Insights into the Transforming Growth Factor superfamily specific modulation: unravelling the impact of winter-hibernating bear serum in primary human muscle cells

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Introduction

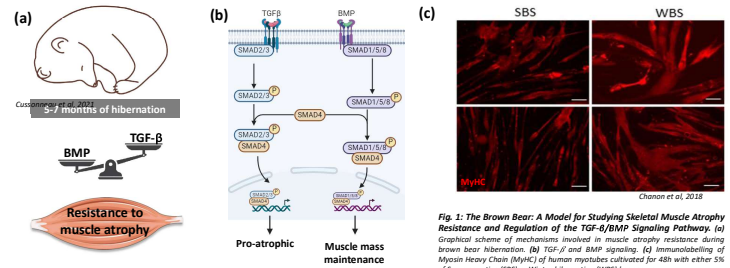

Fig. 1: The Brown Bear: A Model for Studying Skeletal Muscle Atrophy Resistance and Regulation of the TGF-ß/BMP Signaling Pathway. (a) Graphical scheme of mechanisms involved in muscle atrophy resistance during brown bear hibernation. (b) TGF-ß and BMP signaling. (c) Immunolabelling of Myosin Heavy Chain (MyHC) of human myotubes cultivated for 48h with either 5% of Summer-active (SBS) or Winter-hibernating (WBS) bear serum.

Context **Investigating the effects of Winter Bear Serum on human myotubes, focusing on *TGF-ß* and BMP signaling**

Muscle atrophy poses significant challenges in patient care, with **no proven effective treatment available**, despite the huge knowledge acquired using rodent and human models of induced atrophy. We seized opportunity to study a **natural model of resistance to muscle atrophy:** the brown bear. **Despite prolonged fasting and physical inactivity during hibernation**, brown bears do not experience **muscle loss**. This resistance is associated with a shift in the balance between **pro-atrophic TGF-ß signaling and hypertrophic BMP signaling, favoring the latter in muscle from hibernating brown bears** (Fig. 1a,b)**.** Additionnally, bear serum demonstrates **translational effects on human myotubes,** resulting in higher myosin heavy chain protein content in WBS conditions (Fig 1c).

Methodology

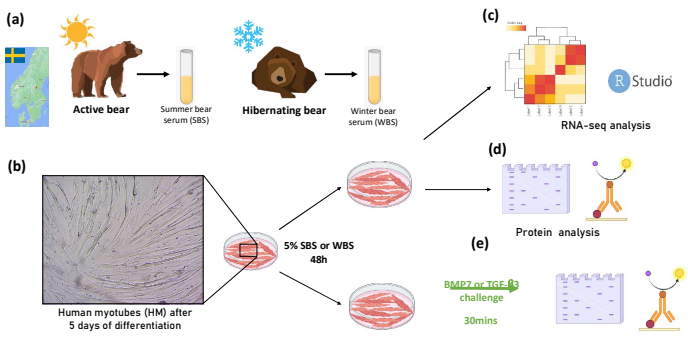

Fig. 2: Experimental procedures. (a) Sampling: Serum samples were collected from paired free-ranging bears during active and hibernation periods (Sweden, Tackåsen). (b) Cell culture: Human myotubes, cultivated in a differentiation medium, were treated for 48h with 5% SBS or WBS. (c) Transcriptomic analysis: Total RNA samples were subjected to DNBSEQ Sequencing (BGI, China). DESeq2 analysis was used to identify differentially expressed genes (DEGs, Padj<0.05), and functional analysis of the DEGs was conducted using Rstudio software. (d) Protein content for some identified DEGs from (c) was assessed using Western blotting. (e) TGF-ß/BMP challenge: Human myotubes cultivated with 5% SBS or WBS for 48h were then treated for 30 min with increasing concentrations of BMP7 (0 to 2000 ng/mL) or TGF-ß3 (0 to 100 ng/mL). SMAD1/5 and SMAD3 phosphorylation (P-SMAD) were analyzed by Western blotting on whole protein content.

Results

Fonctional analysis reveals enrichment of muscle development processes and BMP signaling among DEGs identified in human myotubes in WBS conditions

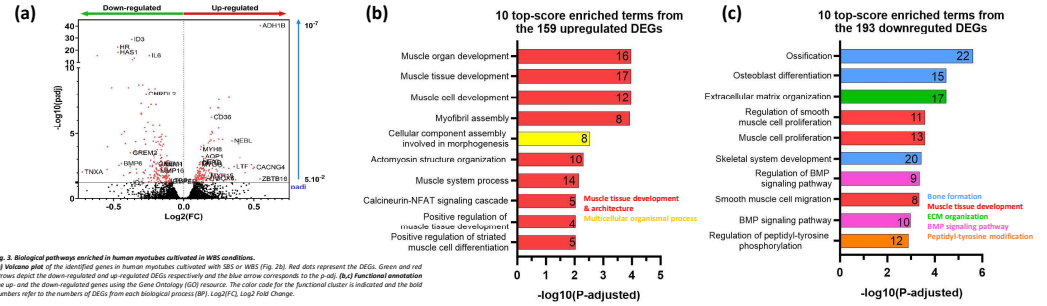

Fig. 3. Biological pathways enriched in human myotubes cultivated in WBS conditions. (a) Volcano plot of the identified genes in human myotubes cultivated with SBS or WBS (Fig. 2b). Red dots represent the DEGs. Green and red arrows depict the down-regulated and up-regulated DEGs respectively and the blue arrow corresponds to the p-adj. (b,c) Functional annotation the up- and the down-regulated genes using the Gene Ontology (GO) resource. The color code for the functional cluster is indicated and the bold numbers refer to the numbers of DEGs from each biological process (BP). Log2(FC), Log2 Fold Change.

Key informations

- Transcriptomic analysis identified **352 DEGs** between human myotubes cultivated with WBS vs. SBS (Fig. 3a). Functional annotation revealed a significant enrichment of multiple biological processes within up and down-regulated genes.
- The **159 upregulated DEGs** are mainly involved in muscle tissue development and sarcomere architecture (Fig. 3b) and the **193 downregulated DEGs** are involved in extracellular matrix organization and BMP signaling pathway regulation (Fig. 3c).

WBS induced an up-regulation of several genes associated with the structural organization of human myotubes

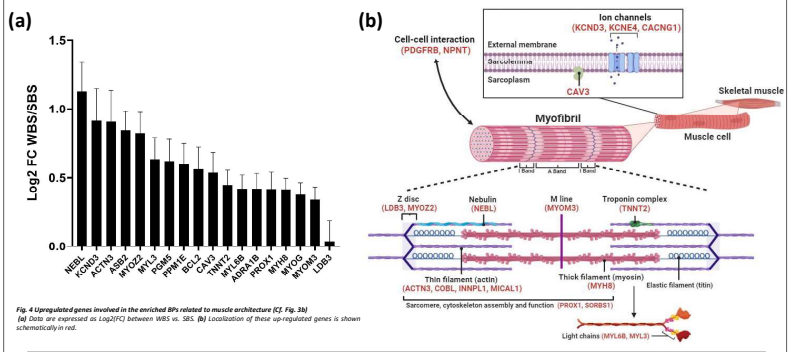

Fig. 4 Upregulated genes involved in the enriched BPs related to muscle architecture (Cf. Fig. 3b) (a) Data are expressed as Log2(FC) between WBS vs. SBS. (b) Localization of these up-regulated genes is shown schematically in red.

These specific genes are involved in the organisation of the sarcolemma and ion exchanges (CAV3, KCND3, KCNE4, CACNG1), sarcomere, cytoskeleton assembly and function (NEBL, MYOM3, TNNT2…), and also in cell-cell interaction (PDGFRB, NPNT).

WBS changes the TGF-β and BMP response to their respective ligand

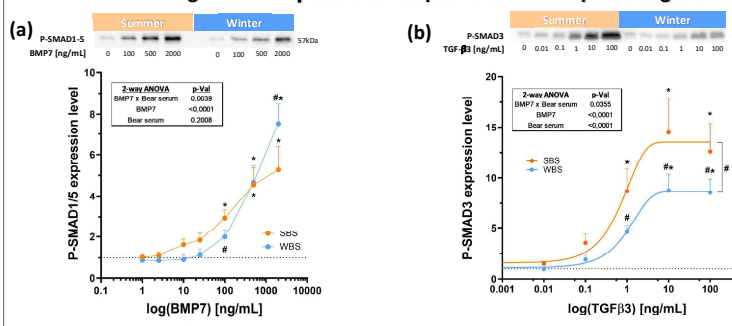

*Fig 6. Effect of bear serum preconditioning on BMP or TGF-ß signalling response to a challenge of human myotubes. TGF-ß/BMP challenge of human myotubes cultured with SBS or WBS were challenged with increasing BMP7 (a) or TGF-ß3 (b) ligands (Fig. 2e). SMAD1-5 (a) and SMAD3 (b) phosphorylation were assessed by Western blotting (upper panels). Signals were quantified and normalized with the amount of loaded proteins (lower panels). *Pval<0.05 vs. untreated myotubes from SBS or WBS conditions; #Pval<0.05 vs. same dose of ligands in SBS condition (n=10-12, 2-way ANOVA, Tukey's post-hoc test). Dot lines are arbitrary set at 1.*

BMP7 and TGF-β3 treatment induces dose-dependent SMAD1/5 and SMAD3 phosphorylation, respectively. BMP7 triggers SMAD1/5 phosphorylation at 100 ng/ml in SBS, but at 500 ng/ml in WBS. However, at high concentration, SMAD1/5 phosphorylation was much higher in the WBS. TGF-β3-induced SMAD3 phosphorylation is consistently lower in WBS compared to SBS.

WBS mainly down-regulates the expression of extracellular and intracellular BMP modulators

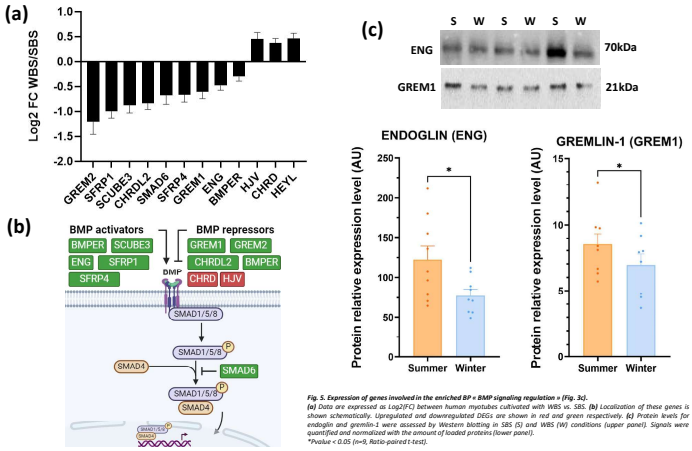

*Fig. 5. Expression of genes involved in the enriched BP « BMP signaling regulation » (Fig. 3c). (a) Data are expressed as Log2FC) between human myotubes cultivated with WBS vs. SBS. (b) Localization of these genes is shown schematically. Upregulated and downregulated DEGs are shown in red and green respectively. (c) Protein levels for endoglin and gremlin-1 were assessed by Western blotting in SBS (S) and WBS (W) conditions (upper panel). Signals were quantified and normalized with the amount of loaded proteins (lower panel). *Pvalue < 0.05 (n=9, Ratio-paired t-test).*

Most BMP signaling inhibitors (e.g. GREM1/2, CHRDL2, SMAD6) or activators (ENG) were downregulated at the mRNA level. Several BMP repressors such as HJV and CHRD were however upregulated (Fig. 5a). Among these regulators, ENG and GREM1 were also downregulated at the protein level.

Conclusion

Overall, our findings indicate a **transcriptomic reprogramming** with an enrichment of biological processes associated to muscle tissue development and **regulation of BMP signaling,** with **down-regulation of several BMP regulators in WBS conditions.**

Challenging the BMP and TGF-β pathways shows a **higher responsiveness of the BMP pathway in myotubes under WBS conditions**, contrarily to the TGF-β signaling which remained consistently weaker.
This suggests that WBS shifts the TGF-β/BMP balance towards the BMP pathway.

Altogether, our results are in agreement with the hypertrophic phenotype observed in human myotubes cultured with WBS *(Chanon et al. 2018)* and the specific TGF-β/BMP balance depicted in atrophy-resistant muscles from the hibernating brown bear *(Cussonneau et al. 2021).*

Further studies will investigate how bear serum induced this reprogramming and how this might influence BMP signaling and/or muscle mass maintenance during catabolic conditions.