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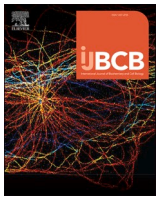

# Hibernating brown bear serum modulates the balance of TGF-β and BMP pathways in human muscle cells

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ABSTRACT

Muscle atrophy is observed in several pathophysiological situations, including physical inactivity, leading to negative health consequences, without any effective treatment currently available. Conversely, brown bears resist muscle atrophy during hibernation, despite prolonged physical inactivity and fasting. We previously reported that hibernating brown bear serum increases protein content in human myotubes and inhibits proteolysis. To go further, we deciphered here the transcriptional effects of brown bear serum in human myotubes using large-scale transcriptomics. After 48 h, the winter-hibernating bear serum (WBS) induced a specific transcriptomic program, affecting mostly biological pathways related to muscle growth and BMP signalling, compared to the summer-active bear (SBS) serum. WBS predominantly reduced, at mRNA and protein levels, activators and inhibitors of BMP signalling, which is associated with muscle mass maintenance. Moreover, BMP activity was more responsive to a stimulation by BMP7 at supra-physiological concentrations in human myotubes cultured in WBS versus SBS conditions. Meanwhile, WBS also up-regulated expression of genes encoding repressors of the pro-atrophic TGF-β pathway, decreased phosphorylated SMAD3 nuclear protein levels, and down-regulated TGF-β target genes. Furthermore, WBS treatment resulted in reduced TGF-β signalling responsiveness in human myotubes stimulated with TGF-β3 at physiological concentrations. Overall, even though WBS induced larger transcriptomic changes in the BMP compared to TGF-β pathway, the functional consequences were more pronounced for the TGF-β pathway with a marked inhibition. This study suggests that bioactive compounds in WBS may protect human muscle cells during catabolic situations, by regulating the TGF-β/BMP balance. These findings open new perspectives for therapies targeting muscle atrophy.

## 1. Introduction

Striated skeletal muscles account for a large proportion of our body and ensure essential functions (e.g., locomotion, thermoregulation, metabolism) (Frontera and Ochala, 2015). Under catabolic conditions in humans, such as ageing, physical inactivity, prolonged bed rest, or certain pathologies, numerous interconnected signalling pathways contribute to the dysregulation of the protein turnover, resulting in skeletal muscle atrophy (Peris-Moreno et al., 2021; Sartori et al., 2021). Muscle atrophy is associated with adverse health outcomes, such as reduced autonomy and increased morbidity and mortality, making it a significant public health issue. However, despite extensive research using human and animal models of induced atrophy, an effective treatment has still not been identified.

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Among the pathways involved in the regulation of skeletal muscle mass and function, the pro-atrophic TGF-β (Transforming Growth Factor)/myostatin/activin and the pro-anabolic BMP (Bone Morphogenic Proteins)/GDF (Growth and Differentiation Factors) pathways are interconnected to control muscle protein turnover (Peris-Moreno et al., 2021; Sartori et al., 2014). Binding of TGF-β and BMP ligands to their receptors triggers phosphorylation of the Suppressor of Mothers against Decapentaplegic (SMAD) transcriptional factors. Once phosphorylated, SMAD2/3 and SMAD1/5/9 for TGF-β and BMP signalling, respectively, interact with the cytoplasmic SMAD4 protein to form a complex that translocates into the nucleus, where it regulates target genes upon binding to SMAD-binding elements in their regulatory regions (Weiss and Attisano, 2013). The therapeutic potential of inhibiting the pro-atrophic TGF-β pathway has been studied for decades as a strategy to fight muscle atrophy in animals and humans (Abrigo et al., 2018; Lan et al., 2024). In addition, promoting BMP signalling during catabolic situations has recently emerged as a promising approach to counteract muscle atrophy (Sartori et al., 2021; Mina et al., 2023). In fact, inhibiting BMP signalling in mouse muscle resulted in muscle atrophy, whereas its promotion led to muscle hypertrophy (Sartori et al., 2013) or prevented cancer-induced muscle atrophy (Sartori et al., 2021; Mina et al., 2023).

Conventional laboratory animal models are used to explore the mechanisms of muscle atrophy and to test potential therapeutic strategies against muscle loss. However, previous studies have also highlighted the benefits of investigating free-ranging animals, as they offer unique insights (Bertile et al., 2021; Stenvinkel et al., 2023; Le Maho et al., 2025). For example, in-depth studies in hibernators, such as brown bears (*Ursus arctos*), have highlighted promising advances in human care for several health problems, including heart failure, atherosclerosis, kidney dysfunction, osteoporosis, and muscle atrophy (Berg von Linde et al.,2015). In fact, hibernating bears exhibit a natural resistance to muscle loss and maintain both muscle fibre type and size despite facing an annual prolonged period of physical inactivity and fasting (Tinker et al., 1998; Harlow et al., 2001; Lohuis et al., 2007; Hershey et al., 2008). We previously reported substantial remodelling of the muscle transcriptome during hibernation in the brown bear. More specifically, we identified a molecular signature suggesting simultaneous inhibition of the pro-atrophic TGF-β pathway and activation of the BMP pathway, which may likely help maintain muscle mass (Cussonneau et al., 2021). Such a concomitant modulation of TGF-β and BMP signalling could be a promising strategy for fighting muscle atrophy. This is particularly important, as inhibition of the TGF-β pathway could also repress the activities of other closely related TGF-β family members, including BMPs, and, result in off-target side effects, such as vascular toxic effects, bone weakness, or formation of benign tumours (Suh and Lee, 2020; Teixeira et al., 2020).

We and others have demonstrated an increase in total protein content in primary human myotubes cultured with hibernating brown bear serum (Chanon et al., 2018; Miyazaki et al., 2022), suggesting the presence of circulating compounds that may act on the protein balance. Mechanisms include decreased muscle protein synthesis and, most importantly, protein catabolism via the lysosomal and proteasomal pathways (Chanon et al., 2018). As TGF-β and BMP signalling both control protein synthesis and degradation (Sartori et al., 2014), compounds in hibernating bear serum may modulate these signalling pathways.

This study investigated the effects of bear serum in primary human muscle cells using large-scale transcriptomics. Incubation of human myotubes with hibernating bear serum reprogrammed their transcriptome, primarily affecting genes involved in muscle growth and BMP signalling. To further validate the actual effects of this reprogramming, we also examined whether the responsiveness of TGF-β and BMP pathways to their ligands was influenced by the presence of hibernating bear serum.

## 2. Materials and methods

### 2.1. Bear serum samples

Free-ranging sub-adult brown bears (*Ursus arctos*; 14 females, 7 males, aged 2–3 years) from Dalarna and Gävleborg counties (Sweden) were captured during winter hibernation (February) and recaptured during their summer active period (June) between 2014 and 2019 for blood collection. The serum was prepared as previously described (Chanon et al., 2018). A pooled serum was prepared by mixing equal amounts of serum from each individual bear captured in either winter (WBS, winter-hibernating bear serum) or summer (SBS, summer-active bear serum) to minimize inter-individual variability. WBS and SBS pools were then aliquoted and stored at −80°C until use. All procedures were approved by the Swedish Ethical Committee on Animal Experimentation (Dnr C 286/12 and C 3/2016), the Swedish Environmental Protection Agency (NV-01758–14 and NV-00741–18), and the Swedish Board of Agriculture (Dnr 5.2.18–3060/17). All procedures complied with Swedish laws and regulations.

### 2.2. Cell culture and treatments

Primary human muscle cells were isolated from vastus lateralis muscle biopsies taken from healthy control donors (Diomed experimental protocol) and frozen in liquid nitrogen. All procedures were approved by the French Ethical Committee SUD EST IV (Agreement #12/111 A 13–02) and conducted in accordance with the French legislation (Huriet's law). Each patient provided written consent after receiving comprehensive information regarding the study's nature, objectives and potential risks. Myoblasts were thawed from liquid nitrogen, seeded into 12-well plates (#25115083, Dutscher, Bernolsheim, France), and maintained at 37°C under 5 % CO2, in Ham's F10 medium (1 g/L glucose) (#L0145–500, Dutscher) containing 1 % L-glutamine (Sigma, Saint-Quentin-Fallavier, France), 100 U/mL antibiotics (penicillin/streptomycin, P06–07100, Dutscher), and 15 % Foetal Bovine Serum (FBS) (#10270106, Fisher Scientific, Illkirch, France). After reaching 80 % confluence, differentiation was induced for five days by replacing the previous medium with standard Dulbecco's Modified Eagle Medium (DMEM, 1.0 g/L glucose) (#P04–01500, Dutscher, France), containing 1 % L-glutamine, 100 U/mL antibiotics, and 2 % FBS. For transcriptome, protein and mRNA analyses, myotubes were then cultured for 48 h in DMEM, in which FBS was replaced with either 5 % SBS or 5 % WBS, as previously described (Chanon et al., 2018). To analyse the effect of bear serum on the responsiveness of BMP or TGF-β signalling, myotubes were first cultured for 48 h with either 5 % SBS or 5 % WBS as described above, and then treated for 30 min with increasing doses of BMP7 (0–10 μg/mL) or TGF-β3 (0–100 ng/mL) (#752304 and # 585802), respectively; BioLegend, Amsterdam, The Netherlands). Biological replicates were performed on cell preparations from different donors, with the exact number specified in the figure legends.

### 2.3. RNA isolation

Total RNA was isolated from human myotubes using the NucleoMag RNA kit for magnetic bead-based RNA purification (Macherey-Nagel, Hoerdt Cedex, France) and the KingFisher™ Duo Prime Purification System (Thermo Fisher Scientific, Wilmington, DE, USA), according to manufacturers' instructions. RNA was quantified by measuring the absorbance at 260 nm on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at −80°C until use for RNA sequencing or RT-qPCR measurements.

### 2.4. High-Throughput sequencing

RNA sequencing was performed by BGI (Beijing Genomics Institute, China) using the DNBseq sequencing technology. Quality control of

samples was assessed with fragment analyser tests to validate that they met the requirements of library construction. The RNA-Seq libraries were constructed with the BGI Optimal Dual-mode mRNA Library Prep Kit. Three samples were sequenced on DNBSEQ-T7 sequencer (pair-end, 100 bp, 50 M clean reads per library). Generated raw reads were filtered using SOAPnuke software developed by BGI to remove adaptor sequences, contamination, and low-quality reads (Chen et al., 2018). Cleaned data were used to align the RNA-Seq reads to the *Homo sapiens* genome (GCF_000001405.40_GRCh38.p14_genomic.fna, from NCBI) with HISAT2. Htseq-count was then used for the quantification step (summarisation) at the gene level. It provides a raw counts matrix for the whole samples. These count data were analysed using R software (v4.3.0) and DESeq2 package (v1.42). The first step includes low counts filtering and normalization at the sample level. Differential analysis using DESeq2 was conducted with the Likelihood Ratio Test (LRT) method. The Benjamini-Hochberg (BH) method was applied to adjust p-values, with a significance threshold set at 0.05 to identify significant genes. Additionally, factorial analysis, including principal component analysis (PCA), was performed using the FactoMineR (v2.11) and factoextra (v1.0.7) libraries on log-cpm transformed data.

### 2.5. Functional annotation analysis

Gene Ontology (GO) enrichment analysis was conducted using the enrichR package (v.1.22.0). Molecular Functions (MF) and Biological Processes (BP) enrichment analyses were conducted using Fisher's exact test, followed by a Benjamini-Hochberg correction to control the false discovery rate. Terms with an adjusted p-value below 0.05 were considered significantly enriched. The org.Hs.eg.db package (v.3.19) from Bioconductor was used for mapping and gene annotation. The data were filtered to eliminate functional redundancy using the clusterProfiler package (v.4.10.1), which computes similarities between GO terms and applies a similarity threshold above 0.7 to avoid redundancy (Schlicker et al., 2006). To ensure that the annotations selected were relevant to muscle tissue, we choose to exclude BP terms corresponding to processes clearly involved in non-muscle cell types. The enrichment factor was determined by calculating the ratio between the number of DEGs in our list that are annotated in a given BP term to the total number of DEGs with BP annotations in the dataset. This value was then normalized by the ratio between the total number of genes associated with this same BP term in the background and the total number of genes with BP annotations that were identified in the background. The BPs were also classified into broad functional categories that encompassed related processes. The transcription factor analysis was performed by submitting the 352 DEGs to EnrichR software (https://maayanlab.cloud/Enrichr/) and selecting the ENCODE and ChEA Consensus TFs from ChIP-X tool. Only transcription factors with an adjusted p-value $< 0.05$ were selected (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021).

### 2.6. Western blots

At the end of bear serum and/or BMP/TGF-β ligand treatments, total myotube proteins were extracted from myotubes directly in the wells of the culture dish, using 100 µL of an ice-cold lysis buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 3 mM KCl, 1 mM MgCl2, 1 % NP40, 0.1 mM SDS, 1 mM DTT, 1 mM Na3VO4, 20 mM NaF, 1X Protease Inhibitor Cocktail (Sigma, Saint-Quentin-Fallavier, France)). To assess the nuclear localization of phosphorylated SMAD3 and SMAD1/5/9, proteins were extracted using the Nuclear Extract Kit (Active Motif, Waterloo, Belgium), following the manufacturer's instructions. The homogenates were diluted in Laemli buffer containing 5 % β-mercaptoethanol, were sonicated for 5 min, and stored at −80°C before use. After denaturation at 95°C for 10 mins, 15 µl of whole protein extracts, or 10 µg of nuclear protein extracts, were resolved on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using TGX™ FastCast™ Acrylamide Kit, 10 % (Biorad, Marnes-la-Coquette, France), and transferred onto a PVDF membrane (0.45 µm, 10600023, Hybond P, Amersham, England) using the Trans-blot® TurboTM Transfer System standard protocol (Biorad, Marnes-la-Coquette, France). Western blots were then blocked (1 h, stirring, room temperature) in Tris Buffered Saline (TBS) buffer with 0.1 % Tween-20 (TBS-T, PH = 7.8) and 5 % bovine serum albumin (BSA) (#367698 and #04–100–812-C, respectively; Euromedex, Souffelweyersheim, France) for all targets, according to the manufacturer's instructions. Blots were then washed thrice and incubated (overnight, stirring, 4°C) with primary antibodies against: (i) Endoglin (CD105), SMAD6, and the phosphorylated forms of SMAD3 and SMAD1/5/9 (#ab169545–1002, #ab273106–1001, #ab52903 and #ab92698, respectively) (Abcam, Cambridge, United Kingdom); (ii) gremlin-1 and total SMAD1/5 (#PA5–79324 and (#PA5–80036, respectively) (ThermoFisher, Courtaboeuf cedex, France); (iii) total SMAD2/3 (#8685) (Ozyme, Saint-Cyr-L'Ecole, France). All primary antibodies were diluted at 1/1000 in TBS-T containing 5 % BSA. Blots were washed thrice and incubated (1 h, stirring, room temperature) with a secondary rabbit-HRP antibody (#7074, Cell Signaling Technology, Saint-Cyr-L'Ecole, France) diluted 1/5000 in TBS-T with 5 % BSA. Blots were washed thrice, and signals were detected after incubation with Luminata Forte Western HRP substrate (Millipore, Burlington, MA, USA). And visualized using G:BOX ChemiXT4 (XL1) system (Syngene, Frederick, MD, USA). Signals were then quantified using ImageJ (v1.48) software and normalized to the total amount of proteins determined by the Biorad's stain-free system. To determine the effect of bear serum preconditioning on the responsiveness of BMP or TGF-β signalling, relative P-SMAD protein levels were normalized to the value obtained without ligand under SBS or WBS conditions.

### 2.7. Id1-luciferase reporter activity

To assess Id1 transcription activity, human myotubes were cultured as described above and were transfected after five days of differentiation with 1 µg of pGL3 BRE Luciferase plasmid DNA, containing two copies of the SMAD binding element present in the ID1 promoter, and cloned into pGL3-MLP-luc minimal promoter vector (gift from Martine Roussel & Peter ten Dijke; # 45126, Addgene, Watertown, MA, U.S.A) using the ViaFect Transfection Reagent (#E4981, Promega, Charbonnières-les-Bains, France). To normalise for intra-well luminescence, 5 ng of renilla pRL-SV40 luciferase plasmid DNA (#E2231, Promega, Charbonnières, France) was added to the transfection mixture. After overnight transfection, human myotubes were cultured in a fresh medium containing 5 % SBS or 5 % WBS for 48 h, with or without a subsequent 24-hour BMP7 treatment (0.5 µg/mL), to assess the effects of bear serum and/or BMP7 ligand on ID1 transcription activity. Cells were then washed with ice-cold 1X PBS and lysed with 250 µL of the passive lysis buffer from Dual-Luciferase® Reporter (DLRTM) Assay System (#E1910, Promega). In the DLRTM system, activities of firefly and renilla luciferase were measured sequentially from a single sample using the plate-reading Synergy™ 2 luminometer (Biotek, Colmar, France) according to the manufacturers' instructions. The firefly luciferase values were normalised by the renilla luciferase values to normalise for translation efficacy.

### 2.8. Statistical analysis

Determination of differentially expressed genes (DEGs) between myotubes cultured with 5 % WBS versus 5 % SBS was conducted using DESeq2, as described above (High-Throughput sequencing paragraph). Except for DESeq2, which was performed using R software (see above), all statistical analyses were performed using Prism 8 (GraphPad Prism 10 v10.3.1, San Diego, CA, USA). Data are presented as means ± SEM with individual values and were analysed for normality of residuals using the Shapiro-Wilk test. No set of data was transformed for non-normal distribution. Protein and mRNA levels in human myotubes cultured with WBS or SBS were analysed using the bilateral paired

Student's *t*-test. Human myotube responsiveness to BMP7 and TGF-β3 ligands, in the presence of WBS or SBS, was analysed using a two-way ANOVA to evaluate the main effects of bear serum type (WBS versus SBS) and ligand treatment (ligands versus untreated), as well as their interaction. Post-hoc analysis was conducted using Tukey's test. Statistical significance was defined for a p-value $< 0.05$.

## 3. Results

### 3.1. Winter bear serum induces transcriptional reprogramming of human myotubes

High-throughput RNA-sequencing was performed on mRNA from primary human myotubes cultured with summer (SBS) or winter (WBS) bear serum. Principal Component Analysis (PCA) shows a good discrimination between the expression profiles of the two groups. Dimension 1 (X-axis) explains more than 50 % of the variance, highlighting a predominant seasonal effect from the bear serum (Fig. 1A). Among the 24,827 transcripts identified in all samples, 352 were differentially expressed genes (DEGs) between human myotubes cultured with WBS and those cultured with SBS (Supplementary Table 1). Among them, 159 were up-regulated, and 193 were down-regulated (Fig. 1B). Gene Ontology (GO) term enrichment analysis revealed 14 molecular functions (MFs) and 104 biological processes (BPs) that were significantly enriched from the DEG list (Fig. 1C-D and Supplementary Tables 2 and 3). Among the identified MFs, "BMP binding" shows the highest enrichment factor. In addition to muscle-related processes, broader categorisation of the enriched BPs also revealed "BMP pathway regulation" and "Response to stimulus" (Fig. 1D). Consensus transcription factors (TFs) analysis highlighted SUZ12, SOX2, CTCF, SMAD4, TP53 and GATA2 as potential regulators of the 352 DEGs (Fig. 1E). Interestingly, within these TFs, SMAD4 is a key regulatory component of TGF-β or BMP signal transduction (Weiss and Attisano, 2013).

### 3.2. Winter bear serum regulates mRNA expression of activators and inhibitors of TGF-β and BMP signalling in human myotubes

We compiled a list of 226 components involved in the TGF-β and BMP signalling pathways from the literature (Supplementary Table 4 and Supplementary References) and explored their expression levels in human myotubes treated with WBS compared with SBS. Overall, DEGs associated with the TGF-β and BMP pathways accounted for less than 5 % and 10 % of the 352 total DEGs, respectively (Supplementary tables 1 and 4). Among the 152 actors of TGF-β signalling, only six genes (~4 %) encoding extracellular and intracellular regulators were differentially regulated between WBS and SBS culture conditions (Fig. 2, Supplementary Table 1, padj $< 0.05$). The antagonists *APOBEC2* and *SESN3,* as well as the transcriptional repressor *HEYL,* were up-regulated by ~1.4-fold, whereas other repressors, such as *PPARGC1A* and the transmembrane co-receptor *ENG*, were down-regulated by ~0.7-fold (WBS/SBS). In addition, the intracellular activator *BTG2 was* up-regulated by ~1.3-fold (WBS/SBS). Among the DEGs, TGF-β target genes were predominantly down-regulated, with 7 genes down-regulated and 4 genes up-regulated in myotubes cultured with WBS versus SBS (Fig. 2, Supplementary Table 1).

Approximately 25 % (25 out of 111) of the BMP pathway regulators compiled above were differentially regulated in human myotubes cultured with WBS compared to SBS (Fig. 3, Supplementary Table 1), with activators and repressors represented in roughly equal proportions. At the extracellular level, most DEGs were down-regulated by 20–50 % in response to WBS versus SBS, including both BMP activators (e.g., *BMP6*, *SFRP1*, *ISLR*) and repressors (e.g., *BMPER*, *GREM1*, *CHRDL2)*. Intracellularly, genes encoding BMP activators were predominantly up-regulated (e.g., *HJV, PARM1*, *FHL3*, and *BTG2*) by approximately 1.3-fold (WBS/SBS), while most genes encoding repressors, including *FMN1*, *SMAD6*, and *HGF*, were downregulated by ~0.7-fold (WBS/SBS). Nevertheless, some activators, such as *ENG* and *SCUBE3*, were down-regulated by 0.5- to 0.7-fold (WBS/SBS), and the *ASB2* repressor was up-regulated by 1.8-fold (WBS/SBS). The BMP target genes were either up- or down-regulated in myotubes cultured with WBS versus SBS (Fig. 3, Supplementary Table 1). We then investigated more specifically mRNA levels for the well-known BMP targets *SMAD6* and *ID3* (Hollnagel et al., 1999; Wu et al., 2021). Fig. 4A-B show that *SMAD6* and *ID3* transcript abundance was lowered by almost 50 % in human myotubes cultured in WBS versus SBS. In addition, the mRNA levels of *ID1*, another well-described BMP target gene, were also reduced (-60 %, $P = 0.053$), as was *ID1* transcription (-34 %) in myotubes cultured in WBS versus SBS (Fig. 4C-D, Supplementary Table 1). We further show that protein levels of the BMP co-receptor ENG and the BMP repressors GREM1 and SMAD6 were also reduced by 40 %, 18 % and 38 %, respectively, in WBS versus SBS conditions (Fig. 5A-D). However, although total and phosphorylated forms of SMAD1/5/9 and SMAD3 remained unchanged in whole-cell extracts (Fig. 6A-E), WBS treatment resulted in a ~50 % reduction in the level of nuclear phosphorylated SMAD3 and, to a lower extent, in the level of nuclear phosphorylated SMAD1/5/9 (-29 %, $P = 0.054$) (Fig. 6-H). Thus, altogether these data show transcriptomic changes related to the BMP and TGF-β signalling pathways under WBS versus SBS conditions.

### 3.3. Winter bear serum reduces TGF-β signalling responsiveness, while maintaining BMP signalling in human myotubes

We then investigated the consequences of this reprogramming on TGF-β and BMP pathways responsiveness to their respective ligands. Human myotubes cultured with WBS or SBS were treated with increasing doses of BMP7 or TGF-β3 ligands. To assess the impact on BMP and TGF-β signalling, the levels of the phosphorylated forms of SMAD1/5/9 and SMAD3 were measured, respectively (Fig. 7A-B). SMAD1/5/9 phosphorylation increased in a dose-dependent manner following BMP7 treatment and showed similar response in both WBS- and SBS-cultured myotubes up to 100 ng/mL. Interestingly, for supra-physiological BMP7 concentrations, SMAD1/5/9 phosphorylation was enhanced in myotubes cultured with WBS, indicating a potentiated response under WBS conditions (Fig. 7A and C). Conversely, the dose-dependent increase in SMAD3 phosphorylation observed in both WBS and SBS conditions remained consistently lower in WBS, even when increasing TGF-β3 concentrations (Fig. 7B and D). Altogether, these data highlight a persistent attenuation of TGF-β signalling response under WBS conditions, while BMP signalling response was preserved.

## 4. Discussion

Hibernating bear serum has been reported to increase protein content and reduce proteolysis in human muscle cells, suggesting that some still non-identified compounds may regulate signalling pathways to prevent muscle atrophy (Chanon et al., 2018; Miyazaki et al., 2022). In this study, we aimed to decipher the mechanisms that may be involved in these changes.

As an initial approach, we performed a large-scale analysis using high-throughput RNA sequencing. Our data revealed a specific transcriptomic program induced in human myotubes cultured in winter-hibernating bear serum, compared to those cultured with summer-active bear serum. Among the 352 Differentially Expressed Genes (DEGs), Hairless (*HR*), a gene involved in heterochromatin maintenance (Liu et al., 2021), was found to be downregulated in human myotubes treated with hibernating bear serum, mirroring its expression pattern in the atrophy-resistant muscle of hibernating brown bear (Cussonneau et al., 2021). Furthermore, our analysis also revealed *SUZ12* and *CTCF* as potential regulators of the 352 DEGs, which is consistent with their established roles in chromatin architecture (Wang et al., 2020). Chromatin organization is a key regulatory process for gene expression

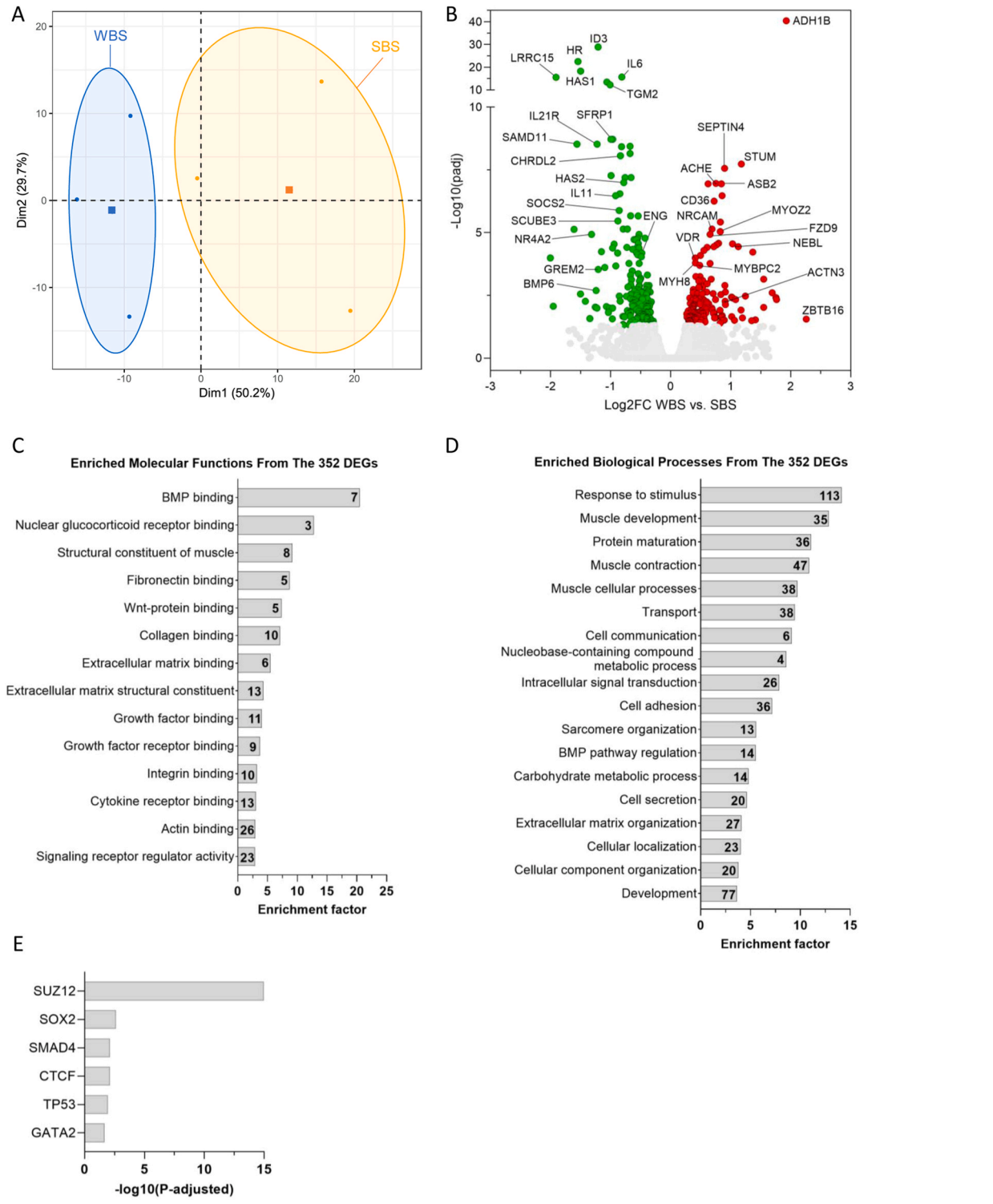



**Fig. 1.** Identification of 352 differentially expressed genes in human myotubes cultured for 48 h in winter-hibernating bear serum. Human primary myotubes from 3 distinct donors were cultured for 48 h with 5 % summer-active bear serum (SBS) or 5 % winter-hibernating bear serum (WBS). (A) Principal Component Analysis of transcriptomic profiles of human myotubes cultured in SBS (orange color) and WBS (blue color) conditions. The barycenter is shown by a square. (B) Volcano plot of the 24,827 identified genes between human myotubes cultured in SBS and WBS. Genes are distributed along the X-axis according to their log2 fold change (Log2FC) and the Y-axis according to the -Log10 of p-adjusted (padj). Differential gene expression was analysed by DESeq2 as described in Materials and Methods and significance was set at padj$< 0.05$. Red dots, up-regulated genes; Green dots, down-regulated genes; Grey dots, $P > 0.05$. (C-D) Graphs representing the enrichment analysis using gene ontology tools performed on the 352 differentially expressed genes (DEGs) (FC W/S $>|1|$ and padj $<0.05$) (Supplementary Table 1) to identify over-represented molecular functions (MF) (panel C, Supplementary Table 2) and biological processes (BP) (panel D, Supplementary Table 3). Bold numbers in the different bars represent the numbers of DEGs found in the enriched terms. (E) Graph representing the transcription factors that potentially regulate the 352 DEGs (EnrichR tool ENCODE and ChEA Consensus TFs from ChIP-X).

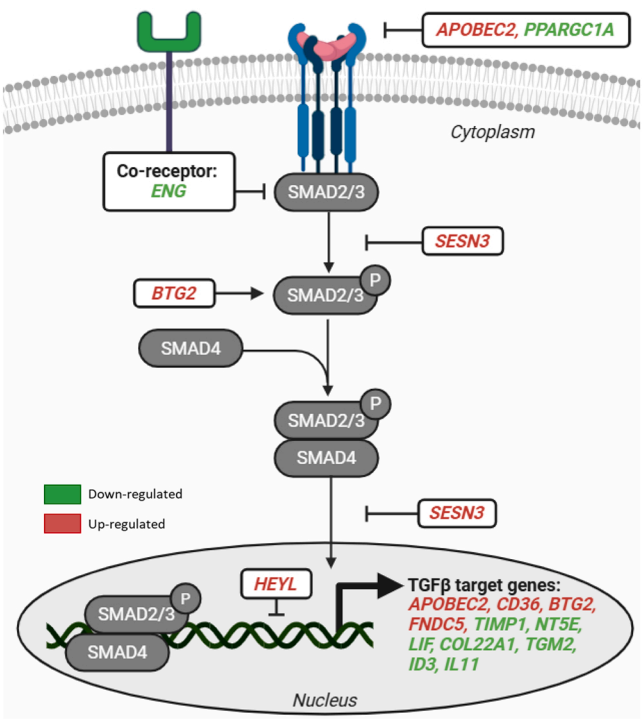



**Fig. 2.** TGF-β signalling components differentially expressed in human myotubes cultured in WBS conditions. Schematic representation of the differentially expressed transcripts (DESeq2, padj <0.05, Supplementary Table 1) involved in the regulation of the TGF-β signalling in human myotubes (n = 3) cultured in WBS versus SBS conditions. Red and green boxes indicate up-regulated and down-regulated transcripts, respectively. Arrows indicate activation; ⊥ bars indicate inhibition. Created with BioRender.com.

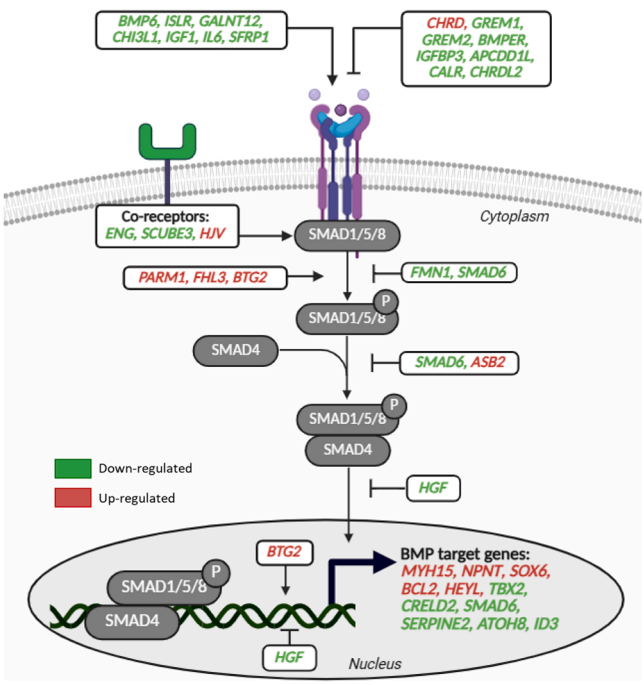



**Fig. 3.** Several BMP activators and repressors were downregulated in human myotubes cultured in WBS conditions. Schematic representation of the differentially expressed transcripts (DESeq2, padj <0.05, Supplementary Table 1) involved in the regulation of the BMP signalling in human myotubes (n = 3) cultured in WBS versus SBS conditions. Red and green boxes indicate up-regulated and down-regulated transcripts, respectively. Arrows indicate activation; ⊥ bars indicate inhibition. Created with BioRender.com.

(Narlikar et al., 2002). Thus, the effect of hibernating bear serum on the transcriptome of human myotubes may involve multiple mechanisms to regulate transcription, including modulation of chromatin structure, as well as additional mechanisms such as mRNA stability and epigenetic modifications, all of which warrant further investigation. All the transcription factors identified as potential regulators of the 352 DEGs were linked to TGFβ and BMP signalling pathways (von Bubnoff et al., 2005; Lee et al., 2006; Dalgin et al., 2007; Van Bortle et al., 2015; Fischer, 2017; Schang et al., 2022; Barbosa et al., 2024). Notably, SMAD4, a central mediator of this signalling, partners with R-SMADs to drive transcriptional changes following phosphorylation (Sartori et al., 2014; Peris-Moreno et al., 2021). Further investigation of DEG promoter regions and factors affecting transcript stability may help uncover the molecular mechanisms driving these transcriptional changes.

For human myotubes exposed to hibernating bear serum, only a few hundred DEGs were present. Thus, the reprogramming of human muscle cells appears to be less pronounced than in the atrophy-resistant muscles of brown bears where thousands of DEGs were found during hibernation (Cussonneau et al., 2021). Unlike hibernating brown bears, which remain inactive for months, human muscle cells in our model were exposed to 5 % bear serum for 48 h as previously described (Chanon et al., 2018). Taken together, the *in vitro* conditions and the interspecies barrier may partly explain the lower number of DEGs. Nevertheless, despite differences in the overall scale of transcriptomic changes, 120 genes were differentially expressed in both the atrophy-resistant muscle of hibernating bears and human myotubes exposed to hibernating bear serum. These shared DEGs represent approximately one third of those identified in human myotubes. Among them, genes up-regulated in human myotubes treated with hibernating bear serum include key components associated with the contractile apparatus (e.g., *MYL6B, ACTN3*, *MYBPC2)* (Dowling et al., 2023), the triad membrane architecture (e.g., *JPH1*, CAV3) (Landstrom et al., 2014) and positive regulators of muscle mass (e.g., *MYOZ2*) (Wang et al., 2018). The shared DEGs also include genes encoding regulators and target genes of the TGF-β and BMP signalling pathways (e.g., *BMPER*, *ENG*, *TIMP1* and *ID3*). This transcriptomic reprogramming in human myotubes therefore echoes our previous findings in the atrophy-resistant muscles of hibernating bears (Cussonneau et al., 2021). These observations align with the previously reported anabolic effect of hibernating bear serum, which was shown to increase protein content in human myotubes (Chanon et al., 2018; Miyazaki et al., 2022).

The fact that the putative transcription factors regulating the 352 DEGs are linked to the TGF-β or BMP signalling reinforces the significant role of these pathways in the effects of hibernating bear serum on human muscle cells. In fact, almost 15 % of the DEGs encode components related to BMP or TGF-β signalling pathways, including activators and repressors of the BMP signalling that are mostly down-regulated. Among DEGs, TGF-β target genes were mostly repressed, whereas BMP target genes were either up- or down-regulated. Some of them are themselves regulators of these signalling pathways, such as the BMP target gene *SMAD6*, which inhibits BMP signalling at multiple levels (Wu et al., 2021) and the TGF-β target gene *APOBEC2*, which is an extracellular repressor of TGF-β signalling (Vonica et al., 2011). Thus, the possibility that amplification loops or feedback mechanisms are induced following exposure of myotubes to hibernating bear serum warrants further investigation. In addition, we show that, beyond the transcriptomic remodelling of TGF-β and BMP pathways, the nuclear localization of the phosphorylated form of the canonical intracellular mediator SMAD3 was markedly reduced by hibernating bear serum, whereas nuclear phosphorylated SMAD1/5/9 was not significantly affected. This is consistent with the predominant down-regulation of differentially expressed TGF-β target genes, in contrast to the nearly equal proportion of down- and up-regulated BMP target genes.

In addition, we had the opportunity to investigate the functional consequences of the regulation of these pathways by bear serum. Upon

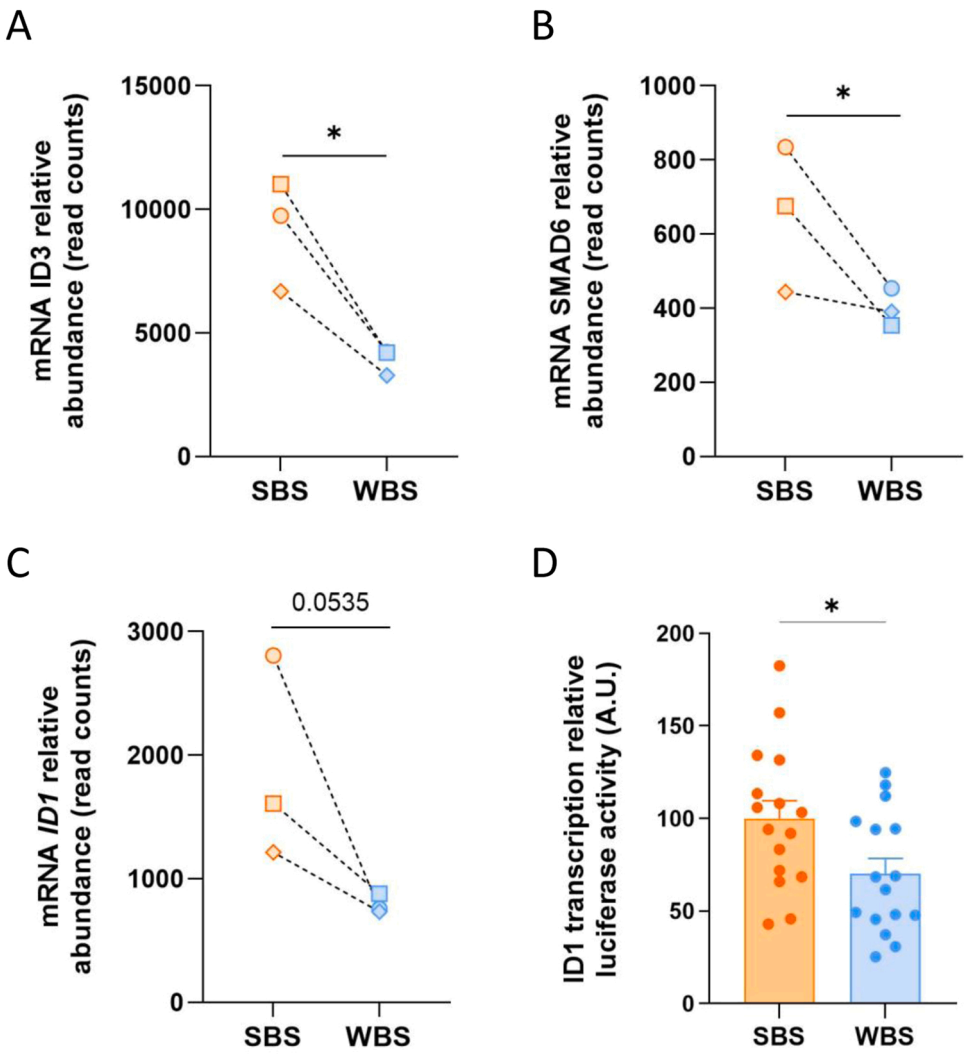



**Fig. 4.** BMP-target genes are downregulated in human myotubes cultured in WBS conditions. (A–C) SMAD6, ID3 and ID1 abundance (read counts) in human myotubes (n = 3) cultured in WBS and SBS conditions. (D) ID1 transcription level assessed in human myotubes (n = 16) cultured in WBS and SBS conditions using the Luciferase ID1 reporting system as described in Materials and Methods. Statistical analysis was performed using DESeq2 analysis for SMAD6, ID3 and ID1 transcript levels (*, p-adj<0.05, panel A-C), and the ratio-paired *t*-test for ID1-luciferase relative activity (*, $p < 0.05$, ratio-paired *t*-test versus SBS, panel D). Data are means ± SEM with individual values. Paired individuals are indicated with different symbols for read counts data. SBS, orange bars; WBS, blue bars. A.U., Arbitrary Units.

stimulation with their respective ligands at physiological concentrations (Grainger et al., 2000; Gore et al., 2014; Olsen et al., 2014; Özdemirel et al., 2022), TGF-β signalling responsiveness decreased in human myotubes cultured with hibernating bear serum, whereas BMP signalling responsiveness was maintained. Again, this is consistent with the changes in nuclear localization of phosphorylated SMAD3 and SMAD1/5/9. We further showed that increasing BMP7 concentration beyond the pathophysiological range enhanced BMP signalling responsiveness in human myotubes cultured in hibernating bear serum conditions. It is noteworthy that supraphysiological concentrations of BMP7 are commonly used across various cell types, including muscle cells (Klatte-Schulz et al., 2016; Egerman et al., 2025). Although supraphysiological blood concentrations of BMP ligands have not been reported under pathophysiological conditions, such levels can be achieved in therapeutic contexts where local administration of BMP ligands is used to promote BMP signalling, for example, to prevent cartilage degeneration or to treat bone repair failure (Calori et al., 2008; Sekiya et al., 2009). Altogether, these data show that even though hibernating bear serum induced larger transcriptomic changes in the BMP pathway versus the TGF-β pathway, the functional consequences were more pronounced for the TGF-β pathway with a marked inhibition. Moreover, although BMP signalling activity appeared slightly reduced by hibernating bear serum, it remained more responsive to stimulation by supra-physiological concentrations of its ligand in myotubes cultured with hibernating bear serum compared to active bear serum. This suggests that the effects of hibernating bear serum on the regulation of TGFβ and BMP pathways involved distinct mechanisms yet to be fully elucidated, such as differences in ligand-receptor affinity and in the levels and activity of extracellular and intracellular inhibitors. In addition, we previously reported that the increased protein content observed in human myotubes cultured with hibernating bear serum was associated with increased AKT phosphorylation (Chanon et al., 2018). AKT has been shown to bind and sequester SMAD3 in the cytosol (Luo, 2017). Thus, increased AKT activity observed following treatment with hibernating bear serum may be a mechanism explaining the reduced TGF-β activity.

TGF-β signalling activation results in muscle atrophy by increasing proteolysis and reducing protein synthesis, while BMP signalling activation has the opposite effect, leading to muscle growth (Sartori et al., 2014). Our data supports the hypothesis that bioactive compounds in

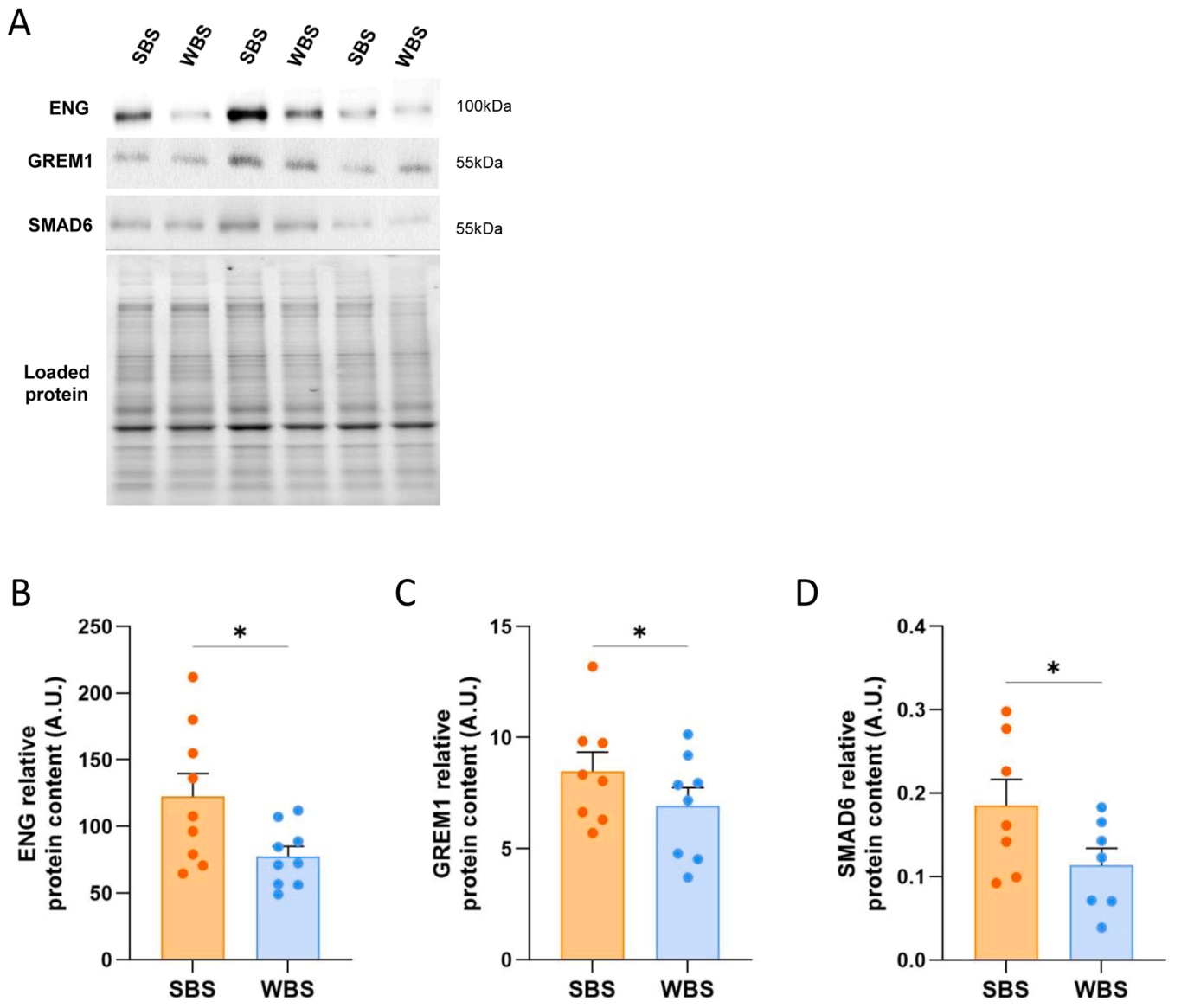



**Fig. 5.** Protein levels for several BMP modulators were lower in human myotubes cultured in WBS conditions. Relative protein levels for ENG, GREM1 and SMAD6 in human myotubes (n = 7–9) cultured in WBS and SBS conditions were measured by Western blotting (A), quantified and normalised to the total protein content (B-D). Total protein loaded is indicated (A, lower panel). Molecular weights are indicated next to the blots. Data are means ± SEM with individual values. *, $p < 0.05$, ratio-paired *t*-test versus SBS. SBS, orange bars; WBS, blue bars. A.U., Arbitrary Units.

hibernating bear serum could (i) limit TGF-β signalling activity under conditions characterized by elevated TGF-β ligand concentrations (e.g., cancers, rheumatoid arthritis, pulmonary arterial hypertension) (Ivanovic et al., 1995; Ivanovicć et al., 2006; Gore et al., 2014; Olsen et al., 2014; Johdi et al., 2017), and (ii) enhance BMP signalling activity in conditions where BMP ligand concentrations are therapeutically elevated beyond the pathophysiological range (Calori et al., 2008; Sekiya et al., 2009). Whether the effects of hibernating bear serum on the regulation of TGF-β and BMP signalling are direct or mediated indirectly through other pathways, remains to be further explored.

Overall, our findings suggest a shift of the TGF-β/BMP balance towards BMP signalling, which may contribute to ensure proteostasis in treated cells, and thus are particularly relevant for future therapeutic applications. Similar mechanisms may occur in vivo in hibernating bears. Pharmacological inhibition of pro-atrophic TGFβ signalling has raised concerns about its applications in humans due notably to a concurrent inhibition of BMP signalling (Suh and Lee, 2020; Teixeira et al., 2020). In addition, the promotion of BMP signalling has gained attention recently, as it has been shown to preserve muscle mass during cancers (Sartori et al., 2021; Mina et al., 2023). Here, our data suggest that compounds within the hibernating bear serum could help mitigate the negative muscular effects observed in clinical trials targeting TGF-β signalling, possibly by preserving BMP activity. Such factors, once identified, may contribute to the development of novel therapies targeting muscle atrophy. The unique modulation of TGFβ and BMP pathways in myotubes exposed to hibernating bear serum could provide a valuable readout for isolating these components. Further investigations would provide significant insight into the underlying mechanisms. Such investigations could include: (i) functional rescue experiments to establish the central role of TGF-β in hibernating bear serum-mediated effects in human myotubes; and (ii) inactivation of TGF-β and/or BMP signalling under hibernating bear serum exposure to help elucidate how these circulating compounds affect muscle protein metabolism. In addition, it would be of interest to determine whether serum from other hibernators, such as ground squirrels, produce comparable effects, potentially revealing a conserved hibernation-related

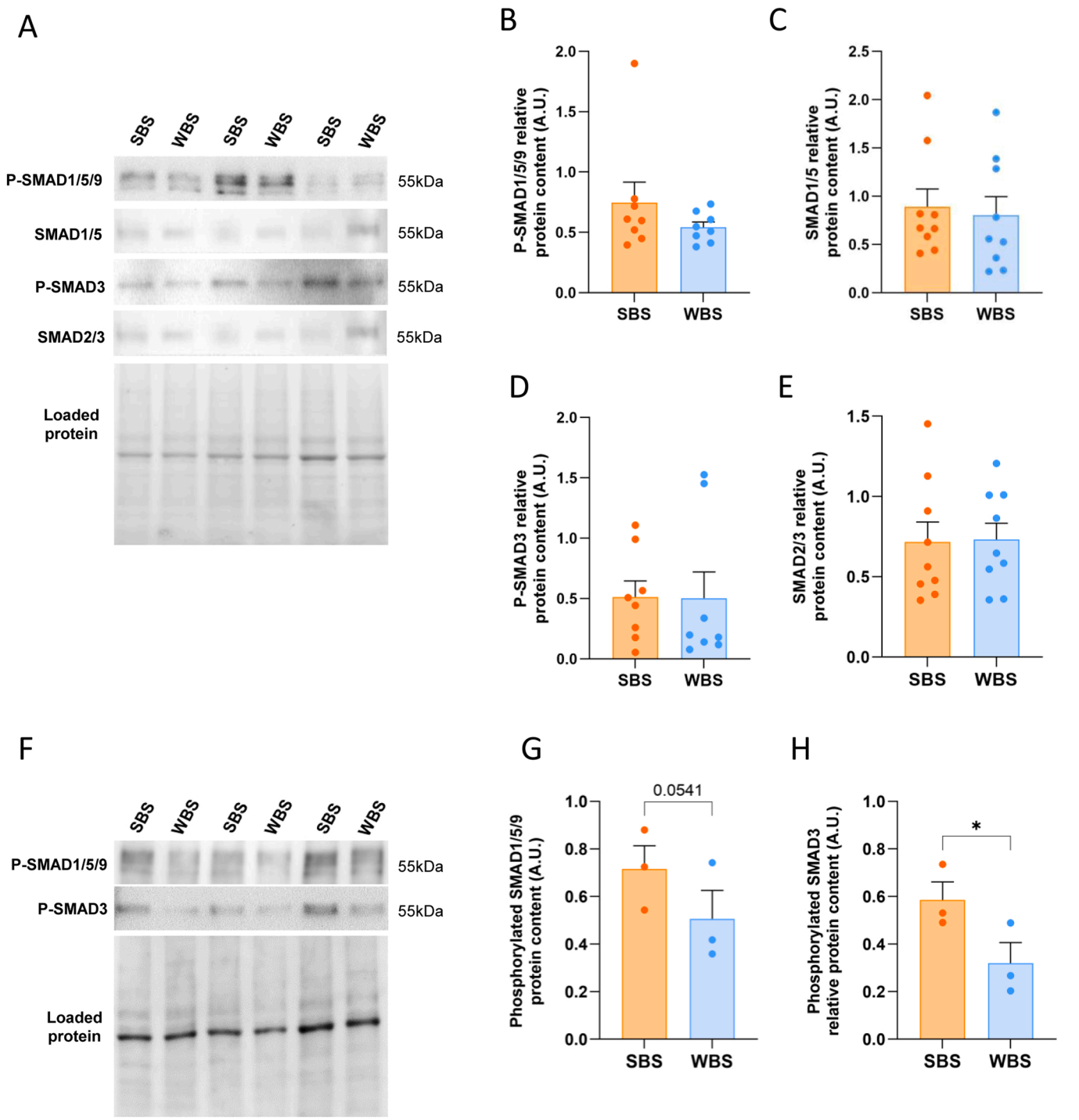
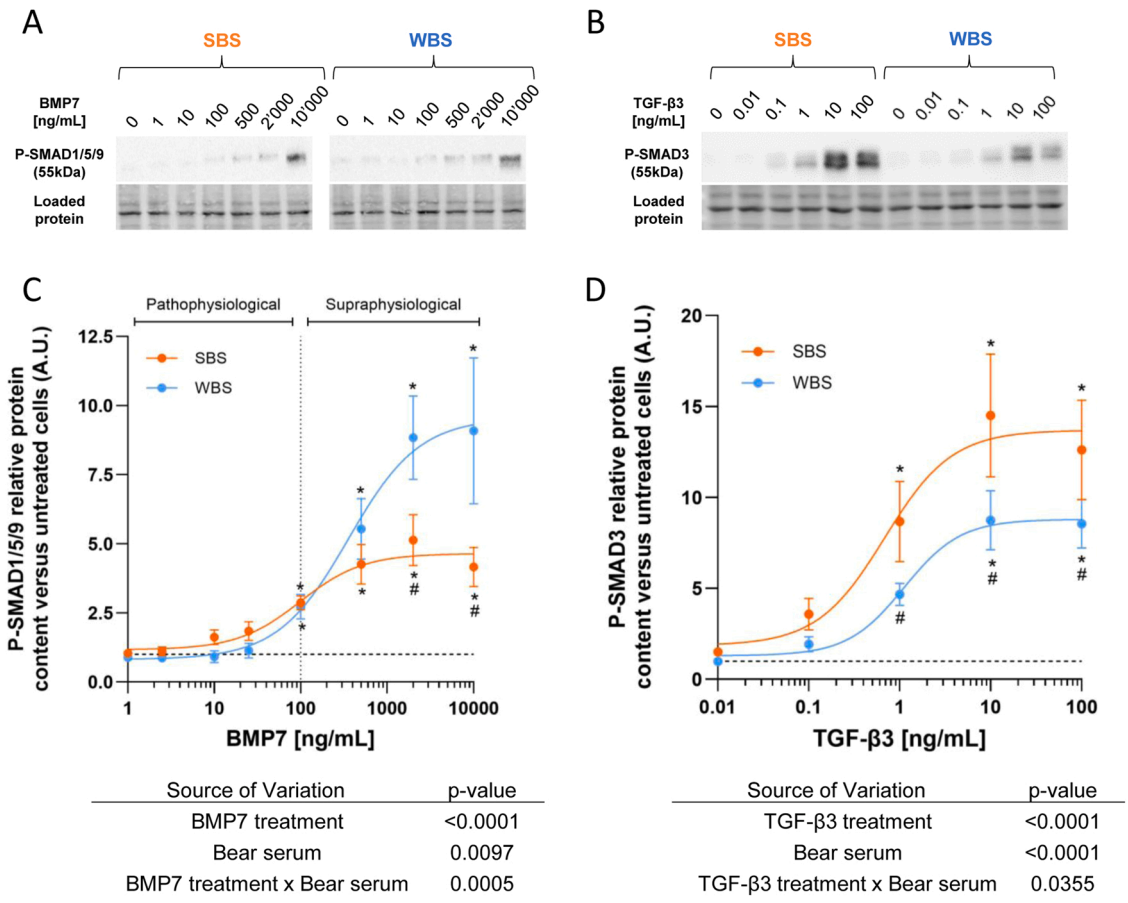



**Fig. 6.** Total and phosphorylated SMADs remained unchanged in human myotubes cultured in WBS conditions. Relative protein levels for phosphorylated SMAD1/5/9 (P-SMAD1/5/9) and SMAD3 (P-SMAD3) in whole-cell extracts (A-E, n = 9) and nuclear extracts (F-H, n = 3), as well as total SMAD1/5 and total SMAD2/3 in whole-cell extracts of human myotubes cultured in WBS and SBS conditions. Protein levels were measured by Western blotting (A, F), quantified and normalised to the total protein content (B-E, G-H). Total protein loading is shown in the lower panels of A and F. Molecular weights are indicated next to the blots. Data are means ± SEM with individual values. *, $p < 0.05$, ratio-paired *t*-test versus SBS. SBS, orange bars; WBS, blue bars. A.U., Arbitrary Units.

signature affecting TGF-β and BMP signalling pathways. Finally, it remains to be determined whether hibernating bear serum can prevent and/or counteract muscle atrophy during catabolic conditions. Addressing these questions will provide important knowledge for the mechanisms of action and the therapeutical potential of hibernating bear serum compounds.

## CRediT authorship contribution statement

**Jonas Kindberg:** Resources. **Lydie Combaret:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Laurent Parry:** Investigation. **Cécile Coudy-Gandilhon:** Investigation. **Fabrice Bertile:** Writing – review & editing, Visualization, Supervision, Project administration,




| Source of Variation | p-value |
|---|---|
| BMP7 treatment | <0.0001 |
| Bear serum | 0.0097 |
| BMP7 treatment x Bear serum | 0.0005 |

| Source of Variation | p-value |
|---|---|
| TGF-β3 treatment | <0.0001 |
| Bear serum | <0.0001 |
| TGF-β3 treatment x Bear serum | 0.0355 |

**Fig. 7.** TGF-β and BMP signalling responsiveness to their ligands is respectively reduced and maintained, or even increased, in human myotubes cultured in WBS versus SBS. Human primary myotubes were cultured for 48 h with 5 % SBS or 5 % WBS and then treated for 30 min with increasing doses of BMP7 (0–10,000 ng/mL) or TGF-β3 (0–100 ng/mL) ligands. (A-B) Relative protein levels for phosphorylated SMAD1/5/9 (P-SMAD1/5/9) and SMAD3 (P-SMAD3) were measured by Western blots to assess for BMP (n = 12) and TGF-β (n = 10) activation, respectively. Total protein loaded is shown. Molecular weights are indicated next to the blots. (C-D) Signals were quantified and normalised to the total protein content. The data for ligand stimulation were normalized to the untreated condition within either the SBS or the WBS culture condition. The horizontal hatched line shows the basal value of 1 in SBS and WBS. The vertical hatched line separates the physiological doses of BMP7 for the supraphysiological ones. Data are represented as dose-response curves for BMP7 and TGF-β3 stimulation in human myotubes pre-conditioned with SBS (orange curve) or WBS (blue curve). All data are means ± SEM. Data were analysed by two-way ANOVA as described in Materials and Methods. "Bear serum effect", "BMP or TGF-β ligand effect" and their interaction are shown in the respective tables below the curves. *, $p < 0.05$, versus untreated cells within either SBS or WBS conditions; #, $P < 0.05$ versus SBS for a same dose of BMP7 or TGF-β3. A.U., Arbitrary Units.

Funding acquisition, Conceptualization. **Gwendal Cueff:** Writing – review & editing, Software, Methodology, Formal analysis. **Etienne Lefai:** Writing – review & editing, Validation, Project administration, Investigation, Funding acquisition, Conceptualization. **Guillaume Fourneaux:** Investigation. **Daniel Taillandier:** Writing – review & editing. **Chloé Richard:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cécile Polge:** Writing – review & editing. **Charlène Pourpe:** Investigation. **Guillemette Gauquelin-Koch:** Writing – review & editing, Supervision, Funding acquisition. **Christophe Tatout:** Writing – review & editing, Software, Methodology. **Evans Alina Lynn:** Resources. **Andrea Miller:** Resources.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.biocel.2025.106864.

## Data availability

Data will be made available on request.

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