO term	Corrected *P* value	Count in selection	Count in total
32354	GO:0072358	Cardiovascular system development	< 0.001	33	756
32355	GO:0072359	Circulatory system development	< 0.001	33	756
30029	GO:0065008	Regulation of biological quality	< 0.001	80	3453
8111	GO:0010941	Regulation of cell death	< 0.001	46	1461
19244	GO:0042127	Regulation of cell proliferation	< 0.001	45	1419
20033	GO:0042981	Regulation of apoptotic process	< 0.001	43	1372
5647	GO:0008083	Growth factor activity	< 0.001	14	159
20115	GO:0043067|GO:0043070	Regulation of programmed cell death	< 0.001	43	1382
5797	GO:0008284	Positive regulation of cell proliferation	< 0.001	30	767
12640	GO:0030154	Cell differentiation	< 0.001	73	3234

GO, gene ontology.
Curated list of 10 most significant GO categories with relevance for our study. *P* value, count of significant genes and total count of genes within each category are presented.

Table 2 Top 10 over-represented GO categories: Summer vs. Control

GO ID	GO accession	GO term	Corrected *P* value	Count in selection	Count in total
8111	GO:0010941	Regulation of cell death	<0.001	37	1461
18436	GO:0036293	Response to decreased oxygen levels	<0.001	17	265
20033	GO:0042981	Regulation of apoptotic process	<0.001	36	1372
20115	GO:0043067|GO:0043070	Regulation of programmed cell death	<0.001	36	1382
30504	GO:0070482	Response to oxygen levels	<0.001	17	284
32354	GO:0072358	Cardiovascular system development	<0.001	25	756
32355	GO:0072359	Circulatory system development	<0.001	25	756
1009	GO:0001666	Response to hypoxia	<0.001	15	260
12640	GO:0030154	Cell differentiation	<0.001	55	3234
22370	GO:0045595	Regulation of cell differentiation	<0.001	32	1408

Curated list of 10 most significant GO categories with relevance for our study. *P* value, count of significant genes and total count of genes within each category are presented.
GO, gene ontology.

Table 3 Top 10 over-represented pathways: Winter vs. Control

Pathway	*P* value	Selected entities in pathway	Total entities in pathway
Hs_Differentiation_Pathway_WP2848_80009	< 0.001	–8	50
Hs_Photodynamic_therapy-induced_HIF-1_survival_signaling_WP3614_84550	< 0.001	+1/–5	37
Hs_Endochondral_Ossification_WP474_80208	< 0.001	–7	64
Hs_Senescence_and_Autophagy_WP615_71375	< 0.001	+2/–6	106
Hs_Cardiac_Progenitor_Differentiation_WP2406_73324	< 0.001	+1/–5	53
Hs_Angiogenesis_WP1539_75222	< 0.001	+1/–3	24
Hs_TGF_Beta_Signaling_Pathway_WP560_68944	< 0.001	+1/–4	55
Hs_Hypertrophy_Model_WP516_71358	0.001	–3	20
Hs_MicroRNAs_in_cardiomyocyte_hypertrophy_WP1544_84696	0.002	+1/–4	104
Hs_Prostaglandin_Synthesis_and_Regulation_WP98_72088	0.004	+1/–2	31

Curated list of 10 most significant pathways with relevance for our study. *P* value, count of entities with significant differential expression (+ stands for upregulated genes and – for downregulated genes) and total counts of entities in each pathway are stated.

cardiovascular system, bone remodeling, metabolism of muscle, and adipose tissue.

Cell growth and differentiation

The transcripts for growth factors *IGF1*, *IER3*, *HMOX1*, *IL11*, and *TGFA*[17] decreased in the category Winter vs. Summer, suggesting that hibernating bear plasma downregulates cell growth. Hibernating bears have decreased plasma levels of *IGF1* compared with active bears.[18] This has an impact on proliferation and differentiation when used to treat ADSCs[6] and could contribute to the differences in expression of growth and differentiation factors between the plasma-treated cells.

Transcripts for the key regulators *IGF1*, *IER3*, *HMOX1*, *IL11*, and also *PGF* and *INHBA* were found downregulated in Winter vs. Control and Summer vs. Control. This indicates decreased cell growth and differentiation of the plasma-treated ADSCs. It is a well-established fact that FBS contains higher levels of growth factors than bovine serum from newborns and grown animals, and, therefore, we could speculate that FBS also contains more growth factors than subadult bears.

Hypoxia, inflammation, and senescence

Pathways and GO terms associated with response to hypoxia, inflammation, and senescence were over-represented. *EDN1* is a gene associated with response to hypoxia and inflammation,[19] and was found downregulated in the categories Winter vs. Control (> 10-fold), Summer vs. Control, and also in Winter vs. Summer. These findings suggest not only that bear plasma suppresses hypoxia response and inflammation, but also that plasma from hibernating bears have a particular effect of downregulating hypoxia response and inflammation on ADSCs compared with plasma from active bears. Additionally, the genes *IGF1*, *IL11*, *PGF*, and *TGFA,* which were all > 10-fold downregulated in Winter vs. Control, are associated with inflammation.[17] Another gene associated with inflammation is *MAPK11*,[20] which was upregulated in Winter vs. Control and Winter vs. Summer. Genes associated with senescence, namely *IGF1*, *IGFBP3*, and *INHBA*,[21] were found downregulated in Winter vs. Control and Summer vs. Control. *SMAD3*, also associated with senescence, was upregulated in Winter vs. Control and Summer vs. Control.

Recent studies have shown suppressed markers of immune defense in plasma from hibernating bears, namely white blood cell count[22] and CRP,[11,22] and also an elevation in levels of antioxidants, such as vitamin C,[23] haptoglobin,[22] and glutathione,[24] which could have influenced the downregulation of genes related to inflammation in Winter vs. Summer. However, genes related to inflammation in the

Table 4 Top 10 over-represented pathways: Summer vs. Control

Pathway	*P* value	Selected entities in pathway	Total entities in pathway
Hs_Differentiation_Pathway_WP2848_80009	< 0.001	–7	50
Hs_Photodynamic_therapy-induced_HIF-1_survival_signaling_WP3614_84550	< 0.001	–5	37
Hs_Senescence_and_Autophagy_WP615_71375	< 0.001	+1/-6	106
Hs_TGF_Beta_Signaling_Pathway_WP560_68944	< 0.001	+1/-3	55
Hs_Endochondral_Ossification_WP474_80208	0.001	–4	64
Hs_Prostaglandin_Synthesis_and_Regulation_WP98_72088	0.001	+1/-2	31
Hs_Signaling_by_VEGF_WP1919_76864	0.002	–2	10
Hs_MicroRNAs_in_cardiomyocyte_hypertrophy_WP1544_84696	0.003	+1/-3	104
Hs_Regulation_of_Insulin_like_Growth_Factor_(IGF)_Transport_and_Uptake_by_Insulin_like_Growth_Factor_Binding_Proteins_(IGFBPs)_WP2799_77094	0.003	–2	13
Hs_Growth_hormone_receptor_signaling_WP2657_83058	0.006	–2	20

Curated list of 10 most significant pathways with relevance for our study. *P* value, count of entities with significant differential expression (+ stands for up-regulated genes and – for downregulated genes) and total counts of entities in each pathway are stated.

plasma-treated cultures were also downregulated compared with controls, even though FBS contains particularly low levels of antibodies and complement factors compared with bovine serum from newborns or adults. Our findings suggest a strong anti-inflammatory effect of bear plasma, regardless if from hibernating or active bears.

Table 5 Representative genes with significant differential expression: Winter vs. Control, Summer vs. Control, and Winter vs. Summer

	Winter vs. Control		Summer vs. Control		Winter vs. Summer	
Gene	Fold change	Corrected *P* value	Fold change	Corrected *P* value	Fold change	Corrected *P* value
ACTC1	–25.3	0.008	–13.1	0.008		
ADM	4.1	0.002	2.0	0.002	2.0	0.002
ANGPTL4	3.6	0.011			3.5	0.011
APOL3	16.1	0.002	4.7	0.002	3.4	0.002
COL10A1	–4.4	0.016	–4.1	0.016		
DEPTOR	6.1	0.002			3.4	0.002
EDN1	–11.7	0.006	–4.9	0.006	–2.4	0.006
HDAC 9	2.6	0.018	3.3	0.018		
HMOX1	–6.1	0.003	–3.0	0.003	–2.0	0.003
IER3	–5.6	0.002	–2.4	0.002	–2.3	0.002
IGF1	–31.0	<0.001	–13.3	<0.001	–2.3	<0.001
IGFBP3	–2.6	0.012	–2.5	0.012		
IL11	–138.6	<0.001	–38.3	<0.001	–3.6	<0.001
INHBA	–6.4	0.002	–3.8	0.002		
MAPK11	4.0	0.008			2.0	0.008
PDK4	4.1	0.004			5.2	0.004
PGF	–13.5	0.002	–8.6	0.002		
RGCC	9.4	0.009			14.1	0.009
SMAD3	3.7	0.016	3.0	0.016		
SORT1	3.6	0.019			2.3	0.019
TGFA	–12.5	0.005			–5.7	0.005
TIMP3	–3.8	0.006			–3.2	0.006
VEGFA	–4.4	0.005	–4.1	0.005		

Curated list of genes representative for overrepresented gene ontology terms and pathways with relevance for adaptive changes in hibernating bears, containing fold change and *P* values. All of the differentially expressed genes within the categories Winter vs. Control, Summer vs. Control, and Winter vs. Summer are presented in **Supplementary Table S1**, **Table S2**, and **Table S3**.
ACTC1, actin, alpha, cardiac muscle 1; ADM, adrenomedullin; ANGPTL4, angiopoietin like 4; APOL3, apolipoprotein L3; COL10A1, collagen type X alpha 1 chain; DEPTOR, DEP domain-containing mTOR-interacting protein; EDN1, endothelin 1; HDAC9, histone deacetylase 9; HMOX1, heme oxygenase 1; IER3, immediate early response 3; IGF1, insulin like growth factor 1; IGFBP3, insulin like growth factor binding protein 3; IL11, interleukin 11; INHBA, inhibin subunit beta A; MAPK11, mitogen-activated protein kinase 11; PDK4, pyruvate dehydrogenase kinase 4; PGF, placental growth factor; RGCC, regulator of cell cycle; SMAD3, SMAD family member 3; SORT1, sortilin 1; TGFA, transforming growth factor alpha; TIMP3, TIMP metallopeptidase inhibitor 3; VEGFA, vascular endothelial growth factor A.

Cardiovascular development

The most significant genes within GO terms and pathways with association to a specific organ were those related to cardiovascular development. Genes specific for angiogenesis are *ADM*, *ANGPTL4*, *APOL3*, and *HDAC9* and all were upregulated in Winter vs. Control. More interestingly, *ADM*, *ANGPTL4*, and *APOL3* also increased in Winter vs. Summer. *RGCC* was another, > 10-fold, upregulated gene in Winter vs. Summer, which regulates cell cycle progression in response to DNA damage.[25] This gene could, together with the upregulated angiogenic genes, serve as a favorable effector after myocardial infarction.[26] The expression of *ACTC1*, *EDN1*, and *VEGFA*, which are specific for cardiovascular development,[27] were decreased in Winter vs. Control and Summer vs. Control.

Bone remodeling

Genes associated with skeletal development were over-represented significantly within GO terms and pathways. *IL11* was the gene with greatest fold change of downregulation in both of the categories Winter vs. Control and Summer vs. Control and was also downregulated in Winter vs. Summer. This gene increases bone resorption by stimulating osteoclastic formation,[28] meaning that a downregulated *IL11* could promote osteogenesis. Specific genes for ossification, namely *COL10A1* and *TIMP3*,[29] were downregulated in Winter vs. Control. *TIMP3* decreased in the category Winter vs. Summer.

Overall, no obvious increased properties of osteogenetic differentiation were seen in Winter vs. Control or Winter vs. Summer. These results do not confirm the findings of osteoblasts cultured in medium with serum from hibernating bears where apoptosis of osteoblast were reduced and markers in bone turnover were decreased.[30] However, difficulties to achieve bone regeneration *in vitro* have been described[31] and could be a possible reason for lack of differentially expressed genes specific for skeletal development.

Metabolism of muscle and adipose tissue

Changes of expression were also seen in genes involved in metabolism of muscle and adipose tissue. The genes *SORT1*[32] and *DEPTOR*[33] are expressed in skeletal muscle and adipocytes and were upregulated in Winter vs. Control and Winter vs. Summer. *PDK4* is involved in regulation of lipid metabolism[34] and was also upregulated in Winter vs. Control and Winter vs. Summer.

Perspectives

In summary, winter plasma-treated ADSCs showed downregulation of genes associated with cell growth and inflammation and upregulation of genes specific for angiogenesis compared with summer plasma-treated cultures. These outcomes coincide with some of the pathways in the signaling cascade of hypoxia-inducible factor (HIF-1). This cascade regulates cellular response to hypoxia, including controlling genes associated with metabolism, erythropoiesis, angiogenesis, and inflammatory response.[35] HIF-1 protein levels have shown significant upregulation in brown adipose tissue and skeletal muscle in hibernating ground squirrels (*Spermophilus tridecemlineatus*).[36] Hibernating bears experience extreme bradycardia, accompanied by extremely low respiratory rates,[37] and to maintain adequate tissue oxygen tension during hibernation, erythrocyte count and hemoglobin increase.[38] The gene targets responsible for the adaption during hibernation is not known. However, a mechanism similar to the HIF-1 signaling cascade seems to be induced.

Strengths and limitations

Differences in levels of parameters in bear plasma during summer and winter are partly known from previous studies,[18,22] but the samples of bear plasma used for our experiments were not tested for any parameters before usage. This was not possible due to the limited amount of plasma available. Plasma volume restrictions forced us to pool plasma from different bears for cell cultures. Ideally, we should have cultured ADSCs with plasma from individual bears obtained during the summer and winter in order to avoid differences due to host factors, such as gender, age, body lean mass, etc. The plasma was collected from two subadult males in summer and two different subadult bears, one male and one female, in winter that could lead to variations of steroid hormone levels. However, the steroid hormones mainly differ after sexual maturity[39] and when comparing other biochemical parameters between males and females most of the variables showed no significant variation.[40] In addition, previous summer-winter comparative studies have shown substantial differences with little or no overlap in parameters measured.[22] It is likely that substances affecting gene regulation are equally different in summer and winter plasma justifying plasma pooling.

Another confounding variable is the different use of anesthetics to immobilize the bears before plasma collection. In winter, a lower dose of medetomidine-tiletamine-zolazepam was used in comparison with summer bears and also ketamine was added to the anesthetic mixture in winter bears.[15] A previous study has shown that hypoxemia could be caused by higher doses of medetomidine-tiletamine-zolazepam.[41] Genes associated with hypoxia response showed a stronger downregulation in Winter vs. Control than Summer vs. Control, which could theoretically have been influenced by the higher dose of anesthetics in the summer bears.

The five patients, from whom the ADSCs were collected, differed in individual variables, such as age and gender. As they are no homogenous cell lines, there could be differences in genetic expression between these cultures even before they were exposed to the different bear plasmas. However, ADSCs from every patient was exposed to both of the bear plasmas and control serum, which means that it is still possible to see different reactions due to the differences in winter and summer plasma in respect to each one of the patients. Using ADSCs in our experiment should reduce the risk of eventual immunological effects from foreign serum complements because they are hypoimmunogenic, suppress T lymphocytes and natural killer cells, and induce a suppressive microenvironment.[42] However,

the primary intention of our experiments was to find expressed genes that could potentially improve ADSCs for therapeutic usage rather than planning to actually add bear plasma to the cells when culturing ADSCs for therapeutic applications.

To keep a broad spectrum regarding the resulting data, RNA sequencing was used in order to examine global changes in gene expression. This method is limited to mRNA expression, which is not equivalent to formation of intact proteins. However, mammals show high correlation between RNA expression and protein quantities.[43] In addition, significant correlation between gene expression at mRNA and protein levels have been found in hibernating and active ground squirrels.[44] Restricted resources prevented us from performing protein expression studies to confirm our findings.

Conclusion

As a conclusion of this study, ADSCs treated with winter plasma seem to feature some promising genes for experimental therapeutic applications for inflammatory diseases and ischemic heart disease. Further investigation on inflammatory and cardiovascular factors is warranted in disease models.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

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