

**UNIVERSITÀ DEGLI STUDI
DI MODENA E REGGIO EMILIA**

Dottorato di ricerca in Molecular and Regenerative Medicine

Ciclo XXXII

**Muscle-to-bone crosstalk: identification of Sclerostin as a
putative new myokine**

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Abstract (Italiano)

L'osso e il muscolo striato sono stati riconosciuti come organi endocrini poiché producono e secernono "fattori simil-ormonali" attraverso i quali possono influenzarsi a vicenda ed agire su altri tessuti, dando vita ad un "crosstalk muscolo-scheletrico".

Nel nostro studio, abbiamo utilizzato una linea cellulare miogenica (cellule C2C12) ed una osteogenica (cellule 2T3) per studiare gli effetti dei fattori prodotti dalle cellule muscolari sul processo di maturazione degli osteoblasti.

Abbiamo evidenziato che il medium miogenico ha effetti inibitori sulla differenziazione degli osteoblasti: questo dato ci ha spinto ad analizzare il contenuto dei terreni condizionati da mioblasti/miotubi e, così facendo, abbiamo identificato Sclerostina come una delle miochine prodotte dalle cellule muscolari.

Sclerostina è una glicoproteina secreta espressa principalmente dalle cellule ossee/cartilaginee ed è considerata un regolatore negativo della crescita ossea poiché agisce come antagonista della via di segnalazione Wnt/ β -catenina. Si è scoperto anche che inibisce le prime fasi del differenziamento degli osteoblasti in pre-osteociti in vitro.

Dato il ruolo inibitorio di questa proteina nell'osso, abbiamo approfondito l'analisi dell'espressione di Sclerostina da parte delle cellule muscolari e come essa possa influenzare la formazione e l'omeostasi dell'osso.

Inizialmente, si è caratterizzata e quantificata la sintesi di Sclerostina in mioblasti murini (C2C12) tramite Western Blot, Real Time PCR, Immunofluorescenza ed ELISA. Successivamente, si è passati all'analisi della produzione di Sclerostina muscolare in vivo. Abbiamo esaminato la sua sintesi in muscoli di topi di età diversa: giovani (6 settimane), adulti (5 mesi) e anziani (18 mesi). Per ogni età abbiamo studiato: i) un muscolo composto da fibre fasiche o glicolitiche (Gastrocnemio); ii) un muscolo composto da fibre toniche o ossidative (Soleo), che sono soggette al carico meccanico e al movimento; iii) un muscolo dorsale (Trapezio), con meno carico in ragione dell'attività fasica degli arti, per verificare se il tipo di lavoro muscolare/carico influisce sulla produzione di Sclerostina. Inoltre, è stato isolato un muscolo dell'arto superiore (Tricipite brachiale), per confrontarlo con i muscoli dell'arto inferiore.

Infine, allo scopo di delineare il ruolo di Sclerostina muscolare abbiamo espresso in modo transiente la proteina nei seguenti muscoli: quadricipite femorale, gastrocnemio e tibiale anteriore di giovani topi (2 settimane di età) attraverso l'elettroporazione di un plasmide contenente il gene SOST. Questo esperimento mira a valutare sia gli effetti paracrini della Sclerostina muscolare sui segmenti scheletrici adiacenti sia la sua azione endocrina sull'intero scheletro.

Abstract (English)

Bone and muscle have been recognized as endocrine organs since they produce and secrete “hormone like factors” that can mutually influence each other and other tissues, giving rise to a “bone-muscle crosstalk”.

In our study, we made use of a myogenic (C2C12 cells) and an osteogenic (2T3 cells) cell lines to investigate the effects of muscle cell-produced factors on the maturation process of osteoblasts.

We found that the myogenic medium has inhibitory effects on bone cells differentiation: this datum prompted us to analyze the content of myoblast/myotube-conditioned media and, by so doing, we identified Sclerostin as one of the myokines produced by muscle cells.

Sclerostin is a secreted glycoprotein mainly expressed by bone/cartilage cells and is considered a negative regulator of bone growth due to its role as an antagonist of the Wnt/ β -catenin pathway. It has also been found to inhibit the early stages of in vitro osteoblasts-to-preosteocytes differentiation.

Given the inhibitory role of this protein in bone, we analyzed deeper the expression of Sclerostin by muscle cells and how it affects bone formation and homeostasis.

Firstly, we characterized and quantified Sclerostin synthesis by a murine myoblast cell line (C2C12) by Western Blot, Real Time PCR, Immunofluorescence and ELISA assay.

Next, we moved on to the in vivo muscular production of Sclerostin. We examined its synthesis in muscles of differently aged mice: young (6 weeks), adult (5 months) and old (18 months). For each age we studied: i) a muscle composed of fast/phasic or glycolytic fibers (Gastrocnemius); ii) a muscle composed of slow/tonic or oxidative fibers (Soleus), which are subjected to mechanical loading and movement; iii) a back muscle (Latissimus dorsi), less loaded according to the phasic activity of the limbs, to check if the type of muscular work/load affects the Sclerostin production. Also, a muscle of the upper limb (Triceps brachii) was isolated, to compare it with the muscles of the lower limb.

Finally, we transiently expressed Sclerostin in Quadriceps femoris, Gastrocnemius and Tibialis anterior muscles of young mice (2 weeks of age) via electroporation of a plasmid containing the SOST gene, in order to investigate the effects of a muscle-specific overproduction of the protein. This experiment was aimed at evaluating the paracrine effects of muscular Sclerostin on the adjacent skeletal segments and its endocrine action on the whole skeleton.

Introduction

1. Bone-muscle crosstalk

The musculoskeletal system is a complex apparatus comprised of the skeletal bones, skeletal muscles, tendons, ligaments, cartilage, joints, and other connective tissues that physically and mechanically interact to assure the integrated and sophisticated motor activity in humans.

The coupling of skeletal muscles and bones has long been considered primarily to be a **mechanical relationship** in which bone provides an attachment site for muscles and muscles apply load to bone. This coupling is necessary to support the motor activity as well as to maintain optimal scaffolding function for muscles and other soft tissues. Indeed, bone adjusts its mass and architecture to changes induced by mechanical loading (a bone property called skeletal homeostasis), and the contraction of skeletal muscles is an important element contributing in bone loading. This mechanical perspective implies that as muscle function declines, this would result in decreased loading of the skeleton and would lead to a decrease in bone mass.

However, in the last decade it was discovered that these two tissues function at a higher level through crosstalk signalling mechanisms that are important, in a paracrine mode, for the function of the concomitant tissue ¹. It is now fully evident that muscle and bone produce factors (named myokines and osteokines respectively) that circulate and can act also as endocrine molecules targeting distant tissues (Fig. 1). Despite being a recently outlined field of study, understanding this putative paracrine/endocrine crosstalk and biochemical coupling is an exciting new avenue of research.

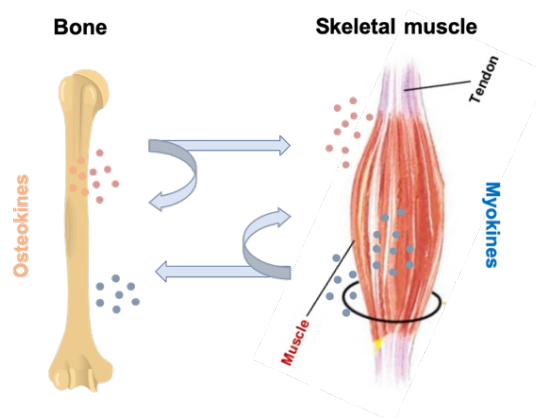


Figure 1. Scheme of the biochemical interplay between bone and muscle

1.1 Bone as a secretory/endocrine organ

The vertebrate skeleton has long been recognized to have important roles such as a structural support essential for motor activity, a protection for the internal organs, a reservoir for calcium and phosphorus and the site of adult haematopoiesis. The skeleton has also been long recognized to be an endocrine target tissue, responding to hormones such as PTH, calcitonin and sex steroids.

However, the concept of the skeleton being an endocrine organ is relatively new. Evidence from several groups has shown that bone cells such as osteoblasts and osteocytes are able to produce and secrete at least three circulating factors: **FGF23**, **osteocalcin** and **lipocalin 2**, which are capable of altering distant tissue function^{1,2}.

Osteocytes appear to regulate phosphate level through molecules such as Phosphate Regulating Neutral Endopeptidase on Chromosome X (PheX), Dentin Matrix Protein 1 (Dmp1), and Fibroblast Growth Factor 23 (FGF23)³. **FGF23** is a key player in the regulation of phosphate and Vitamin D levels in the circulation through its endocrine actions on the kidney to suppress 1,25-dihydroxyvitamin D production⁴. Both Dmp1 and PheX downregulate FGF23 in osteocytes allowing reabsorption of phosphate by the kidney to maintain sufficient circulating phosphate for normal bone mineral content. **Osteocalcin**, produced by osteoblasts, is another bone derived endocrine factor that seems to play an important role in energy metabolism. It is produced uniquely by bone, it targets numerous tissues and is therefore considered as an hormone⁵.

Osteocalcin is produced by the late osteoblast before embedding into osteoid and by embedded osteocytes. Once secreted into the bloodstream, it stimulates insulin secretion in the pancreas, increases insulin sensitivity in adipose tissue and testosterone production in the testis. In addition, osteocalcin promotes monoamine neurotransmitter synthesis and cognitive functions in the brain (Fig. 2).

As far as the skeletal muscle is concerned, the osteocalcin signalling in myofibers promotes the uptake and catabolism of glucose and fatty acids during exercise.

Finally, another bone-specific factor is **Lipocalin 2**, which binds to the melanocortin 4 receptor in neurons of the hypothalamus to inhibit food intake.

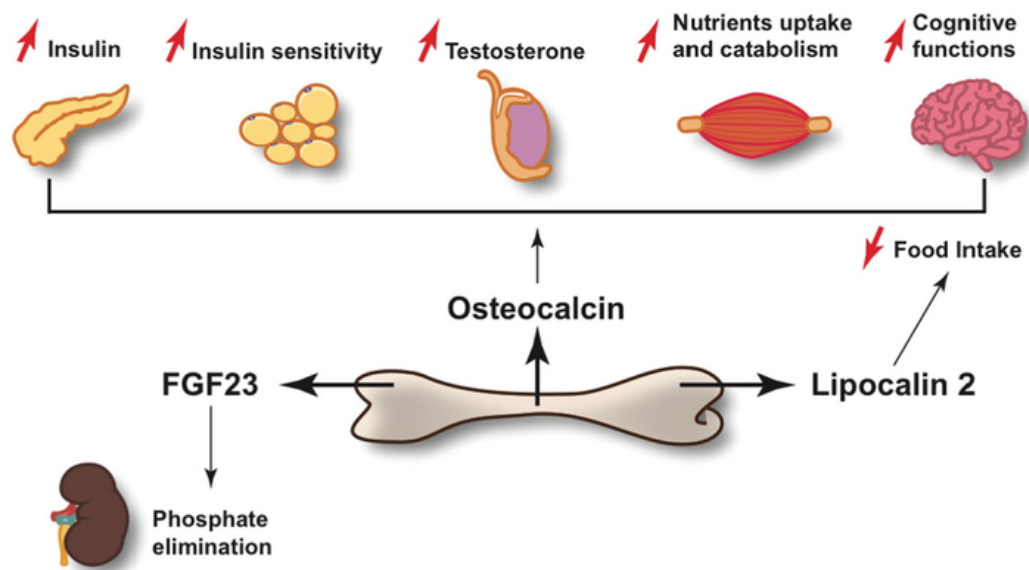


Figure 2. Schematic representation of the physiological functions affected by osteocalcin, lipocalin 2 and FGF23, the three known bone cells-derived hormones.

1.2 Skeletal muscle as an endocrine organ

Muscle fiber is the principal unit of the skeletal muscle and it is able to shorten its length under nervous stimulation. Its development requires the involvement of several proteins and it is promoted by the differentiation and fusion of muscle cell progenitors into myotubes⁶. During physical activity, these cells are subjected to energetic (metabolic) and mechanical (contractile) stimuli that promote the release of myokines, that may alter the function of other tissues⁷ (such as adipose tissue, liver, and pancreatic cells)⁸.

Several myokines are synthesized and secreted during contraction like Fibroblast Growth Factor 21 (FGF-21), Interleukin-6 (IL-6), Interleukin-15 (IL-15), Leukaemia Inhibitory Factor (LIF), Irisin, Myostatin, Angiopoietin-like 4 (ANGPTL4), Brain-Derived Neurotrophic Factor (BDNF), Follistatin-like 1 (FSTL1) and Vascular Endothelial Growth Factor (VEGF). Their expression in skeletal muscle is generally very low, but the levels of some of these myokines increase considerably during muscle contraction (FGF-21, IL-6, IL-15, irisin and BDNF among others)⁹ (Fig. 3).

Exercise and myokine signalling have been linked to brain neurogenesis and cognitive functions through the overexpression of the Brain derived neurotrophic factor (BDNF) responsible for the neurogenesis process^{10,11}.

Myokines that influence adipogenesis include **IL-6**, **myostatin**, **Myonectin**, and **Irisin** and these factors likely exert both local and long-range effects¹².

One of the best-studied metabolic myokines is **IL-6** that seems to promote glucose production in the liver, favours lipolysis in adipose tissue¹³ and promotes pancreatic beta-cell viability and insulin secretion¹⁴.

Irisin is another exercise-induced myokine that influences adipogenesis and metabolism¹⁵. Irisin improves obesity by stimulating white adipose tissue browning and activation of thermogenesis to promote energy expenditure and it has thus been proposed as a therapeutic target for obesity and type 2 diabetes¹⁶.

Myonectin (CTRP15) is a member of the C1q/TNF-related protein (CTRP) family that is secreted by skeletal muscles in response to exercise and nutrients: this factor links the skeletal muscle energy state to lipid homeostasis through its effects on both liver and adipose tissue¹⁷.

As for the myokines that have an impact on the heart, **follistatin like 1** (FSTL1) could have multiple therapeutic effects on the cardiovascular system. Other myokines reported to have salutary effects include **fibroblast growth factor 21** (FGF21), **musclin**, and **apelin** which have been linked to cardioprotection, protection from atherosclerosis and control of blood pressure respectively¹⁸. However, again, future studies are required to determine if skeletal muscle is a major contributor to the circulating levels of these factors.

Very recent studies indicate that also the skin responds to myokines. Physical activity induces **IL-15** release from skeletal muscles that modulates mitochondrial function in skin. Interestingly, IL-15 injections into mice mimic anti-aging effects of exercise both in muscle and skin. Overall, this pioneering study describes a mechanism that explains, at the molecular level, how physical activity slows down skin aging through IL-15-dependent processes¹⁹.

The crosstalk between muscle and bone is very well established as exercise stimulates bone formation and, inversely, muscle loss causes bone loss. Studies have shown that the growth of muscle and bone is controlled by bi-directional biomechanical, paracrine and endocrine signals²⁰. **Insulin like growth factor-1** (IGF1), **fibroblast growth factor-2** (FGF2), **IL15**, **matrix metalloproteinase-2** (MMP2) are released from muscles in response to contraction and positively

contribute to bone formation and maintenance. On the other hand, muscle injury induces release of **myostatin** that in turn impairs bone repair and healing²⁰.

One of the most interesting myokines involved in muscle-to-bone communication is Irisin. Conditioned media from myoblast cultures enhances osteoblast differentiation *in vitro* and this effect is dependent on Irisin²¹. Injection of recombinant Irisin in mice increases cortical bone mass and strength through the suppression of Sclerostin, which is an inhibitor of Wnt signaling²¹.

Despite the big steps ahead research has made in this field, the extent to which muscle-to-bone communication is responsible for relevant conditions such as the twin syndrome of osteoporosis-sarcopenia and aging, still has to be explored.

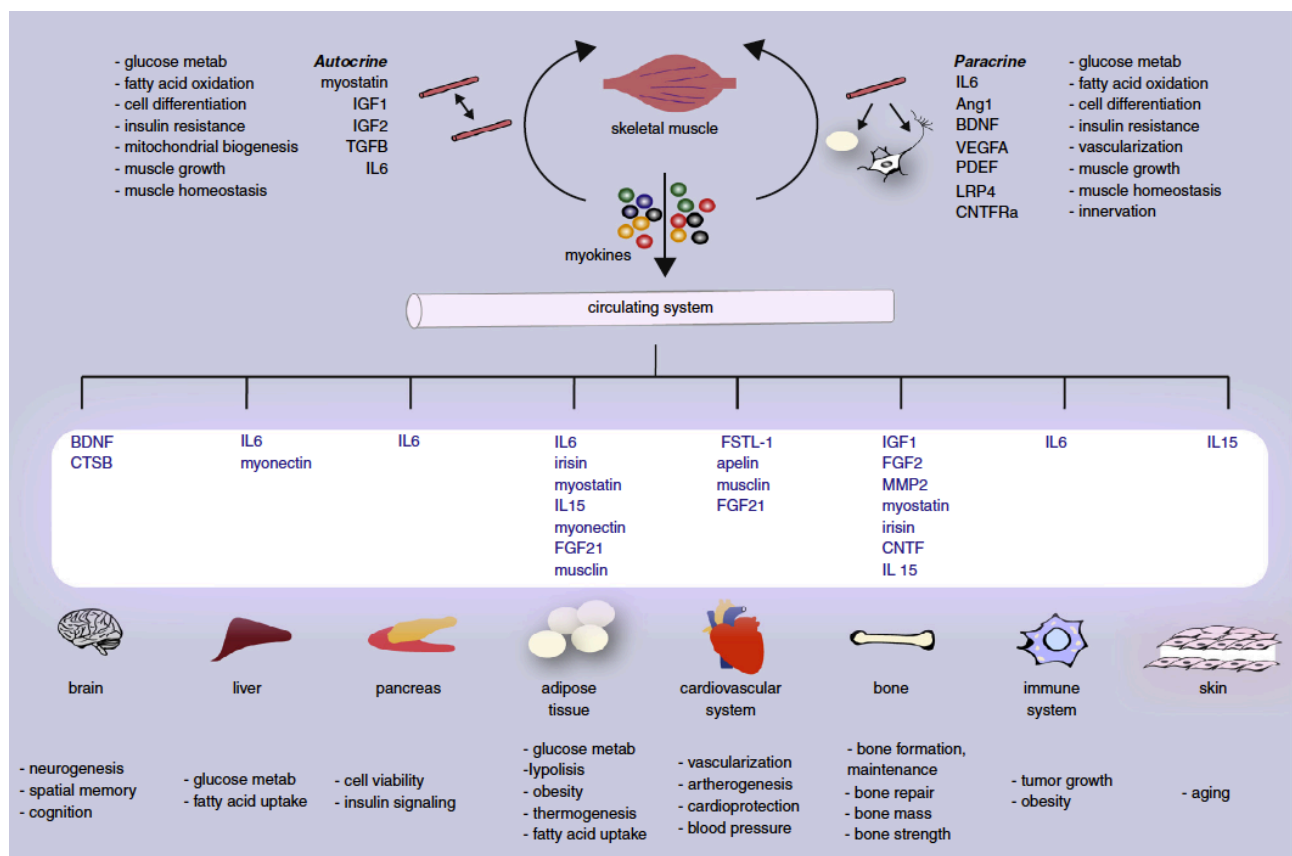


Figure 3. Skeletal muscle as a secretory organ: muscle-derived factors exert autocrine, paracrine or endocrine effects.

2. Musculoskeletal pathologies

Loss of bone and muscle with advancing age represents a huge threat to loss of independence in later life²². **Osteoporosis** is defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture. **Sarcopenia** corresponds to a progressive and generalized loss of muscle mass with either a loss of muscle strength or a loss of physical performance. Notwithstanding a single consensual definition of sarcopenia is lacking. Nevertheless, there is a wide consensus to consider that consequences of sarcopenia, including physical disability, depression, hospitalizations and mortality are linked to direct healthcare costs.²³

During the last decade, bone and muscle were increasingly recognized as interacting tissues, therefore, in this perspective, the “bone–muscle” unit would be the site of privileged exchanges in which the two tissues communicate via paracrine and endocrine signals to coordinate their development and adapt their response to loading and injury^{24,25}.

Growing evidence shows that sarcopenia and osteoporosis share many common pathways including the sensitivity to reduced anabolic hormone secretion, increased inflammatory cytokine activity, and eventually, reduced physical activity.

At this stage, no randomized controlled trial assessing the concomitant effects of the new chemical entity (NCE) on bone and muscle is published. Girgis et al. (2014) suggest that better understanding the interconnected biology of bone and muscle may address the treatment paradigm for musculoskeletal disease to “Kill two birds with one stone” (i.e. treat frail or sarcopenic patients and prevent fractures)²⁴. As potential strategies, the authors recommend either to target pathways that centrally regulate both bone and muscle (e.g. growth hormone, androgens, vitamin D, etc.) or investigate newly emerging pathways that might facilitate the communication between the two tissues (e.g. activin signaling inhibitors, including myostatin-neutralizing antibodies, recombinant follistatin, follistatin derivatives and soluble activin receptors or myokines).

Regular exercise and adequate nutrition are also crucial to optimize bone mass amount and maintain bone and muscle health throughout life.

In this perspective, two intervention trials should be mentioned. Bauer et al. (2015) report, in a 13-week randomized controlled trial, the beneficial effects of a vitamin D and leucine enriched whey protein dietary nutraceutical supplement on muscle mass and lower-extremity function among sarcopenic older adults²⁶. Knowing the importance of vitamin D and proteins for bone health, this nutritional supplement could also be efficient in treating osteoporosis.

In conclusion, the evidence of biochemical and molecular interactions between the two tissues need to be further explored for the development of new chemical entities against these twin conditions of osteoporosis and sarcopenia. Besides, the importance of physical exercise (*i.e.* long-term resistance training) and the need for a balanced diet providing sufficient amounts of proteins, calcium, vitamin D and various micronutrients should not be underestimated.

3. Sclerostin

Sclerostin, the *SOST* gene product, is a 190-amino acid long secreted glycoprotein, which is highly conserved across vertebrate species. *SOST* is expressed in several tissues including bone, cartilage, kidney, liver, heart, lung, pancreas and skeletal muscle²⁷. Despite the fact that *SOST* has been detected in several non-osseous tissues, the last decade of research has primarily focused on *SOST* expression in bone and its function in the skeleton.

In bone, Sclerostin is primarily secreted by osteocytes; however, osteoblasts and osteoclasts also express protein at low levels. Consistent with being a secreted protein, Sclerostin is detected in osteocyte lacunae and along osteocyte canaliculi. *SOST*/sclerostin is a marker of mature osteocytes and its expression progressively increases as osteocytes mature and acquire their full molecular signature²⁸. Sclerostin protein expression is also detected in several other cell types including mineralized hypertrophic chondrocytes, odontoblasts and cementocytes.

3.1 Biochemistry and structure

Following discovery of the *SOST* gene, analysis of the 213 long primary amino acid sequence of its protein product identified Sclerostin as a potential new member of the Dan/Cerberus family of bone morphogenetic protein (BMP) antagonists²⁹. In common with other family members, sclerostin is a

secreted glycoprotein that contains a cysteine knot motif. Sclerostin contains two potential N-linked glycosylation sites, which are conserved across species, and it contains many positively charged lysine and arginine residues.

Expression in mammalian systems yields a mature 190 amino acid protein, which runs as a doublet on SDS-PAGE, likely reflecting differences in the extent of glycosylation³⁰.

The principal features of the Sclerostin structure include N- and C-terminal arms, which are disordered in the free solution state, and three loops which are formed around the cysteine knot motif (Fig. 4).

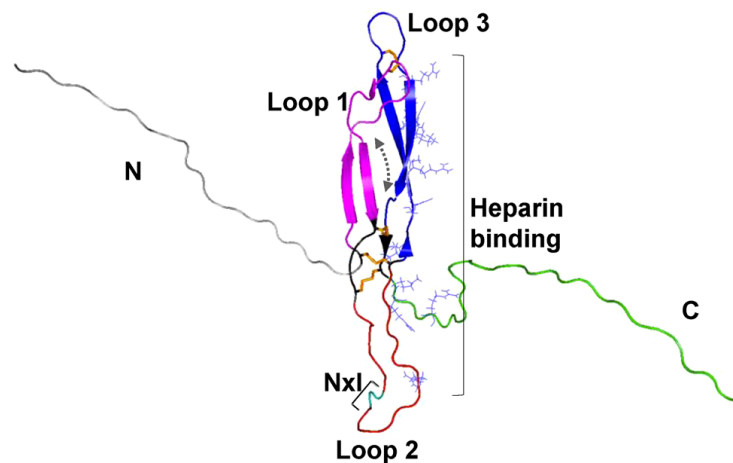


Figure 4. Ribbon cartoon of the sclerostin protein.

3.2 Sclerostin inhibits bone formation through the Wnt/ β -catenin pathway

Sclerostin is a negative regulator of bone formation and it functions by inhibiting Wnt/ β -catenin signaling, which is a major regulator of bone development and metabolism. When Sclerostin binds to the LRP5/6 and Frizzled coreceptors on the cell surface of osteoblasts, Wnt/ β -catenin signaling is inhibited, thereby inhibiting osteoblast differentiation, proliferation, and activity, resulting in reduced osteoblastic bone formation³¹.

The intracellular abundance and location of β -catenin is a central switch in the canonical Wnt signaling pathway. Figure 5 presents a simplified overview which summarizes the key components of the canonical Wnt/ β -catenin signaling pathway in the activated ('Wnt ON') and basal/inhibited ('Wnt OFF') states.

The fate of cytoplasmic β -catenin is controlled by the actions of the multi-protein β -catenin-containing destruction complex, in which AXIN and adenomatous polyposis coli (APC) are associated with disheveled (DVL), β -catenin, casein kinase 1 (CK1) and glycogen synthase kinase 3 beta (GSK3 β), which are constitutively active enzymes that phosphorylate β -catenin.

Canonical Wnt signaling occurs in an autocrine or paracrine fashion and is triggered by binding of Wnt ligand to the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors and Frizzled (FZD) to initiate the formation of a ternary complex ('Wnt ON' in Fig. 5), leading to phosphorylation of the cytoplasmic domain of LRP5/6. Interactions between the intracellular regions of LRP5/6 with AXIN, and FZD with DVL, respectively, localize the destruction complex to the cell membrane, inhibiting the activity of the associated kinases. Non-phosphorylated β -catenin accumulates in the cytoplasm and translocates to the nucleus, where it associates with DNA-bound T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to initiate transcription of *Wnt* target genes³².

In common with other morphogenic pathways, tight control of Wnt pathway activation is required, and one way in which this is achieved is via the existence of multiple secreted antagonists. These may be subdivided into two main classes: those that bind Wnt ligands or those that bind Wnt co-receptors. Sclerostin falls into the latter category.

In the basal or sclerostin-inhibited state ('Wnt OFF' in Fig. 5), β -catenin is constitutively produced and degraded through the actions of the destruction complex. Phosphorylation of β -catenin by CK1 and GSK3 β labels it for ubiquitination and proteasomal degradation, preventing its accumulation and nuclear translocation. In the absence of nuclear β -catenin, TCF/LEF is associated with the transcriptional repressor Groucho and is maintained in an inactive state.

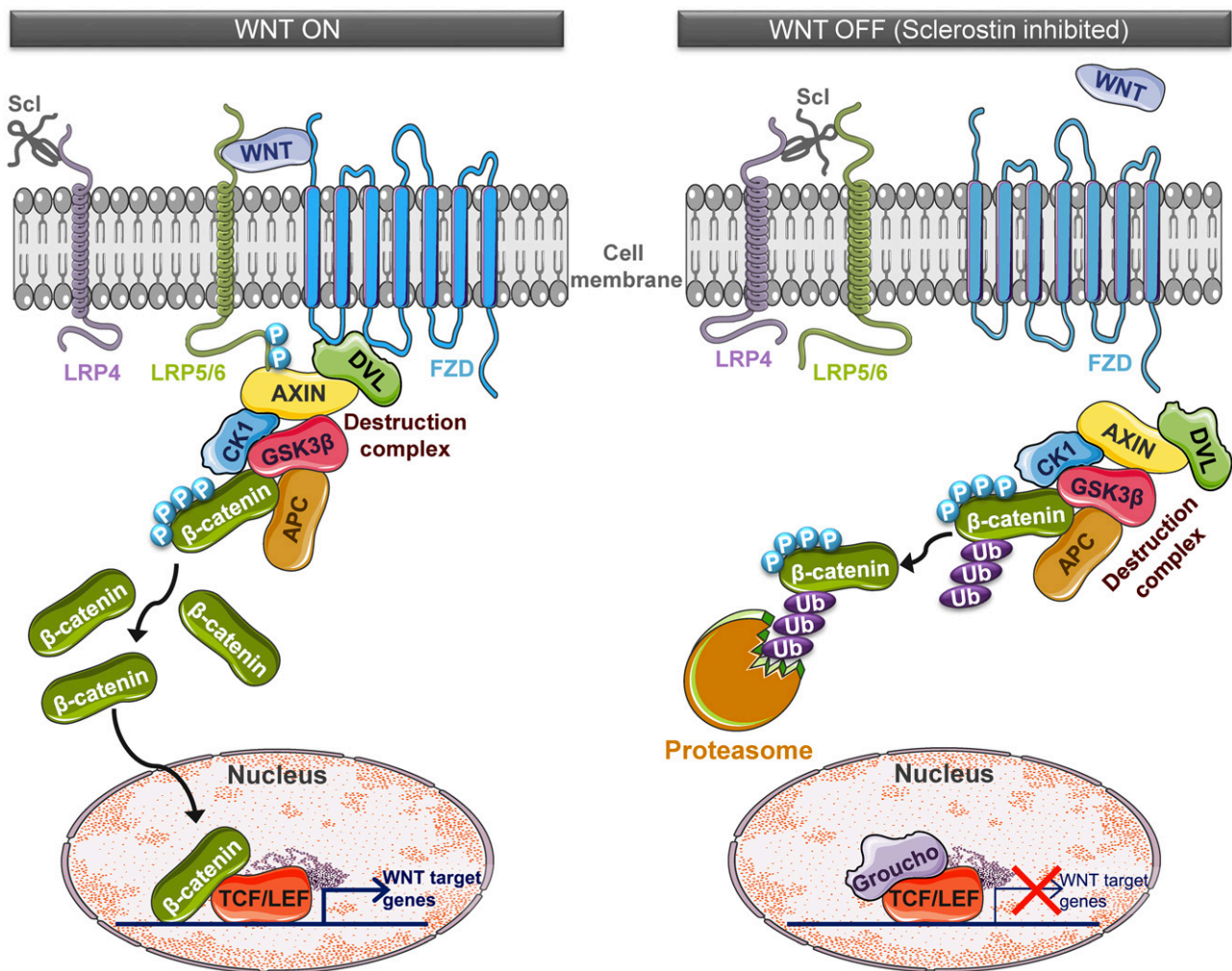


Figure 5. A simplified overview of the canonical WNT signaling pathway, and its inhibition by sclerostin³².

3.3 Cellular level actions of Sclerostin revealed from *in vitro* and *in vivo* studies

Sclerostin directly modulates the osteoprogenitor population and regulates osteoblast lineage function. *In vitro*, Sclerostin inhibits osteoblast proliferation, decreases alkaline phosphatase activity and mineralization and increases apoptosis in osteogenic cultures of mouse and mesenchymal stem cells or human primary osteoblasts³³.

Insights into Sclerostin's action as a regulator of osteoprogenitor differentiation, recruitment and mineral apposition have come from rodent studies using Scl-Ab. Neutralization of Sclerostin enhances proliferation and recruitment of osteoprogenitor cells to active surfaces, increases bone formation and osteoblast number, leading to increased bone formation per osteoblast³⁴. The

transition of late osteoblasts to early osteocytes is inhibited *in vitro* by Sclerostin, suggesting a role for Sclerostin as a regulator of osteocyte maturation.

The study of Sclerostin biology in osteocytes is challenging and the nature of the osteocyte makes it difficult to best study it as a cell within its lacuna, surrounded by its native 3D mineralized environment. Isolation and culture of primary osteocytes from bones is technically difficult and typically yields few viable cells; at the same time, the few immortalized osteocyte cell lines currently available show variable Sclerostin expression.

The early osteocyte cell lines, MLO-Y4 and MLO-A5 were derived from a transgenic mouse, which expressed large T-antigen under the control of the osteocalcin promoter. Sclerostin is a late osteocyte marker and is not highly expressed in MLO-Y4 and MLO-A5 cells. Two additional cell lines, IDG-SW3 and Ocy454, were isolated from double transgenic mice expressing green fluorescent protein (*Gfp1*) under the dentin matrix acidic phosphoprotein 1 (*Dmp1*) promoter as well as a thermolabile large T-antigen. Importantly, both cell lines during the osteogenic differentiation resemble late osteocytes and hence express high levels of late osteocyte markers, including Sclerostin³⁵.

Sclerostin is a regulator of osteocyte apoptosis. *In vivo*, loss of *SOST* leads to enhanced canonical Wnt signaling and decreased apoptosis in osteocytes.

The ability of osteocytes to resorb mineral matrix is termed “osteocytic osteolysis”. Although this remains a controversial topic, studies in lactating animals suggest that osteocytes may be able to remove the peri lacunar matrix thereby mobilizing calcium. Sclerostin has been reported to upregulate expression of several proteins involved in this process. The treatment of primary human osteocyte-like cells or mouse MLO-Y4 cells with Sclerostin increased expression of transcripts encoding carbonic anhydrase 2 (*Ca2*), cathepsin K (*Ctsk*) and tartrate-resistant acid phosphatase form 5b (*Acp5*) in an LRP4/5/6-dependent manner whilst addition of Sclerostin to *ex vivo* explant cultures of human cancellous bone increased lacunar size³⁶.

The importance of Sclerostin in the maintenance of osteocyte morphology and connectivity is indicated from studies using genetic or antibody approaches. Mice lacking the matricellular protein Periostin display increased Sclerostin expression and round-shaped osteocytes with decreased dendrite number and length. Restoration of osteocyte morphology to a well-connected almond-like

morphology was observed when the *SOST* gene was deleted or Scl-Ab was used to neutralize Sclerostin function³⁷.

Together, these findings indicate that the action of Sclerostin as an inhibitor of bone formation is achieved through the osteoblast lineage: control of osteoprogenitor cell proliferation and recruitment, inhibition of osteogenic commitment and differentiation, negative regulation of osteoblast activity, suppression of late osteoblast differentiation into osteocytes and regulation of osteocyte longevity, shape and connectivity.

While Sclerostin does not seem to directly regulate the differentiation or activity of osteoclasts, studies of cells isolated from animals deficient in Sclerostin or treated with Scl-Ab prove that Sclerostin does have important indirect effects on the osteoclast lineage.

The binding of RANKL to its cognate receptor, receptor activator of nuclear factor- κ B (RANK) on osteoclast precursors promotes osteoclastogenesis: Sclerostin has also been shown to stimulate the production of RANKL from osteocytes, thereby enhancing osteoclastogenesis³⁸.

Differentiation of MSCs into chondrocytes is inhibited when canonical Wnt signaling is activated. The effect of Sclerostin on chondrocytes is relatively poorly studied; however, it is known that the protein is expressed by hypertrophic chondrocytes in the growth plate and by articular chondrocytes³⁹.

The role of Sclerostin in regulating bone formation and resorption is detailed in Fig. 6, where its effects on the different bone cell types are outlined.

The function of Sclerostin as an inhibitor of bone formation has been further demonstrated in transgenic mice. Sclerostin knock out (SOST-KO) mice display a high bone mass with increased bone formation and bone strength⁴⁰, whereas overexpression of Sclerostin in mice results in low bone mass with decreased bone formation and bone strength⁴¹.

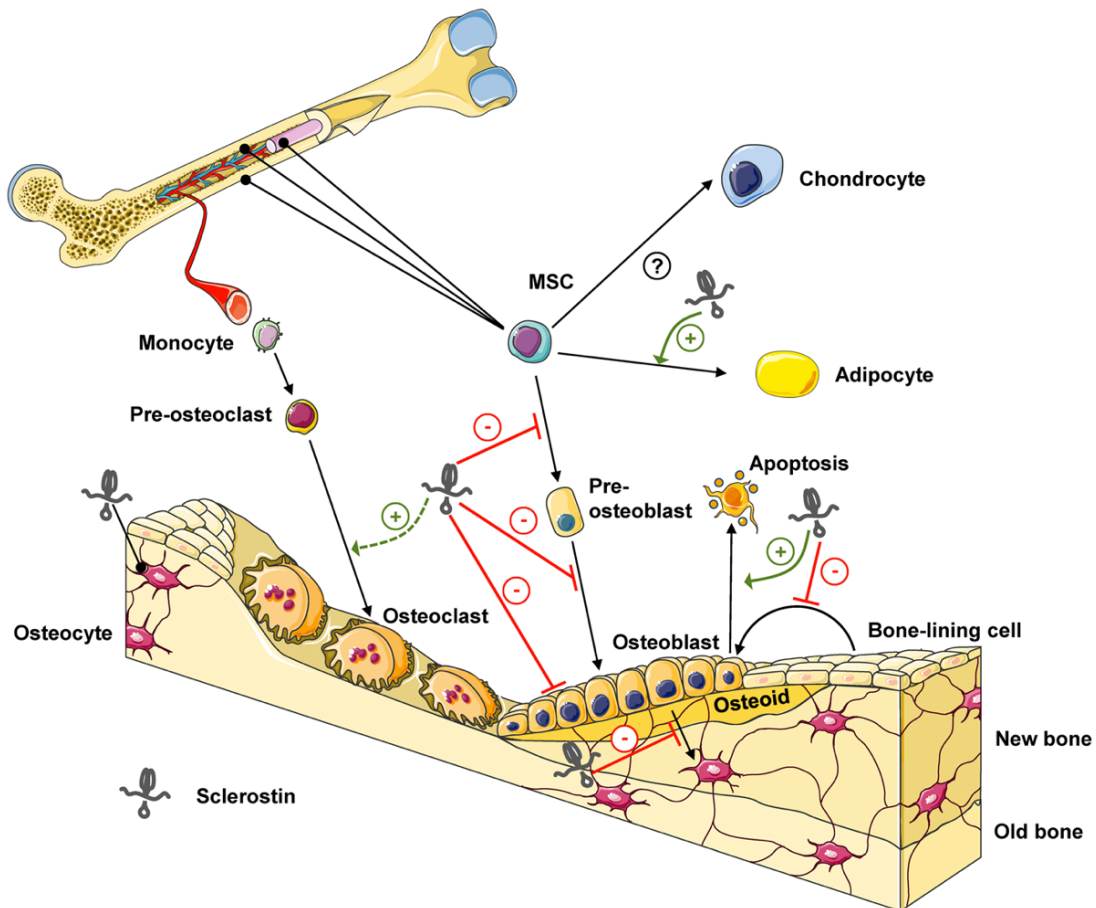


Figure 6. Effects of Sclerostin on bone formation and resorption.

3.4 Human conditions related to mutation of the *SOST* gene

Sclerosteosis is a very rare autosomal recessive disorder that principally affects Afrikaners in South Africa, with a few sporadic cases reported in other world regions⁴². It is characterized by progressive bone thickening, tall stature, syndactyly, enlargement of the skull, and foraminal stenosis resulting in entrapment of cranial nerves with facial palsy, with increases in intracranial pressure. Affected individuals usually appear normal at birth, with the possible exception of syndactyly; other manifestations begin early in life.

It is caused by loss of function mutations of *SOST*, a gene located on the chromosomal region 17q12-21, resulting in decreased production of Sclerostin by osteocytes. As a consequence, osteoblastic bone formation is increased due to enhancement of Wnt/ β -catenin signaling.

Van Buchem disease is a rare related autosomal recessive genetic disorder, principally reported in a fishing village in The Netherlands. It is caused by a 52 kb deletion in the same chromosomal region

as *SOST*, resulting in downstream impairment of *SOST* function and defective Sclerostin production⁴³. The skeletal phenotype of individuals with van Buchem disease is similar but less severe than those with sclerosteosis, with normal stature and no syndactyly⁴⁴.

4. Therapeutic applications of sclerostin inhibition

Evidence from human disorders of Sclerostin, such as sclerosteosis and van Buchem disease, and findings obtained by animal studies investigating the role of Sclerostin in Wnt/ β -catenin signaling, have led to consideration of **Sclerostin** as a potential target for the treatment of osteoporosis and other skeletal diseases associated with low bone mineral density (BMD) and increased fracture risk. This is based on the concept that an anti-Sclerostin compound would block an inhibitor of Wnt signaling, thereby acting to enhance Wnt signaling and stimulate osteoblastic bone formation (Figure 7). Investigational humanized Sclerostin monoclonal antibodies of particular interest include **romosozumab** (codeveloped by Amgen, USA, and UCB, Belgium) and **bloszumab** (Eli Lilly and Company, USA). **BPS804** (Novartis, Switzerland) is another anti-Sclerostin compound that is in the early stages of development.

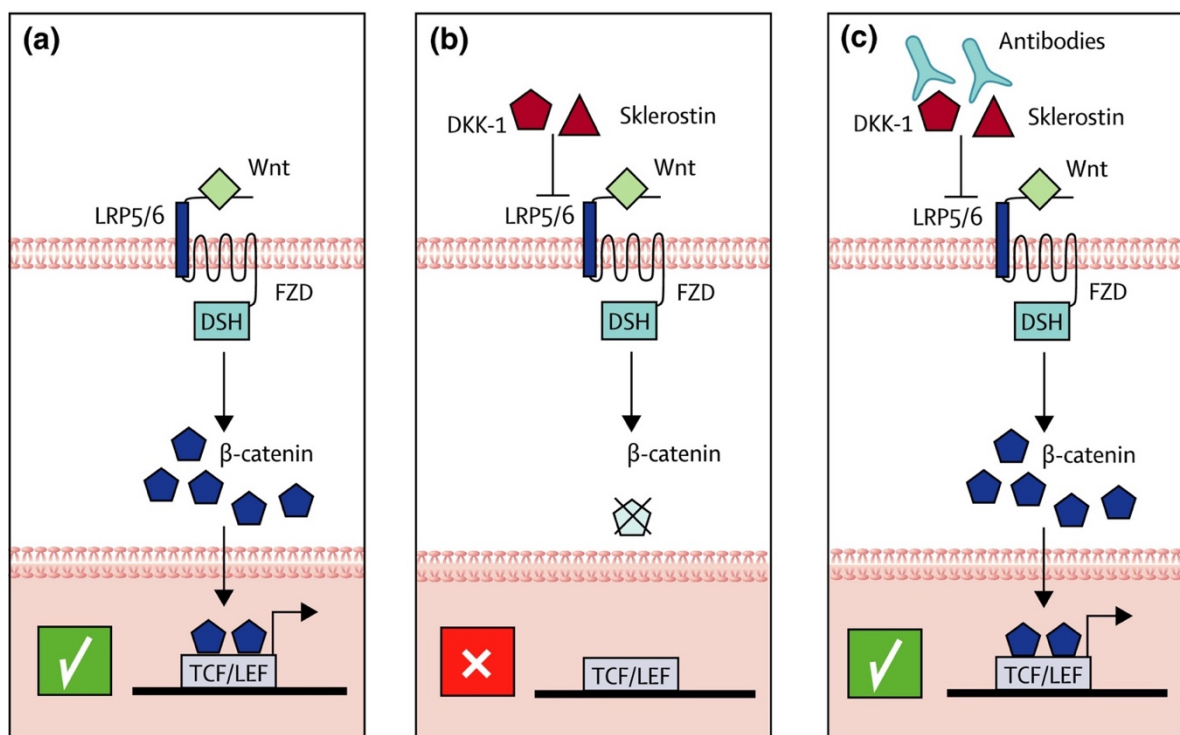


Figure 7. The mechanisms of action for sclerostin antibody inhibition. **A)** β -catenin translocates into the nucleus once Wnt binds to the LRP5/6 receptor. Translocated β -catenin then binds to transcription factors TCF/LEF and stimulates transcription of osteoblast genes, leading to stimulated bone formation. **B)** Endogenous Wnt inhibitors sclerostin and DKK-1 prevent Wnt signal transduction, leading to inhibition of β -catenin translocation to the nucleus, leading to reduced bone formation. **C)** Antibodies to Sclerostin and/or DKK-1 neutralize Wnt inhibitors, leading to increased osteoblastic activity and restored bone formation.

4.1 Preclinical studies

The therapeutic potential of antibodies directed against Sclerostin was first evaluated in preclinical studies. The administration of an anti-sclerostin antibody over 5 weeks in ovariectomized rats led to pronounced increases of bone mass and strength at several skeletal sites⁴⁵. These effects were reproduced in ovariectomized cynos treated with two once-monthly injections of an anti-Sclerostin antibody⁴⁶. Another study in male cynos highlighted the potential of anti-Sclerostin antibodies to improve trabecular microarchitecture⁴⁷. These animal studies have contributed to the elucidation of anti-Sclerostin antibodies' mechanism of action.

4.2 Romosozumab

Romosozumab is a monoclonal antibody against Sclerostin. The antibody is humanized, that is non-human, but the amino acid sequence is modified to increase the similarity with a human antibody. This drug is administered subcutaneously and it represents a promising approach for the prevention of fractures.

Its clinical potential for fracture prevention in postmenopausal osteoporotic women has been investigated in two recent phase III clinical trials, **FRAME** (Fracture Study in Postmenopausal Women with Osteoporosis)⁴⁸ and **ARCH** (Active-Controlled Fracture Study in Postmenopausal Women with Osteoporosis at High Risk)⁴⁹.

Another clinical trial, (named **STRUCTURE**), compared the effects of Romosozumab and Teriparatide on BMD and bone strength in postmenopausal women previously treated with bisphosphonates.

In summary, current efficacy data of Romosozumab appear convincing, and the utilization of this drug followed by a potent antiresorptive compound will probably ameliorate existing therapeutic options for severe osteoporosis.

4.3 Additional Sclerostin antibody trials

Additional Sclerostin antibodies are in clinical development and have been tested in early clinical trials.

BPS804 is a fully human, neutralizing monoclonal antibody directed against Sclerostin. This compound has been investigated in patients with hypophosphatasia. Eight patients were treated with three ascending doses of **BPS804** and followed for 16 weeks after the last dose. Treatment was generally well tolerated and transient increases in bone formation markers alongside with transient decreases in bone resorption markers were seen. At the end of the study, BMD at the lumbar spine had increased by 3.9%⁵⁰. In another phase II trial **BPS804** was tested in nine patients with moderate osteogenesis imperfecta and compared to five control patients. At day 43, all measured markers of bone formation were significantly increased to baseline. Lumbar BMD was increased by 4% at day 141 ($P = 0.038$)⁵¹.

Blosozumab is another sclerostin antibody that has been tested in a phase II trial⁵². In this dose-ranging study, increasing subcutaneous doses of **Blosozumab** were given. Dose-dependent increases in BMD were seen in spine, femoral neck and total hip BMD as compared with placebo. There is currently a clear unmet need for additional bone anabolic agents for the treatment of osteoporosis. With **Romosozumab** and **Abaloparatide** having successfully completed phase III trials in postmenopausal osteoporosis, these agents are in the process of obtaining approval or, in the case of Abaloparatide, have already been approved by the FDA. All presented trials have transitioned into an antiresorptive sequence therapeutic regimen, and there is a general consent that an antiresorptive therapy is required to maintain and stabilize bone improvements gained under anabolic therapy.

Further studies will help better define the safety profiles and long-term effects of these agents, as well as their efficacy in settings other than postmenopausal osteoporosis.

To this end, Scl-Ab could prove useful for stimulating bone formation in Multiple Myeloma (MM), a disease characterized by the uncoupling between bone resorption and osteoblast formation that results in severe inhibition of bone formation⁵³.

Numerous reports demonstrated that Sclerostin plays a pivotal role in MM bone alterations, thus supporting the use of anti-Scl therapy for the treatment of skeletal disease⁵⁴. Indeed, a combination therapy of Scl-Ab and zoledronic acid and other anti-MM drugs has been tested. Delgado-Calle et al. (2017) demonstrated that Scl-Ab therapy did not impact negatively the anti-MM efficacy of Bortezomib and Dexamethasone *in vitro*⁵⁵, while others reported a superior effect of Scl-Ab combined with Zoledronic Acid in increasing bone volume and resistance to fracture *in vivo*⁵⁶.

These data underline the importance of targeting Sclerostin to improve bone disease in patients with MM and emphasize the efficacy of therapies combining Scl-Ab, anti-MM drugs and antiresorptive agents, that should to be further evaluated in clinical studies.

Aim of the work

Bone and skeletal muscle are tightly coupled, both mechanically and biochemically and several efforts have been made to model *in vitro* the communication between these two tissues. To date, the bi-directional flow of signals between bone and muscle has been investigated experimentally by differentiating bone progenitor cells in a medium conditioned by myotubes⁵⁷ or by differentiating skeletal muscle progenitor cells in a medium conditioned by osteocytes⁵⁸. These studies have demonstrated that osteocyte- and myotube-secreted factors (osteokines and myokines, respectively) have a reciprocal inhibitory influence on myogenesis and osteogenesis, since they decrease the majority of the mRNA levels of genes associated with differentiation.

However, these works identified some myokines/osteokines that only partially mimic the inhibition of osteogenesis/myogenesis seen with conditioned media. Therefore, further investigation is needed to expand our knowledge on the molecular mechanisms through which bone and skeletal muscle communicate.

In particular, since there is a growing urgency to identify myokines so that these factors may be exploited therapeutically to benefit both the muscle and skeletal systems, particularly in old age²⁰, we decided to focus on the signaling of myokines on bone progenitors.

In the first part of this study, we propose to model the muscle-to-bone crosstalk by differentiating 2T3 osteoblastic cells in a medium conditioned by early (day 1), mid (day 7) or late (day 14) C2C12 muscle cells, to establish the effect of sequential patterns of myokines.

Next, the study proceeds with the identification of the proteomic profile of the conditioned medium, to identify the myokines most abundantly expressed during C2C12 myogenic differentiation. Interestingly, we found that one of the myokines is **Sclerostin**, a secreted glycoprotein that has been known to be secreted by terminally differentiated cells embedded within a mineralized matrix (i.e. osteocytes in bone), hypertrophic chondrocytes in cartilage and cementocytes in teeth⁵⁹.

Besides, *SOST* mRNA expression in unmineralized tissues has been detected during mouse embryogenesis in the otic vesicle and peridigital or interdigital regions of the limb buds⁶⁰; whereas in humans, *SOST* mRNA is expressed in the heart, aorta, and liver, with high levels in the kidney⁶¹;

but no sclerostin protein has been detected in these organs. Therefore, there is a clear need to further investigate the SOST/Sclerostin transcription and synthesis in the various parts of the body, especially in skeletal muscle, which is tightly coupled to bone in health and disease.

To this end, the second part of the study aims to characterize the expression of muscular SOST/Sclerostin both *in vitro* and *in vivo*. We start by a characterization and quantification of Sclerostin synthesis first by a murine myoblast cell line (C2C12) and then by primary myoblasts (C57 cells). Next, we move on to the *in vivo* muscular production of Sclerostin. We analyze its presence in mice of three different ages (young, adult and old), isolating muscles with distinct metabolic profiles and subjected to a high/low mechanical load, to investigate if the type of muscular activity/load affects Sclerostin production.

Finally, we transiently express Sclerostin, by electroporation, in muscles of young mice to understand the effects mediated by a muscle-specific Sclerostin overproduction on the skeleton.

In this Thesis, expression of Sclerostin by skeletal muscle is extensively described for the first time. Given the tight interplay -both mechanical and biochemical- between bone and muscle, the identification of muscular Sclerostin may represent an additional therapeutic target for the treatment of musculoskeletal disorders. The further elucidation of its mechanisms of action both paracrine and endocrine and how it impacts on bone will allow to discover new therapeutic avenues for anti-sclerostin monoclonal antibodies and to improve the knowledge about possible drugs treating musculo-skeletal diseases more effectively.

Materials and Methods

1 Cell cultures

1.1 C2C12 myoblasts

C2C12 myoblasts (CRL-1772; ATCC) were cultured in growth medium (GM) composed by Dulbecco's modified Eagle's medium (DMEM, AU-L0101-500, Aurogene), containing 10% fetal bovine serum (ECS0170DI, Euroclone) with 1% penicillin-streptomycin (AU-L0022-100, Aurogene) and 2mM L-Glutamine (AU-X0550-100, Aurogene), in 5% CO₂ atmosphere at 37 °C. To induce myogenic differentiation, the cells were plated on tissue culture plates and grown to 95% confluence, as described by Wei et al.⁶², before switching to differentiation medium (DM) (DMEM with 2% horse serum; or DMEM with 5 µg/ml insulin). The cells were replenished with fresh DM every 48 hours to induce differentiation until 14 days.

1.2 2T3 osteoblasts

Osteoblastic cell line 2T3⁶³, provided by Dr. Lynda Bonewald (Indiana University School of Medicine, Indianapolis, USA), was cultured in complete medium with a composition of Alpha Minimum Essential Medium (L0476-500, Biowest) supplemented with 10% Fetal Bovine Serum (ECS0170DI, Euroclone), 2 mM L-glutamine (AU-X0550-100, Aurogene) and 1% penicillin/streptomycin (AU-L0022-100, Aurogene). For osteogenic differentiation, a complete medium supplemented with 50 µg/ml ascorbic acid (A4544, Sigma) and 4 mM β-glycerophosphate (G9422, Sigma) was used. The differentiation medium was changed every two days of culture. Intermediate time points were collected at different times depending on assay typology and each differentiation experiment was performed in triplicate.

Where indicated, the differentiating medium was added to medium conditioned by C2C12 cells at various stages of differentiation in a ratio of 1:4.

1.3 C57 cell culture

C57 primary myoblasts, kindly donated by S. Biressi (CIBIO, University of Trento, Italy) were isolated from limb muscles of young (14- to 30-d-old) mice previously injured with a 30-gauge needle and were dissociated to isolate pure populations of myoblasts as described previously ⁶⁴.

Primary cultures were plated on 5 µg/ml laminin-1 (Invitrogen)/collagen-coated dishes and amplified in growth medium (GM) consisting of Ham's F-12 (L0136-500, Biowest) supplemented with 20% fetal bovine serum (ECS0170DI, Euroclone), 2.5 ng/ml basic fibroblast growth factor (Promega), 2 mM L-glutamine (AU-X0550-100, Aurogene) and 1% penicillin/streptomycin (AU-L0022-100, Aurogene).

To induce differentiation, myoblast cultures were seeded on coated dishes at 60% confluency and the growth medium was switched to differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum, L-Glutamine and penicillin/streptomycin. The time points analyzed were day 1, 3 and 6 of differentiation.

2 Animal procedures

2.1 Characterization of Sclerostin expression in mice of different ages

Eighteen male C57BL/6 mice at the age of 6 weeks (young; $n=6$), 5 months (adult; $n=6$) and 18 months (old; $n=6$) were purchased from Janvier Labs (Saint Berthevin, France). The animal experiments were conducted at the Animal Facilities of the University of Bordeaux, France. All animal procedures were performed in accordance with the European Guidelines for Care and Use of Laboratory Animals. Animal housing and caretaking were provided by the animal facility in accordance with the national guidelines.

All animals were kept under a 12 h–12 h light/dark cycle and the temperature was controlled. After the first week of adaptation, animals were euthanized by cervical dislocation and the following muscles were isolated: Gastrocnemius, Soleus, Trapezius and Triceps brachii; Femur and Skin were taken as positive and negative controls, respectively, for the synthesis of Sclerostin.

One tissue and the contralateral were withdrawn: one was snap-frozen for Molecular Biology assays and the others was fixed in 4% Paraformaldehyde (PFA) for histology; trapezius and skin were cut in two pieces.

2.2 In vivo gene transfer and electroporation procedures

Eight male C57BL/6 mice (Charles River) all belonging to the same progeny were housed in the institutional animal facility (CIBIO, University of Trento, Italy) and maintained on standard chow ad libitum. Three animals (n=3) were electroporated with the SOST vector, three (n=3) with the empty vector and two (n=2) were non-electroporated (CTRs). The pCMV6 expression plasmid (MR222588, Origene) containing the SOST gene tagged with Myc-DDK was amplified in *E. coli*.

Eleven days after birth, mice were anesthetized by inhaled isoflurane, and plasmid DNA was injected into the tibialis anterior (TA), quadriceps (Q) and gastrocnemius (GA) muscles with a 0.3-ml insulin syringe through a 31-gauge needle, in a constant volume of ~40 μ l. Each animal received ~240 γ of plasmid DNA (80 γ /site). A pair of stainless-steel plates was then applied over the muscle to encompass the injection area. In all cases the distance between the electrodes was 4-4.5 mm. Current was delivered 5 min after DNA injection as a constant current, square-wave pulse with a digital Stimulator (Panlab 3100, Biological Instruments). The characteristics of the electric field applied were 200 V/cm, 20 ms amplitude, 1 Hz, eight consecutive pulses. The animals were then kept warm until recovery.

The first electroporation was performed at 11 days of age, followed by a second one 6 days later; the animals were then sacrificed at 25 days of age. At each time point, animal sera were collected. Shortly after sacrifice, animals were X-ray scanned using an In-Vivo Xtreme-Bruker instrument. Animal studies were approved by the Institutional animal use and welfare committee.

3 Molecular techniques

3.1 Western Blot

For western blot analysis, proteins obtained from cell pellet were extracted by addition to the cell pellet of Lysis buffer (10 mM Tris-Cl, pH 7.4-7.6, 1% NP-40, 150 mM NaCl, 10% glycerol, 10 mM EDTA, 20 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, and freshly added Sigma Protease Inhibitor Cocktail) at 4 °C for 20 min.

Proteins from animal tissues were extracted by addition of Lysis buffer, sonicated 3 times for 10 s each in buffer and incubated for 4 hours (muscle) or 24 hours (bone) at 4°C with agitation.

Lysates were cleared by centrifugation and the total protein content was quantified by Bradford staining. Next, total lysates were added to Laemmli sample buffer (25 mM TrisHCl pH 6.8, 1.5 mM EDTA, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% Bromophenol blue) and boiled for 5 min at 100°C. Protein samples were resolved by SDS-PAGE, then immunoblotted on 0.2 μm Nitrocellulose membranes (Bio-Rad Laboratories) and probed by Western blotting with the following antibodies: anti-Actin (A5060, Sigma), anti-β catenin (8480, Cell Signaling Technology), anti-DDK (TA50011, Origene), anti-Phopsho β-catenin (orb222964, Biorbyt), anti-Sclerostin (ab63097, Abcam), anti-Tubulin (T6199, Sigma). Other antibodies used in this study are from Santa Cruz Biotechnology: anti-Desmin (sc-23879), anti-MyoD (sc-760), anti-Osteopontin (sc-21742), anti-Osteocalcin (sc-376726), anti-Runx2 (sc-101145), anti-MEPE (sc-73635), anti-Myogenin (sc-12732), anti-Lamin A/C (sc-517580).

Mouse (31430, Thermo Scientific), rabbit (31460, Thermo Scientific) and goat (31402, Pierce) HRP-conjugated secondary antibodies were used.

Bands were detected using Clarity and Clarity Max Western ECL Substrate (Bio-Rad) according to manufacturer's protocol, with a ChemidocTM Imaging System (Bio-Rad). Quantification was performed digitally using Image LabTM Software (Bio-Rad); where indicated, band signals were normalized to the digitally quantified housekeeping proteins.

3.2 Conditioned media processing

Conditioned media (CM) were collected from three independent experiments of muscle cell maturation and from two trials with two differentiation protocols: [DMEM, 2% HS, 1% Glu, 1% P/S] and [DMEM, 5 µg/ml Insulin, 1% Glu, 1% P/S].

The media were collected in differentiation conditions at diverse time points after induction of the myogenic program. The CM were then concentrated using spin columns with a cutoff of 3 kDa (Amicon® Ultra-4 centrifugal filter devices, Merck). The secreted proteins were quantified, added to Laemmli sample buffer and boiled for 5 min at 100°C.

Next, samples were separated with SDS-PAGE on Mini-PROTEAN® TGX™ 4-15% Precast gels (Bio-Rad Laboratories) or home-made acrylamide gels. The resulting gels were fixed with a fixing solution (40% Ethanol, 10% acetic acid, 50% H₂O) for 1 hour and then stained with Coomassie Brilliant Blue (Sigma) overnight at 4°C. Where indicated, the bands of interest were excised and processed for Mass Spectrometry analysis.

3.3 RNA extraction and quantitative Real Time-PCR

Total RNA was extracted with RNeasy kit (Qiagen) and quantified by spectrophotometry with NanoDrop 2000 (Thermo Scientific). 500 ng of total RNA were reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). mRNA levels were quantitatively determined on a CFX96 Touch™ Real-Time PCR Detection System using SsoFast™ EvaGreen Supermix according to the manufacturer's instructions (Bio-Rad Laboratories). PCR primer sequences were: *SOST* forward primer 5'-TGAGAACCAACCAGACCAT-3', *SOST* reverse primer 5'-ACATCTTTGGCGTCATAG-3'; *RUNX2* forward primer 5'-TTAATCCACAAGGACAGA-3', *RUNX2* reverse primer 3'-GTAAGACTGGTCATAGGA-5'; *WISP2* forward primer 5'-TGTGACCAGGCAGTGATG-3', *WISP2* reverse primer 5'-AGTGACAAGGGCAGAAAGT-3'; *Hprt* forward primer 5'-GGCATTGCTCTCTCAATGACAA-3', *Hprt* reverse primer 5'-ATGTAGGCCATGAGGTCCAC-3'.

As for the "In vivo *SOST* overexpression" experiment, the animal tissues (muscles) were probed with: *Sclerostin* forward primer 5'-AACCAACCAGACCATGAAC-3', *Sclerostin* reverse primer 5'-TACTCGGACACATCTTTG-3' (to detect the endogenous *Sclerostin*); *Sclerostin Myc-Tag* forward

primer 5'-CGGAGCTGGAGAACGCCT-3', *Sclerostin Myc-Tag* reverse primer 5'-AATCCAGGATATCATTTGCTGCC-3' (to detect the exogenous, Myc-tagged Sclerostin); *Hprt* forward primer 5'-TCA GAC CGC TTT TTG CCG CGA-3', *Hprt* reverse primer 5'-ATC GCT AAT CAC GAC GCT GGG AC-3'. Relative gene expression values were calculated by applying the $2^{-\Delta\Delta C_t}$ method⁶⁵: using this method, we obtained the fold changes in gene expression normalized to the internal control gene *Hprt*.

3.4 Alizarin Red assay

Matrix mineralization was evaluated by Alizarin Red-S (AR-S) staining. The cells were fixed in 4% Paraformaldehyde for 20 min at room temperature. After this time, fixative solution was removed, fixed cells were washed in Phosphate Buffered Saline (PBS) and plates were stored at 4 °C until sample processing. PBS was removed from the stored plates and the cell layer was stained with 40 mM Alizarin-S (A5533, Sigma) at ~pH 4.2 for 5 min under gentle agitation. Cell preparations were washed with PBS to eliminate non-specific staining. To quantify calcium deposition, the dye was removed from the monolayer by the addition of 10% cetylpyridinium chloride solution. A volume of 200 μ L of the solution (in duplicate) was transferred to a clean 96-well plate. Optical density was measured by spectrophotometry at 570 nm, using 10% cetylpyridinium chloride as a blank reference.

3.5 Alkaline Phosphatase (ALP) assay

Alkaline phosphatase (ALP) activity was measured with the Alkaline Phosphatase Assay Kit (Colorimetric) (ab83369; Abcam), according to the manufacturer's protocol. The cells were re-suspended with cold PBS and centrifuged for 5 min at 1000 rpm. Then the supernatant was discarded, and 50 μ L of ALP assay buffer was added, followed by centrifugation at 13,000 rpm at 4 °C for 15 min. The supernatant was collected in a new tube. To prepare the sample, 50 μ L of collected supernatant was mixed with 30 μ L of ALP assay buffer, transferred into a 96-well plate, and 50 μ L of pNPP was added to each well. Three replicates were prepared for each condition. The plate was incubated at 25 °C in the dark for 60 min. After the reaction, 20 μ L stop solution was added and the

absorbance was determined at Optical Density 405 nm using an iMark™ Microplate reader (168-1135, Bio-Rad Laboratories).

3.6 Enzyme-linked immunosorbent assay (ELISA)

Sclerostin levels in blood serum samples, animal tissues and cell lysates were determined with a quantitative sandwich ELISA. Either Mouse SOST (EM1815, FineTest) or Mouse Sostdc1 (Sclerostin domain-containing protein 1) (EM0392, FineTest) ELISA kits were used according to the standard protocols provided by the manufacturer. All samples were assayed in duplicate.

4 Morphological techniques

4.1 Immunofluorescence

C2C12 and C57 cells grown on glass coverslips were fixed in 4% paraformaldehyde at 4 °C for 20 min, blocked in 4% (w/v) Bovine Serum Albumin (BSA)-PBS and incubated with primary antibody anti Sclerostin (ab63097, Abcam) overnight at 4 °C. Coverslips were next washed with PBS and incubated with fluorescence-labeled secondary antibodies (Thermo Scientific) for 1 h at room temperature. Slides were washed and mounted with DABCO 10% solution and were observed by a Nikon A1 confocal laser scanning microscope. The confocal serial sections were processed with ImageJ software to obtain three-dimensional projections, and image rendering was performed using Adobe Photoshop CS 8.0 software (Adobe Systems, San Jose, CA, USA). All the images shown in this paper are representative of at least 3 independent experiments carried out under the same conditions.

4.2 Histological staining and immunohistochemistry

All animal tissues (muscle, bone, skin) were fixed with 4% PFA overnight. Bone tissues were decalcified for 7 days in Microdec (F/D0053, MM France) under stirring. Next, tissues were processed for paraffin embedding (Leica EG1150 H) following the procedure here described: 70% ethanol for 30 min, three times in 95% ethanol (1 hour each), three times in 100% ethanol (1 hour

each), three times in toluene (1 hour each) and two times in paraffin, 1 h for each. After paraffin embedding, 5-7 μm sections were obtained from each sample.

Hematoxylin and Eosin (H&E) staining was performed on murine muscles and bone tissues. Briefly, two slides per muscle [Quadriceps (Q), Gastrocnemius (GA) and Tibialis anterior (TA)] of one animal representative of each group (SOST-electroporated, Empty vector-electroporated, non-electroporated) were deparaffinized by means of xylene (two times for 5 min each), hydrated through a graded series of alcohol (100%, 95%, 80% and 70%) for 5 min each and rinsed first with tap water and then with deionized water for 1 min each.

After the dehydration procedure, the slides were stained with Carazzi hematoxylin solution (C0203, DiaPath), rinsed with tap water, stained with alcoholic 0,5% eosin Y (C0353, DiaPath) and then rinsed with deionized water for 1 min each.

Histomorphometry analysis was conducted on the right and left femurs. Briefly, femurs were removed from each animal, deprived of soft tissues, fixed in sodium phosphate-buffered (PBS) 4 % paraformaldehyde pH 7.4, dehydrated in graded ethanol, and paraffin embedded. The femurs were sagittally cut at the distal metaphyseal level and transversally cut at the mid-diaphyseal region to obtain serial 5- μm -thick sections for histomorphometric analysis. The sections were stained with H&E to measure the following static histomorphometric parameters: trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp) of the distal femoral metaphyses, as well as the cortical bone area (Ct-B-Ar) of femoral middiaphyses. The areas in which the measurements were performed were selected as previously described.

The immunohistochemical analysis (IHC) were conducted on muscles, skin and bone tissues. Slides were deparaffinized by means of toluene (three times for 3 min each), hydrated through a graded series of alcohol (100% for two times of 3 min each, 95%, 70% and 50% for 3 min each) and rinsed with distilled water for 3 min. After that, antigen retrieval was performed by dipping the slides in a buffer composed of 0,1 M citric acid anhydrous and 0,1 M trisodium citrate dihydrate, for 2 hours at room temperature. Then, endogenous peroxidase was quenched in 3% H_2O_2 , in PBS, for 10 min. After washing with PBS, slides were blocked using 5% (w/v) BSA in PBS with 0,3% Triton for 30 min. For Sclerostin immunolabeling, incubation was performed overnight with a goat anti-

sclerostin primary antibody (dilution 1:200; AF1589, R&D systems) at 4°C. After two washes with PBS, the slides were incubated with the HRP-conjugated secondary antibody according to the manufacturer's instruction (Anti-goat ImmPRESS® kit; Vector Labs), and then revealed using Impact DAB solution (Vector Labs). Staining was stopped in distilled water; some samples were then counterstained in Methyl Green (sc-215348, Santa Cruz Biotechnology) and washed with distilled water 3 times for 30 sec. Finally, samples were dehydrated and mounted using Pertex.

All stained sections were evaluated under a Nikon Eclipse Ni microscope (Nikon) equipped with a DS-Fi2 camera (Nikon) and processed by means of NIS-Elements D 5.11.00 software (Nikon).

5 μ CT measurements

μ CT scanning and analysis was performed using an X-ray tube KEVEX PXS10 130kV-0.5 mA and a CCD camera Photonic Science VHR1 fiber optic plate with scintillator (GOS) 4000 x 2600 (9 μ m pixel) for in vivo measurements of cortical and trabecular bone. Isometric voxel size was 4.5 μ m and X-ray tube potential was 70 kVolt.

For each tibia, axial sections [from distal epiphysis to middle diaphysis] were acquired and stacked in a single DICOM file. The acquisition volume was reconstructed from each DICOM file and the coronal sections were then extracted, on which the analysis of the epiphysis, metaphysis and epiphyseal plate was performed. The most informative coronal section was selected and analyzed together with the previous 5 and the subsequent 5, for a total of 11 coronal sections per sample. For each section, the epiphysis, metaphysis and epiphyseal plate were segmented separately and automatically; the epiphyseal plate was also segmented manually by a biologist. In each of the 11 sections, the metaphysis was then further segmented through the subdivision of metaphyseal trabeculae into 16 different types, or classes, on the basis of the combination of two values: the thickness of the trabeculae and its angle of inclination with respect to the epiphyseal plate. The thickness, in the case of our classification, can have a value of 2 or 3 pixels (therefore of about 10 or 15 μ m), while the angle of inclination can have the following values: 0 22.5 45 67.5 90 112.5 135 157.5 degrees. So, the classes of metaphyseal trabeculae are defined as follows:

Class	Thickness (pixel)	Angle (degrees)
1	2	0,0
2	3	0,0
3	2	22,5
4	3	22,5
5	2	45,0
6	3	45,0
7	2	67,5
8	3	67,5
9	2	90,0
10	3	90,0
11	2	112,5
12	3	112,5
13	2	135,0
14	3	135,0
15	2	157,5
16	3	157,5

For epiphysis and metaphysis (not divided into classes of trabeculae) the gray levels of the images (to be considered as a proxy measure of the Bone Mineral Density), the trabecular number and thickness and finally the spacing between them were analyzed. For the individual classes of metaphyseal trabeculae, the trabecular number, thickness, shape and area were considered. For the epiphyseal plate, thickness was analyzed, both in the case of automatic segmentation and in the case of assisted segmentation. For the diaphysis, the cortical thickness was analyzed (starting from the acquired axial sections, analyzing the first 10 sections beginning with the most central region of the bone).

The statistical analysis was performed pooling the left and right tibiae of a same group of treatment (untreated (CTR), empty vector-treated (EV-ET) and sclerostin vector-treated (SOST-EV)). Each of the classes of trabeculae identified was analyzed separately. First, the 3 groups of tibiae were compared to each other in pairs. Then, for each pair of groups analyzed, the individual tibias were compared, considering all the possible pairs of inter-group comparison, in order to verify whether what is observed from the comparison between the groups can be valid also for the individual tibias that compose them. For each statistical comparison, the Wilcoxon-Mann-Whitney test was used, considering significant p-values <0.05, with Bonferroni correction.

6 Mass spectrometry analysis, protein identification and annotation

Conditioned media were concentrated, quantified and added to Laemmli sample buffer (25 mM TrisHCl pH 6.8, 1.5 mM EDTA, 20% glycerol, 2% SDS, 5% β -mercaptoethanol, 0.0025% Bromophenol blue). For protein separation, equivalent amounts of extracts were resolved by SDS-PAGE on Mini-PROTEAN® TGX™ 4-15% Precast gels (Bio-Rad Laboratories); the resulting gels were stained overnight with Coomassie Brilliant Blue and the bands of interest were excised for Mass Spectrometry analysis.

Protein digestion was performed following a protocol reported in literature ⁶⁶. Briefly, Coomassie-stained bands were diced into 1 mm³ cubes, destained for 30' with 50% acetonitrile in 100 mM ammonium bicarbonate buffer, dehydrated with 100% acetonitrile for 10 minutes, reduced with 10 mM DTT (1,4-Dithiothreitol, Sigma Aldrich) for 30 minutes at 56 °C and alkylated with 55 mM iodoacetamide (IAA, Sigma Aldrich) for 30 minutes in the dark. After alkylation, gel pieces were dehydrated with 100% acetonitrile and digested overnight with MS-grade trypsin (13 ng/ μ l in ammonium bicarbonate buffer 10nM with 10% acetonitrile; 90057, Pierce) at 37 °C. The digestion products were extracted with a solution of 5% formic acid and acetonitrile 1:2 for 15 minutes at 37°C in a shaker and were then lyophilized using a SpeedVac Concentrator (Savant).

Dry peptides from bands were resuspended in 40 μ L of a mixture of water: acetonitrile: formic acid 97:3:2, sonicated for 10 min at room temperature and centrifuged at 12100 rcf for 10 min. Analyses were performed on an ESI Q Exactive Mass spectrometer (Thermo Scientific), controlled by Xcalibur (v. 29 build 2926) and interfaced with an Ultimate 3000 UHPLC pump.

The column (Zorbax SB-C18 RRHT, 2.1x50mm, 1.8 μ particle size, Agilent Technologies) was equilibrated with 0.3 ml/min of water 0.1% formic acid (A) with 2% acetonitrile (B); after sample injection (18 μ l), B% was raised from 2→3%, then linearly increased from 3→21% in 19 min; B% was then brought to 90% in 4 min and kept at 90% B for three min, before the reconditioning step. The total runtime was 35 min.

ESI source was operated in positive mode; probe was heated at 290°C, capillary temperature was set at 270 °C; the following nitrogen flows (arbitrary units) were used to assist the ionization: Sheath

Gas 40, Aux Gas 30, Sweep Gas 3; capillary voltage was set to 3.8 kV, S-Lens RF level was set at 45 (arbitrary units).

Profile MS and MS² spectra were recorded from 200 to 2000 m/z in FULL MS/dd-MS² (TOP5) mode, at a resolution of 70000 and 17500, respectively. The five most intense multi-charged ions were selected for MS² nitrogen-promoted collision-induced dissociation (NCE=28). A precursor active exclusion of 20 seconds was set; peptide-like isotope pattern ions were preferred. The mass spectrometer was calibrated before the start of the analyses.

Raw data, converted into mascot generic format using MsConvert (v. 3.0.10730, ProteoWizard tools), were searched against Swiss-Prot (accessed June 2018; 16,977 sequences for *Mus musculus*) for peptide sequences and C-RAP for contaminants with MASCOT (Version 2.4, Matrix Science).

Trypsin as proteolytic enzyme and carbamidomethyl cysteine as fixed modification were set in search parameters. Deamidated (NQ) and oxidated (M) were set as variable modifications. One missed cleavage was allowed. Mass tolerances were set at 10 ppm for the precursor ions and 0.05 Da for the product ions. Automatic decoy database search was used to estimate the false discovery rate, which was adjusted to $\leq 1\%$.

All proteins identified are listed in Table 1. GO annotation was used to identify protein clusters, this was obtained using Protein Analysis Through Evolutionary Relationships⁶⁷ (PANTHER version 14.1), STRING database⁶⁸ (version 11.0) and the DAVID Bioinformatics Resources database⁶⁹ (version 6.8).

7 Statistical analyses

All data were analyzed using Prism version 6.0e (GraphPad Software). *In vitro* data are presented as the mean $\pm$ standard deviation (SD) from three independent experiments. Data for animal experiments are expressed as means $\pm$ standard deviation. All statistical analyses were performed using one-way ANOVA followed by Dunnett's, Sidak's or Tukey's multiple-comparison tests. Differences between groups were considered statistically significant at $P < 0.05$.

Results

1. Analysis of the effects mediated by myogenic medium on osteoblast differentiation

1.1 C2C12 differentiation setup

The first part of our work was devoted to establishing a protocol for an efficient differentiation of C2C12 myoblasts and 2T3 osteoblasts, which represent the two cell types we used to model *in vitro* the communication from muscle to bone. C2C12 cells were differentiated into myotubes and their maturation was monitored by Western Blot (WB) and immunofluorescence (IF) analysis of marker proteins of differentiation (Fig. 8, 9).

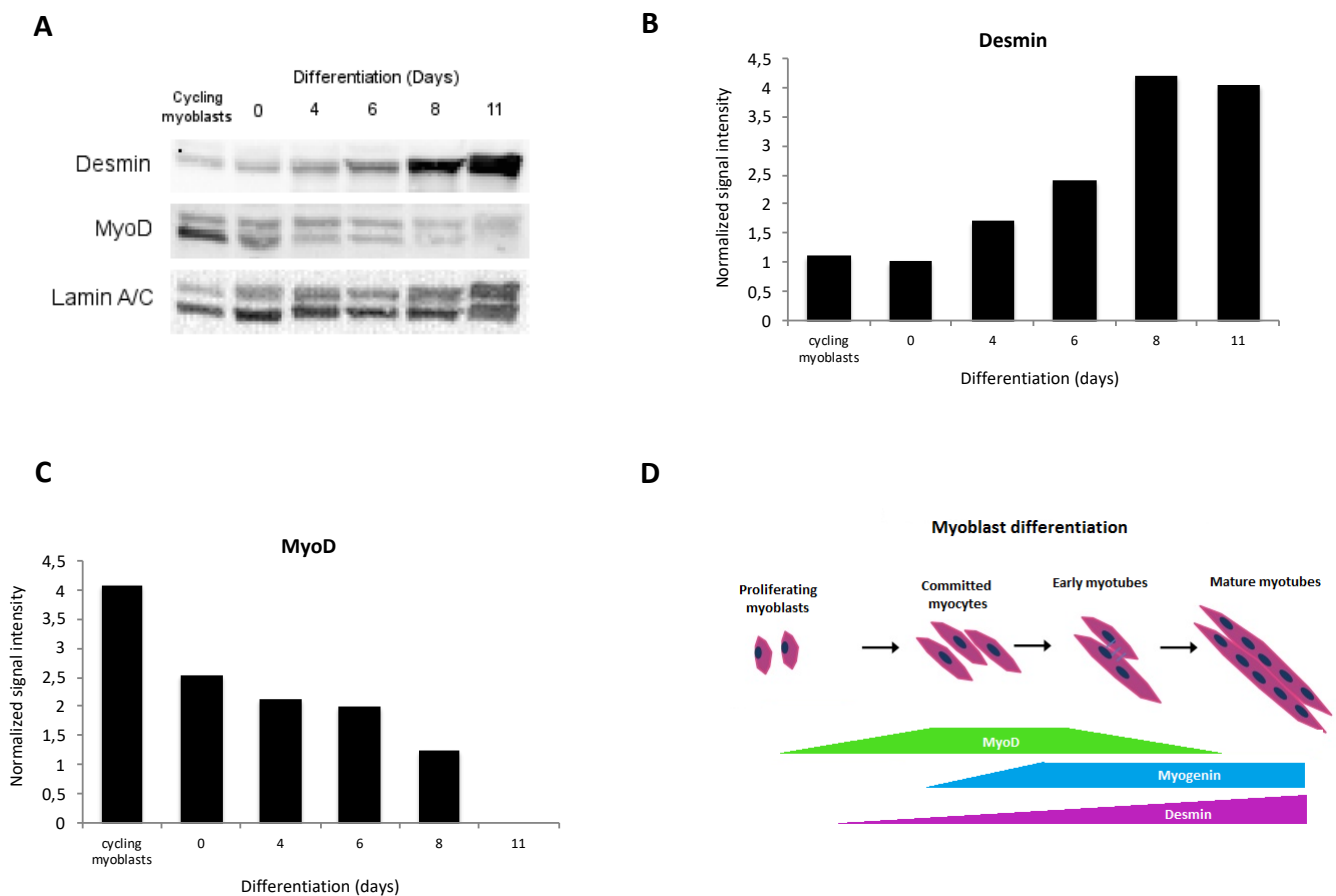


Figure 8. A) Western Blot of Desmin, MyoD and Lamin A/C in myoblasts differentiated for 11 days and collected at different time points. B, C) Normalized signal intensity on LAMIN A/C reported in bar chart. D) Schematic model outlining the temporal gene expression profiles of MyoD, Myogenin and Desmin during C2C12 myogenic differentiation.

Western Blot bands and their relative intensities show that Desmin expression consistently increased over time (Fig. 8 A, B), in accordance with the fact that it is a component of myotube cytoskeleton and is essential for myoblast fusion and myotube formation⁷⁰ (Fig. 8 D).

Conversely, MyoD levels decreased along differentiation until becoming undetectable (Fig 8 A, C), according to its role in muscle differentiation as a repressor of cell renewal and a promoter of terminal differentiation⁷¹ (Fig 8 D). The immunofluorescence staining showed that MyoD signal occurs at nuclear level and its intensity was reduced with differentiation (Fig.9).

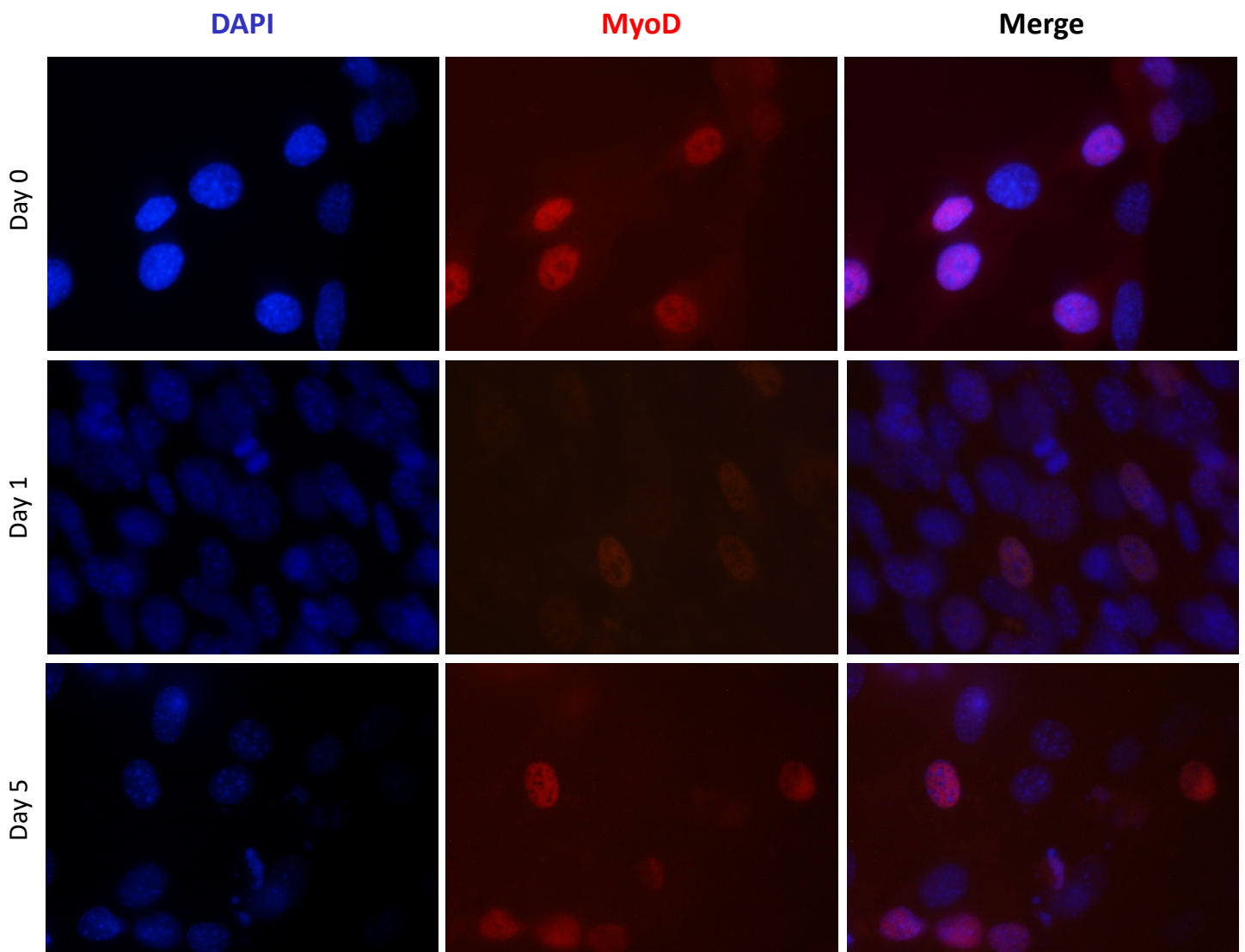


Figure 9. Immunofluorescence staining of MyoD in differentiating myoblasts/myotubes.

1.2 2T3 differentiation setup

After having established a protocol for an efficient differentiation of C2C12 myoblasts, we characterized the maturation process of 2T3 osteoblasts.

2T3 osteoblasts were differentiated for 8-9 days and their maturation was assayed under both the functional and molecular viewpoints. The mineralization, assessed by Alizarin Red-S staining, and the activity of Alkaline Phosphatase (ALP) enzyme both increased throughout differentiation, being significantly higher than the value of the first time point, which was set as control (Fig. 10 A, B). Based on these findings, we decided to shorten to 5 days the duration of differentiation for the subsequent trials.

Next, we analyzed the mRNA and protein levels of *SOST*/Sclerostin and *Runx2*, two key genes for osteogenic differentiation, by quantitative Real Time PCR and Western Blot (Fig. 10 C, D). The amount of *SOST* transcript increased linearly throughout differentiation, in accordance with its specific expression in osteocytes and its role as a suppressor of new bone formation, whereas *Runx2* mRNA did not display significant variations, so we did not investigate the protein counterpart.

Finally, we determined the protein expression of Sclerostin, Osteopontin (OPN) and Osteocalcin (OCN) (Fig. 10 D). Sclerostin and OPN increased during differentiation, both being proteins secreted in the matrix with the function of inhibiting new bone formation (Fig 10 E)^{72,73}. Differently, OCN levels peaked on day 3 of differentiation, suddenly dropping on day 7, as a result of an enrichment in the osteocyte population; such trend mirrors OCN physiological expression, which is produced solely by osteoblasts⁷⁴ (Fig 10 E).

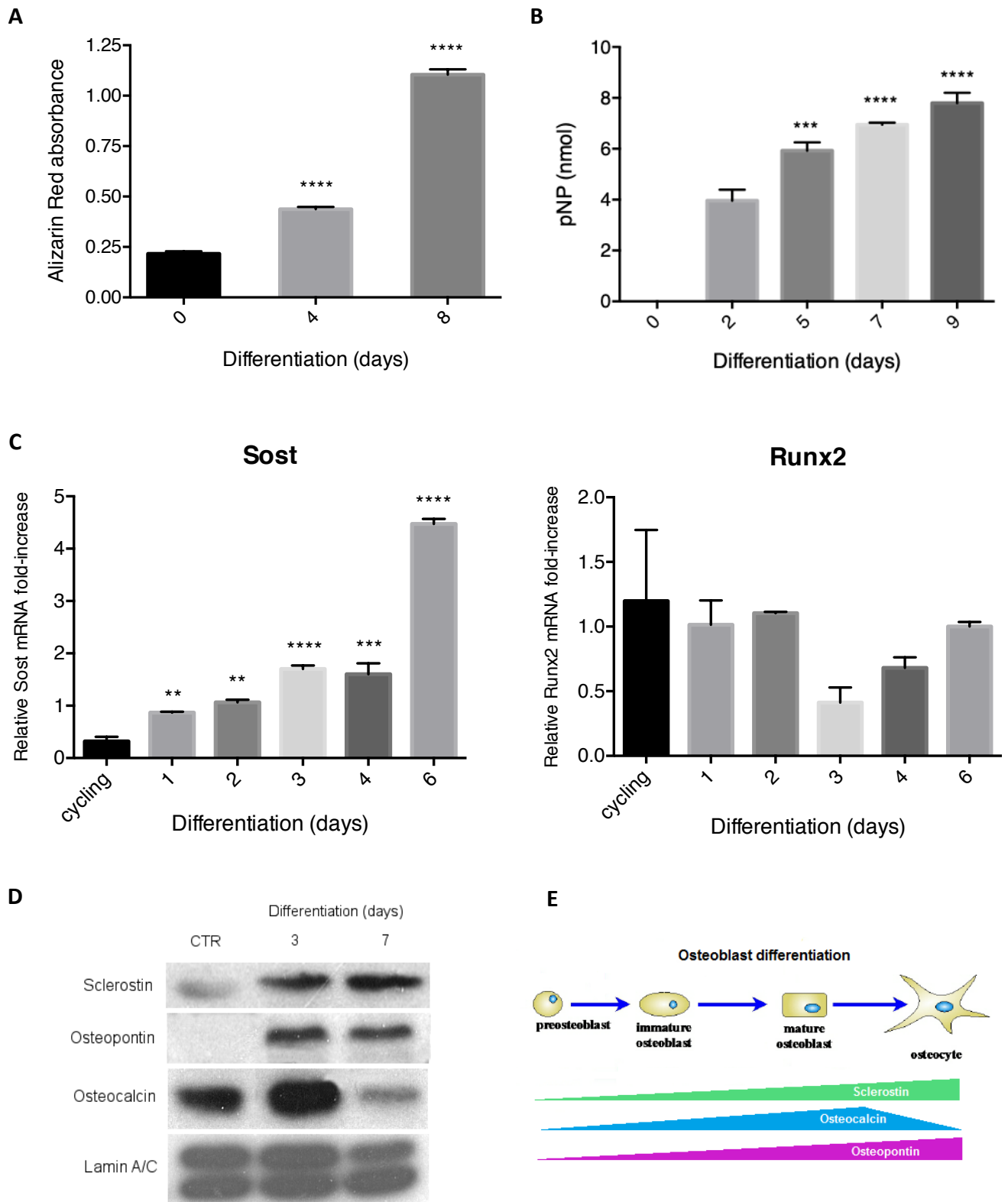


Figure 10. A) Alizarin Red staining of 2T3 osteoblasts during differentiation. B) ALP assay. C) RT-PCR analysis of expression of SOST and Wisp2. D) Western Blot analysis of osteogenic proteins. E) Schematic representation of the temporal gene expression profiles of Sclerostin, Osteopontin and Osteocalcin [picture from Kong et al., 2012⁷⁵]

1.3 Myogenic modulation of osteoblast differentiation

The following set of experiments aimed at assessing the effect of media conditioned by myogenic C2C12 cells at diverse maturation stages onto osteoblast differentiation. We differentiated myoblasts into myotubes along 14 days, collecting the conditioned medium on day 1 (CM 1), 7 (CM 7) and 14 (CM 14), as representative of an early, mid and late cell differentiation degree, respectively. We then tested the effects of C2C12 culture supernatants on osteoblast differentiation (5 days) by adding 25% CM to the osteogenic differentiation medium (Fig. 11).

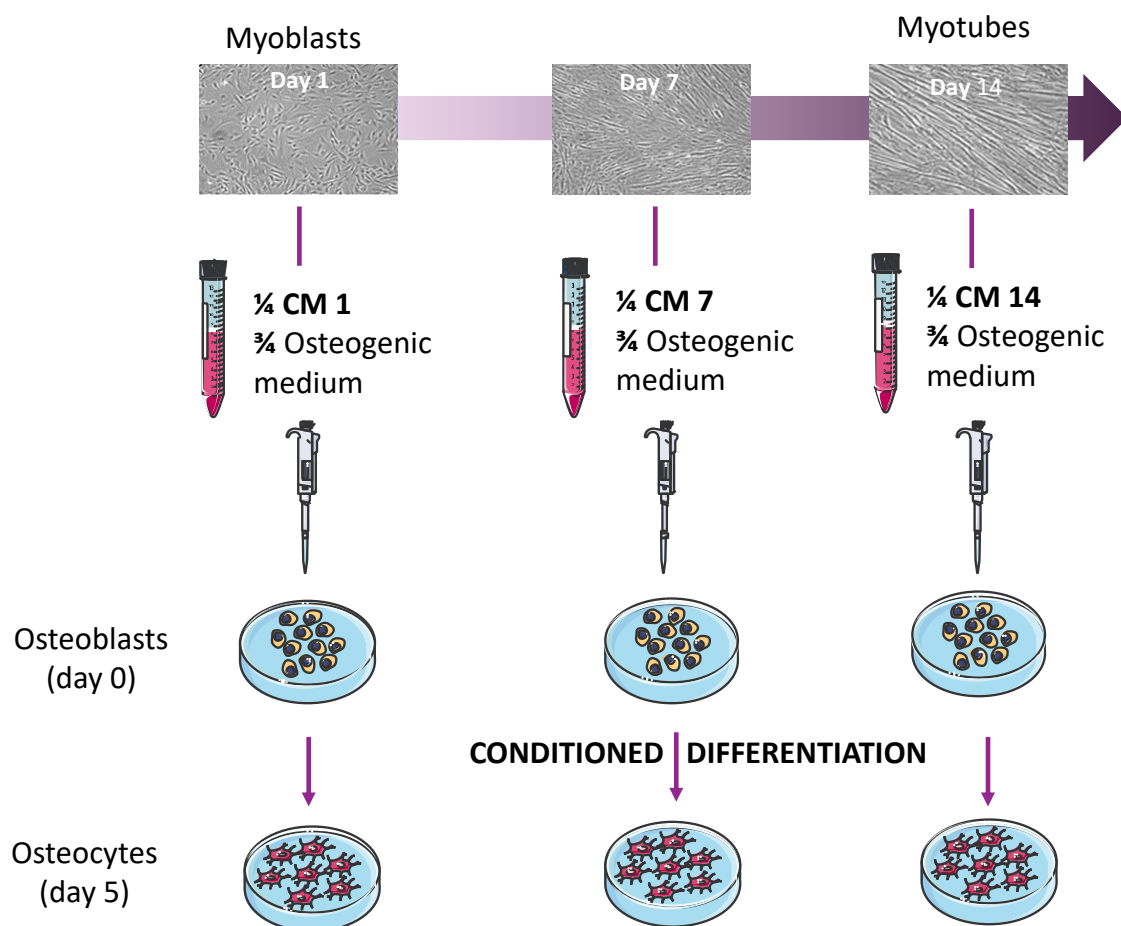


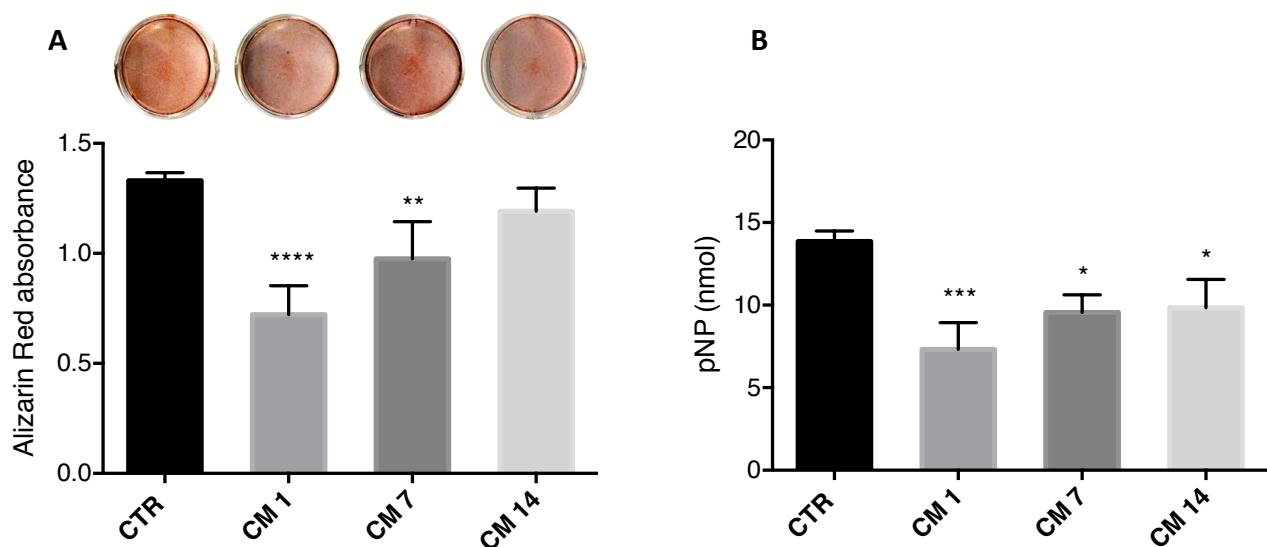
Figure 11. Schematic representation of the experimental workflow of osteoblast conditioned differentiation.

We set up a control condition by adding $\frac{1}{4}$ unconditioned myoblast differentiation medium to the osteoblast maturation medium. The alizarin staining revealed a significant decrease in mineralization (*i.e.* maturation) of osteoblasts differentiated with CM 1, compared to CTR, a progressive increase in the CM 7 condition (still significantly lower than CTR), going back to the CTR

level in the CM 14 condition (Fig. 12 A). The ALP activity assay revealed a similar trend, showing CM 1, 7 and 14 conditions significantly lower than CTR, among which the CM1 appears the most significant (Fig. 12 B). Therefore, the factors secreted in CM 1 altered significantly the functional maturation of osteoblasts.

Next, from the RT-PCR analysis of osteogenic genes emerged a significant increase of *SOST* transcription only in those cells treated with CM 1. Differently, the transcription level of *Wisp2* decreased progressively from the CM 1 to the CM 14 condition: since *Wisp2* acts as a mediator of the canonical WNT pathway activation, its decrease is suggestive of the fact that C2C12 conditioned media may modulate this pathway (Fig. 12 C).

Finally, we detected the variations in the synthesis of osteogenic proteins (the MLO-Y4 osteocyte protein extract served as a positive control). *Runx2*, which is normally upregulated in immature osteoblasts and downregulated in mature osteoblasts, was not affected by the treatment. Whereas *MEPE*, usually expressed by differentiated osteoblasts and osteocytes, showed a trend similar to the mineralization amount: it visibly diminished in CM 1 compared to CTR as an index of reduced maturation (Fig. 12 D).



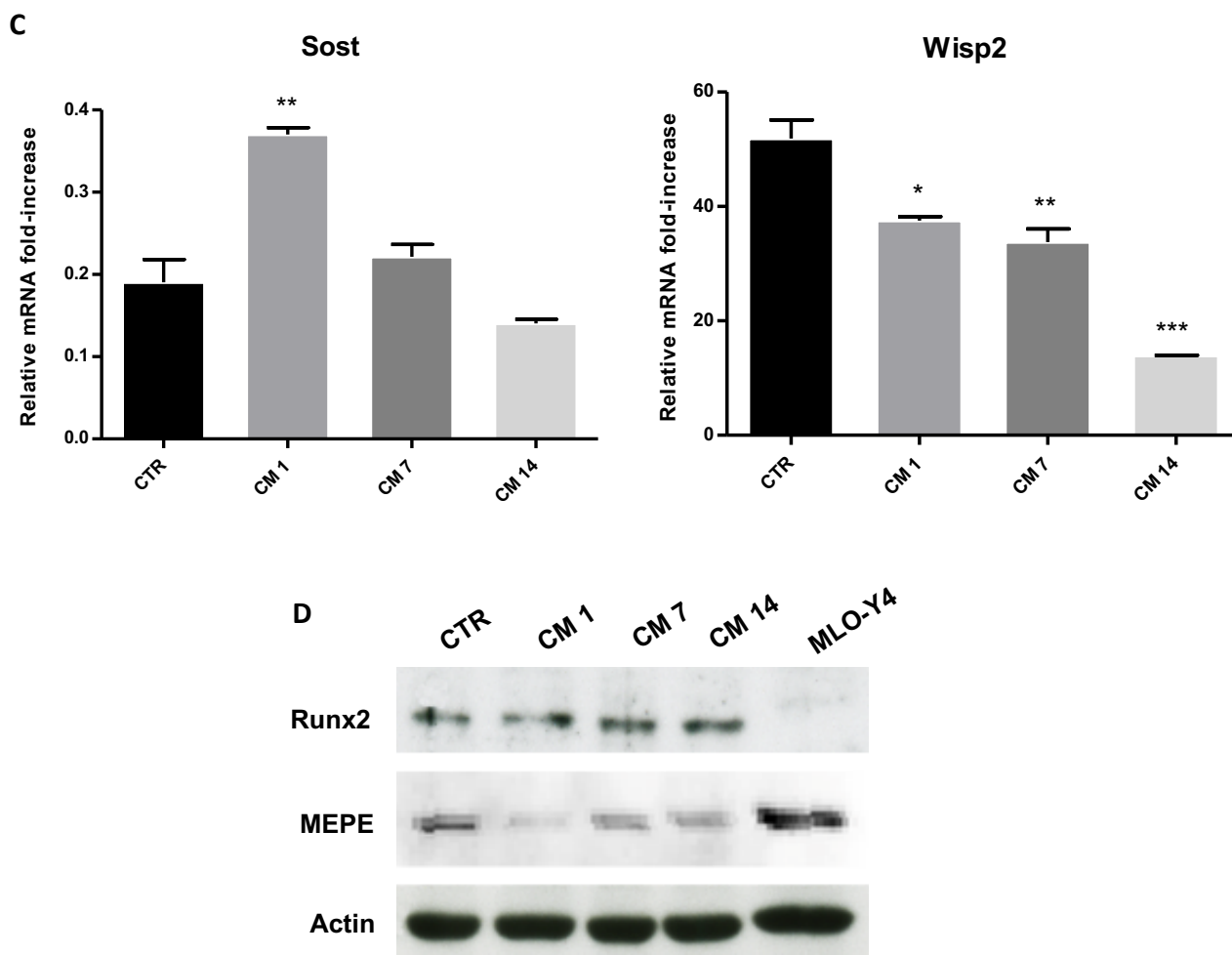


Figure 12. A) Alizarin Red staining of differently conditioned 2T3 osteoblasts. B) ALP assay. C) RT-PCR analysis of SOST and Wisp2 expression. D) Western Blot analysis of osteogenic proteins.

2. Muscle-specific Sclerostin production

2.1 Sclerostin characterization in C2C12 muscle cells

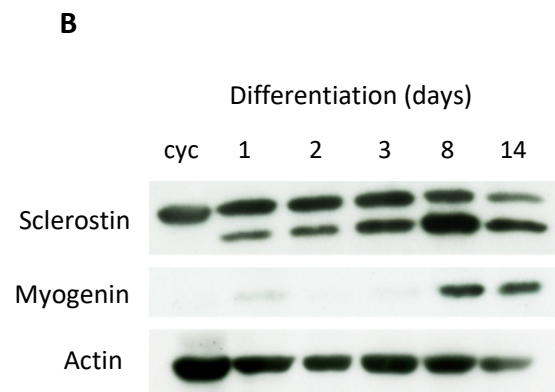
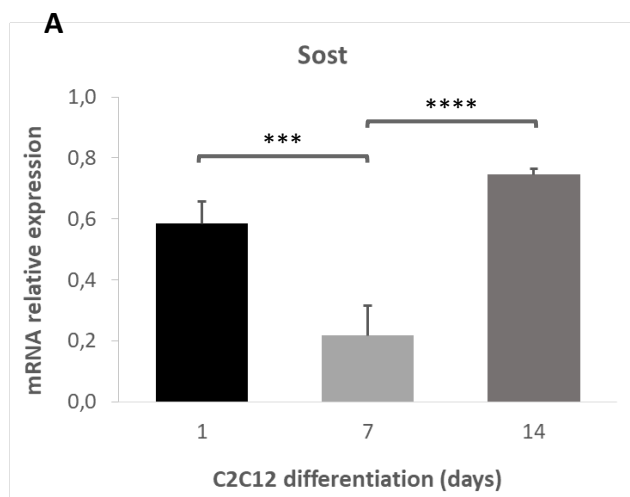
In order to find the agent putatively responsible for the slowdown of osteogenic maturation induced by muscle cells, thanks to our expertise in the field of bone regenerative processes, we hypothesized that a possible candidate could be Sclerostin. Indeed, Sclerostin is the main inhibitor of new bone formation. To date, Sclerostin had always been considered an osteocyte-specific protein, since *SOST* mRNA and Sclerostin protein were detected in mouse and human bones and the expression was restricted to osteocytes²⁹. However, *SOST* mRNA expression by RT-PCR was found in other organs, including skeletal muscle⁷⁶.

Therefore, we supposed that it may be involved in the effects seen by muscle cells-conditioned medium onto osteoblast differentiation and we started investigating the potential synthesis and secretion of Sclerostin by muscle cells Sclerostin by muscle cells, so far never shown.

Interestingly, we found the presence of Sclerostin at the mRNA and protein level (Fig. 13 A, B). The RT-PCR analysis showed that SOST mRNA is highly expressed at the moment of the myogenic commitment (day 1), then decreases significantly at day 7, to increase above the level of day 1 at the end of maturation (day 14). Western blot revealed that Sclerostin is expressed by cycling myoblasts and by differentiating cells from day 1 to 14, displaying two bands with fluctuating intensities, that may correlate to the two possible N-glycosylation sites of mouse Sclerostin⁷⁷ (Fig. 13 B). The expression of Myogenin served as a control of differentiation.

The expression of Sclerostin was also confirmed by immunofluorescence (Fig. 13 C), that showed its localization in secretory vesicles in the cytoplasm, according to its nature of secreted glycoprotein.

PROTEIN EXTRACTS



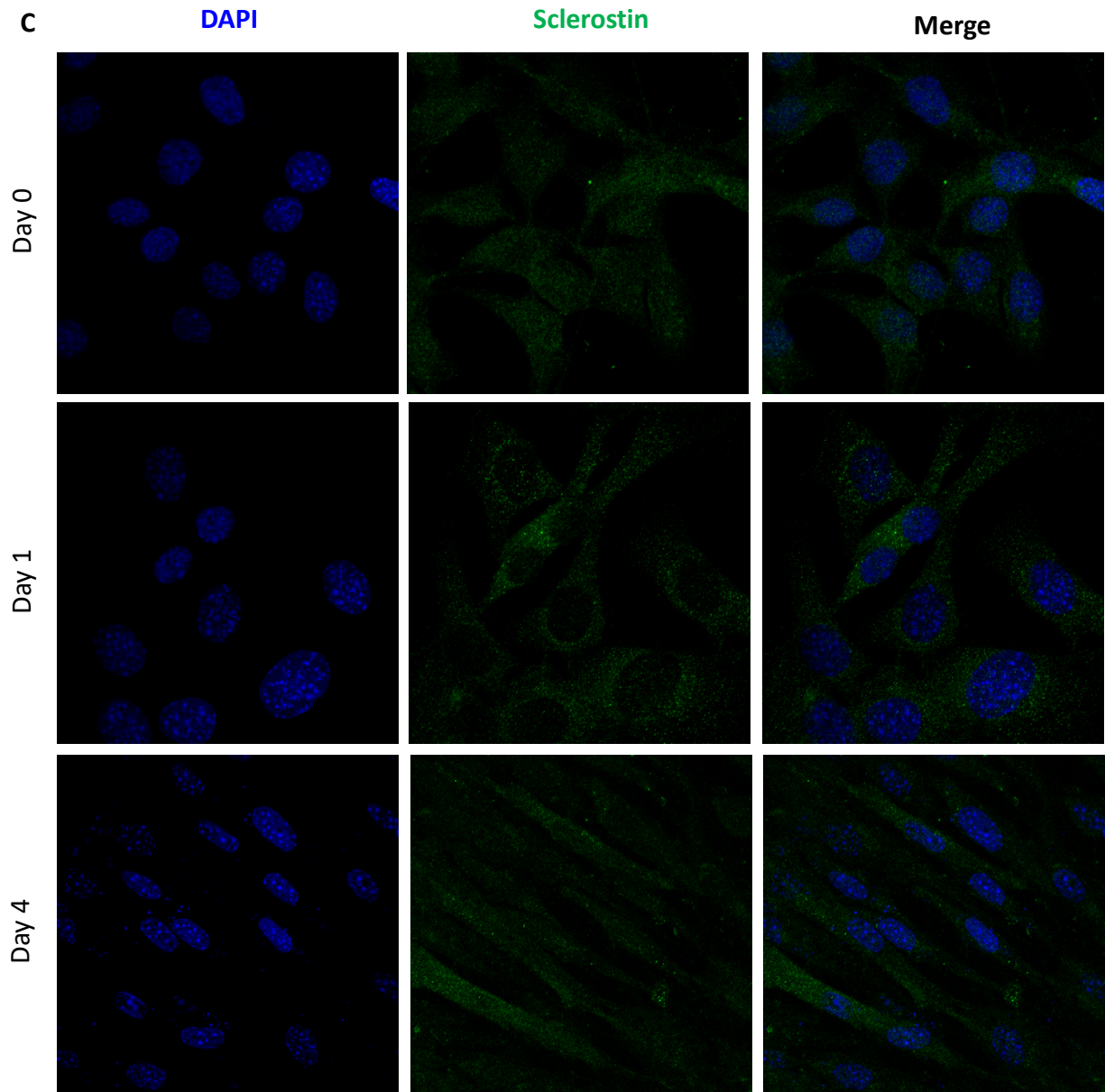


Figure 13. A) Real-time PCR. B) Western blot on C2C12 protein extracts. C) Immunofluorescence staining of Sclerostin.

MEDIUM

The media conditioned by C2C12 cells on day 1, 7 and 14 of differentiation were afterwards analyzed with the ELISA assay (Mouse Sostdc1 ELISA Kit) (Fig. 14), that revealed the highest quantity of the protein on **day 1** (1145,2 pg/ml) and a decrease on **day 7** (273,29 ng /ml), which became undetectable on **day 14**. Therefore, we demonstrated that SOST/Sclerostin is expressed by C2C12 cells at the mRNA and protein level in early, mid and late stages of differentiation and it is secreted in the conditioned medium.

In the skeleton, Sclerostin plays an important role in the regulation of bone remodeling: when it binds to its receptors on the cell surface of osteoblasts, a downstream cascade of intracellular signaling is triggered, with the ultimate effect of inhibiting osteoblast bone formation³³. Since our data clearly demonstrate that SOST/Sclerostin is expressed by a muscle cell line, it could be one of the factors affecting the osteogenic differentiation.

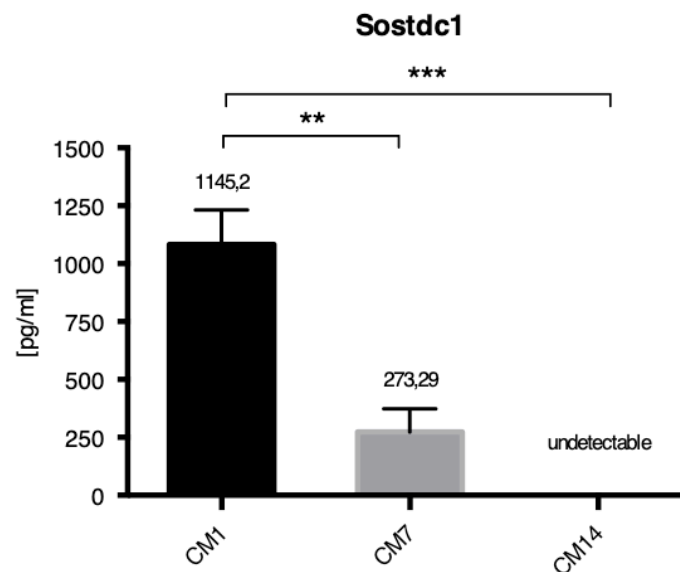


Figure 14. ELISA assay: Sostdc1 quantification in conditioned media.

2.2 Sclerostin characterization in C57 primary muscle cells

After having reported the synthesis and secretion of Sclerostin by the C2C12 myoblast cell line, we moved on to a primary cell type, to investigate a cellular model that better recapitulates the *in vivo* phenotype of muscle cells expresses the protein.

From the Real Time PCR analysis emerged that only cycling (i.e. undifferentiated) myoblasts express SOST mRNA, while cells differentiated at day 1 and 3 have progressively low levels of the transcript until it becomes undetectable at day 6. 2T3 osteoblasts were taken as a control (Fig. 15A).

In the Western Blot experiment, adult mouse tissues were taken as a reference: the tibia, since bone is the tissue of choice to study Sclerostin, and the quadriceps, to compare the potential muscular band with the one of C57 cells. Sclerostin is detectable in cycling and day 3 mouse myoblasts, partially mirroring the mRNA expression, although at a lower molecular weight than the bone

isoform (Fig. 15B). Mouse quadriceps also synthesizes sclerostin, as can be seen by the presence of a band at the expected molecular weight and two lower bands, index of glycosylation.

The immunofluorescence staining images presented in Fig. 15C indicate that Sclerostin is expressed by the majority of cycling myoblasts; at day 1 of differentiation the signal is weak, then at day 4 only few myotubes are positive.

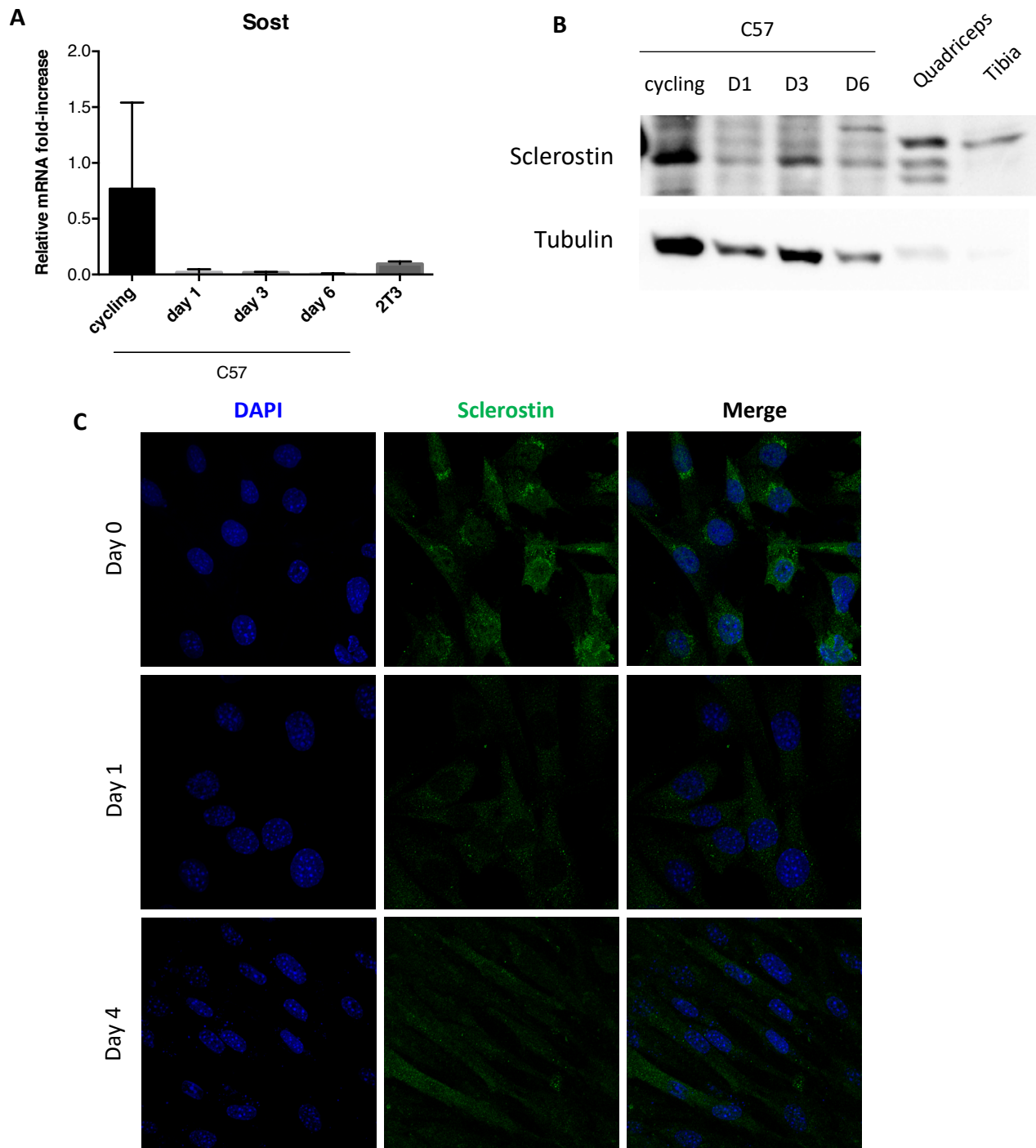


Figure 15. A) RT-PCR analysis of SOST expression. B) Western Blot analysis of Sclerostin in C57 cells and adult mouse tissues (quadriceps and tibia). C) Immunofluorescence staining of Sclerostin.

3. Mass Spectrometry analysis of the myogenic medium

We performed a broad analysis of the secretome produced by C2C12 muscle cells, in order to find other factors potentially involved in the modulation of osteogenic differentiation. Firstly, we performed three independent experiments of C2C12 differentiation with DMEM, 1% L-GLU, 1% P/S and 5 µg/ml Insulin, collecting the conditioned media (CM) on day 1, 3, 7, 11 and 14 after induction of the myogenic program. The CMs were then concentrated, separated by SDS-PAGE and the gels were stained with Coomassie Brilliant Blue.

The Coomassie-stained gel revealed several bands differentially expressed during muscle cell differentiation, in a reproducible manner in the three trials (Fig. 16). Among these, 9 bands were more clearly visible and showed fluctuations in their intensity in the course of maturation. These bands represent groups of proteins secreted by myoblasts/myotubes. More specifically, proteins 1, 3, 8 and 9 were secreted at every differentiation time point but with a fluctuating trend. Conversely, proteins 4, 5 and 6 were classified as “early” because they are present in the media collected from early stages of differentiation (day 1 and 3) and decrease or even disappear in later stages. Finally, proteins 2 and 7 were considered “late”, since they are absent 24 hours after the induction of differentiation (day 1), and secreted later, from day 3 onwards, until the end of maturation (day 14), the earlier with an increasing trend and the latter with a fluctuating trend.

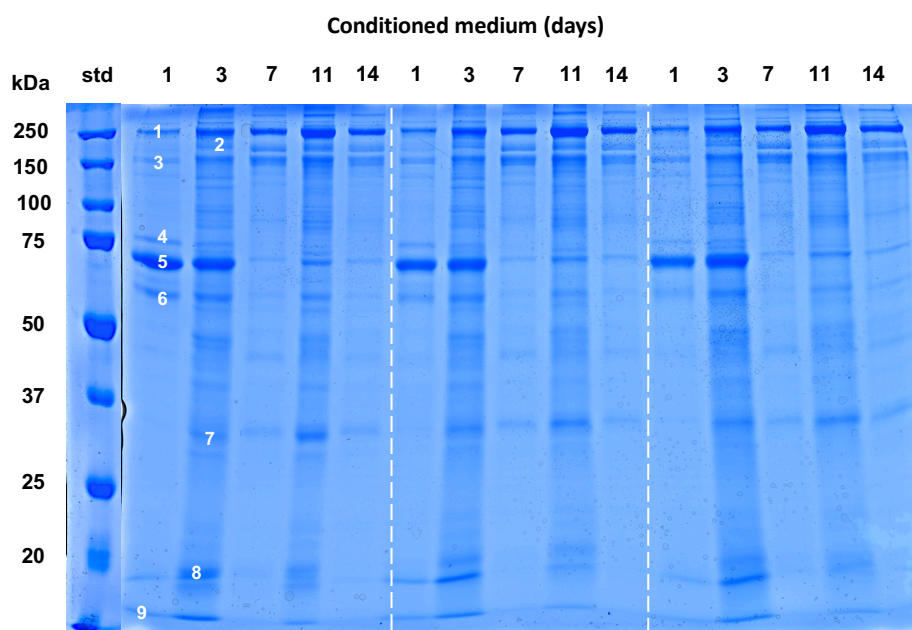


Figure 16. Coomassie-stained gel of the media conditioned by C2C12 cells in three independent experiments of differentiation.

Next, we performed Mass Spectrometry on media conditioned by C2C12 cells differentiated with two protocols: DMEM with 2% Horse serum (Fig. 17 A) and DMEM with 5 μ g/ml insulin (Fig. 17 B). We identified eleven bands of interest, according to the criterion of bands less intense and differentially expressed along maturation. The bands highlighted with dashed rectangles were labeled with the sample code (A-F; G-M) and a progressive number (1-11), excised from the gel and processed for Mass Spectrometry analysis.

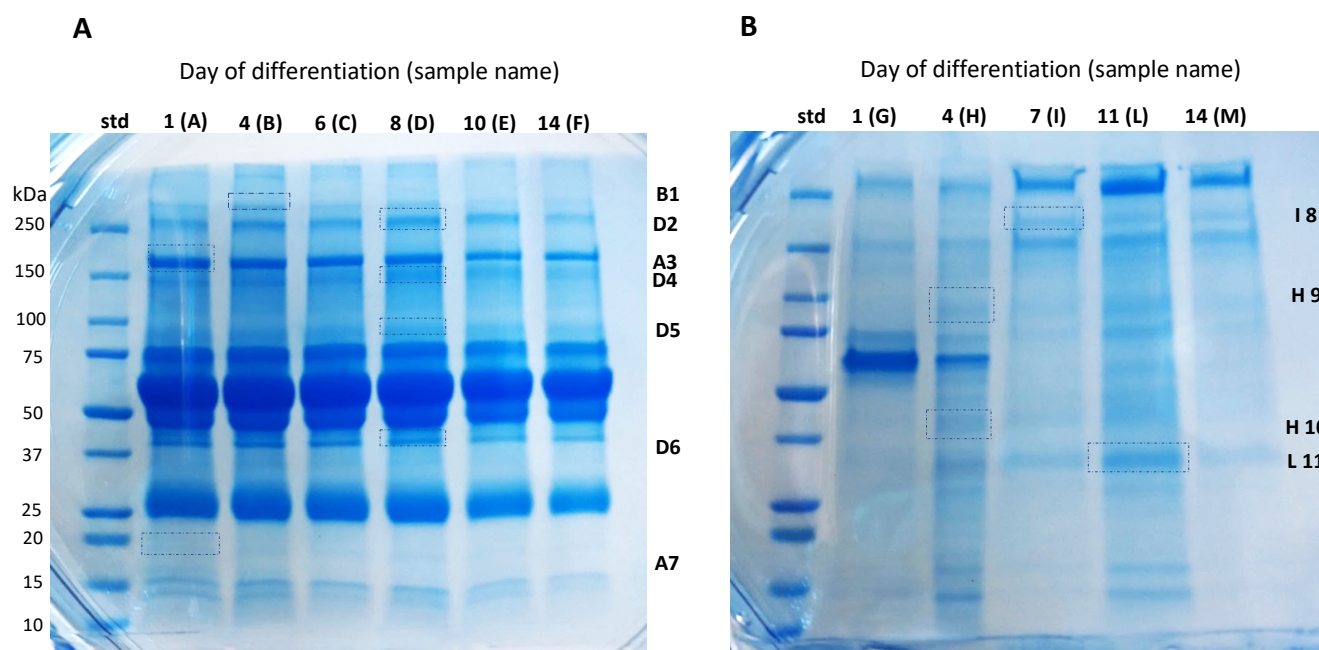


Figure 17. Representative scheme of the bands excised for Mass Spectrometry.

Through the Mass Spectrometry analysis, we found that every band contains several proteins with relative abundances; all the proteins identified in [DMEM-2% Horse Serum] and [DMEM-5 μ g/ml Insulin] differentiation protocols are listed in table 1 and table 2, respectively. All proteins were categorized in GO annotations analyzing molecular functions and biological processes (Fig. 18 A, B). In Fig. 18 A, it is clearly visible that the represented Gene Ontology categories are the same in the two procedures, except that in the Insulin protocol appear two additional functions (“transcription regulator activity” and “transporter activity”), though being represented by really few proteins (1.6% each). The majority of myogenic proteins found are involved in binding and catalytic activity. As for the biological processes, figure 18B shows that the proteins belong again to the same pathways, except for an additional process that appears only in the Insulin protocol: “developmental

processes”, though with only 1.4% total proteins. In both protocols, the majority of the proteins take part in cellular and metabolic processes, with a discrete fraction that is involved in the response to stimuli. Other represented activities are biological regulation, localization and multicellular organismal processes.

Horse Serum			
ID Uniprot	Protein name	Mass (kDa)	MASCOT Protein score
FINC_MOUSE	Fibronectin	276,017	652
VIME_MOUSE	Vimentin	53,712	552
PZP_MOUSE	Pregnancy zone protein	167,116	273
CO1A2_MOUSE	Collagen alpha-2(I) chain	129,992	188
A2MG_MOUSE	Alpha-2-macroglobulin-P	165,674	186
CO3A1_MOUSE	Collagen alpha-1(III) chain	140,112	182
CATB_MOUSE	Cathepsin B	38,168	128
CO4B_MOUSE	Complement C4-B	194,447	123
ALBU_MOUSE	Serum albumin	70,7	119
GELS_MOUSE	Gelsolin	86,287	105
CERU_MOUSE	Ceruloplasmin	121,872	102
PGS2_MOUSE	Decorin	40,126	77
PGS1_MOUSE	Biglycan	42069	75
PZP_MOUSE	Pregnancy zone protein	167,116	65
ACTB_MOUSE	Actin, cytoplasmic 1	42,052	64
FINC_MOUSE	Fibronectin	276,017	63
SPRC_MOUSE	SPARC	35,283	62
HPT_MOUSE	Haptoglobin	39,241	60
A2MG_MOUSE	Alpha-2-macroglobulin-P	165,674	58
EAF6_MOUSE	Chromatin modification-related protein MEAF6	21,636	55
NID1_MOUSE	Nidogen-1	139,302	51
TIMP2_MOUSE	Metalloproteinase inhibitor 2	24,996	49
CFAH_MOUSE	Complement factor H	143,722	48
MYPT2_MOUSE	Protein phosphatase 1 regulatory subunit 12B	109,326	47
NOS2_MOUSE	Nitric oxide synthase, inducible	131,861	46
MIME_MOUSE	Mimecan	34,333	44
FSTL1_MOUSE	Follistatin-related protein 1	35,672	41
TETN_MOUSE	Tetranectin	22,642	39
ATLA1_MOUSE	Atlastin-1	63,736	31
CYTC_MOUSE	Cystatin-C	15,749	29
PPIC_MOUSE	Peptidyl-prolyl cis-trans isomerase C	22,894	25

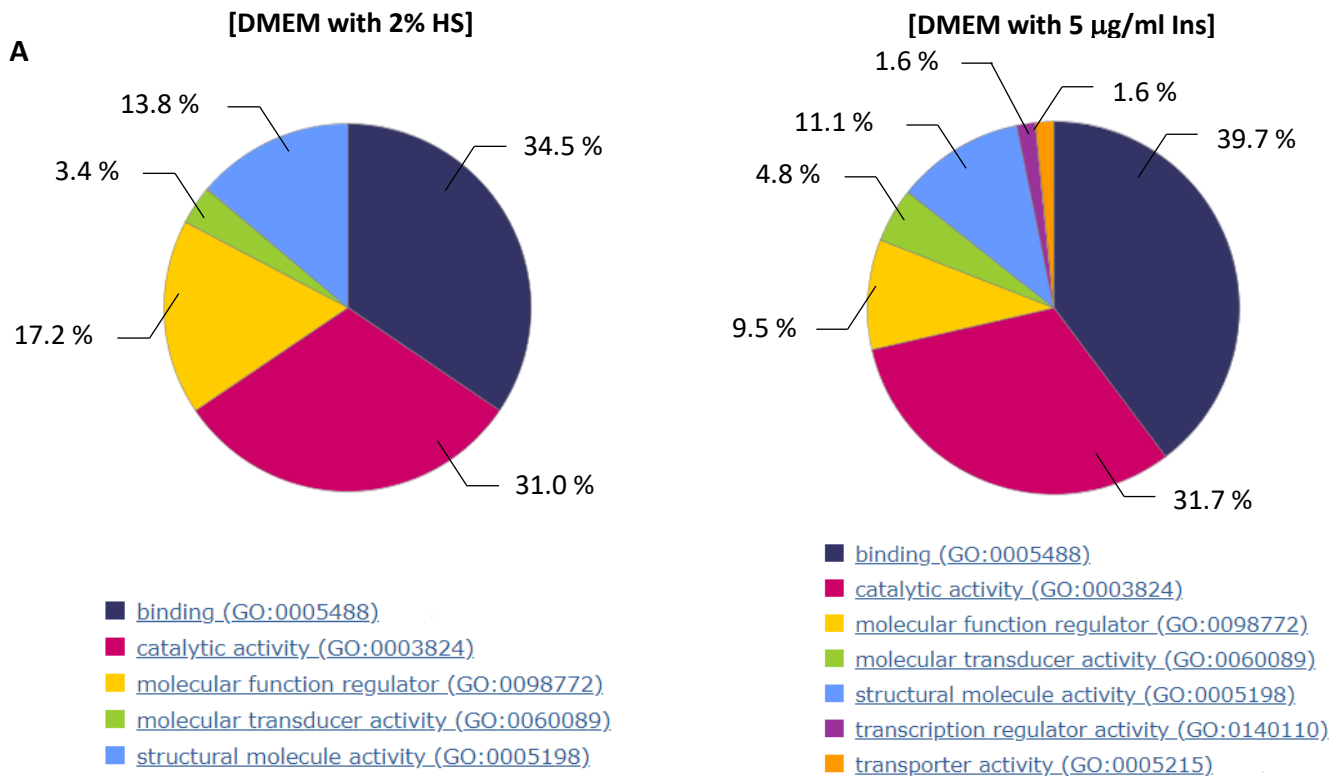
Table 1. List of the proteins secreted by C2C12 cells differentiated with DMEM-2% Horse serum and identified by MASCOT. Proteins have been ordered based on the MASCOT protein score.

Insulin			
ID Uniprot	Protein name	Mass (kDa)	MASCOT Protein score
CO3A1_MOUSE	Collagen alpha-1(III) chain	140,112	1969
CO1A2_MOUSE	Collagen alpha-2(I) chain	129,992	1316
CO1A1_MOUSE	Collagen alpha-1(I) chain	138,974	501
LDHA_MOUSE	L-lactate dehydrogenase A chain	36,817	318
ANXA1_MOUSE	Annexin A1	38,995	307
ALDOA_MOUSE	Fructose-bisphosphate aldolase A	39,787	283
PGK1_MOUSE	Phosphoglycerate kinase 1	44,921	192
ANXA2_MOUSE	Annexin A2	38,937	168
CO3A1_MOUSE	Collagen alpha-1(III) chain	140,112	144
CO1A1_MOUSE	Collagen alpha-1(I) chain	138,974	140
ACTB_MOUSE	Actin, cytoplasmic 1	42,052	140
FPPS_MOUSE	Farnesyl pyrophosphate synthase	40,898	132
CS1A_MOUSE	Complement C1s-A subcomponent	78,347	89
SFRP2_MOUSE	Secreted frizzled-related protein 2	34,473	79
SPB6_MOUSE	Serpin B6	42,913	73
LEG3_MOUSE	Galectin-3	27,612	64
PGS2_MOUSE	Decorin	40,126	61
SAHH_MOUSE	Adenosyl homocysteinase	48,17	59
CAPG_MOUSE	Macrophage-capping protein	39,501	59
CO5A1_MOUSE	Collagen alpha-1(V) chain	184,248	59
LMNA_MOUSE	Prelamin-A/C	74,478	58
TERA_MOUSE	Transitional endoplasmic reticulum ATPase	89,95	57
CATB_MOUSE	Cathepsin B	38,168	56
HS90B_MOUSE	Heat shock protein HSP 90-beta	83,571	55
EF2_MOUSE	Elongation factor 2	96,222	52
SPRC_MOUSE	SPARC	35,283	52
HS90A_MOUSE	Heat shock protein HSP 90-alpha	85,134	51
SODE_MOUSE	Extracellular superoxide dismutase [Cu-Zn]	27,717	50
AATC_MOUSE	Aspartate aminotransferase, cytoplasmic	46,504	46
MSLN_MOUSE	Mesothelin	70,177	46
ANXA3_MOUSE	Annexin A3	36,533	46
FINC_MOUSE	Fibronectin	276,017	45
ILEUA_MOUSE	Leukocyte elastase inhibitor A	42,719	45
NID2_MOUSE	Nidogen-2	156,61	44
AEBP1_MOUSE	Adipocyte enhancer-binding protein 1	129,026	43
1433E_MOUSE	14-3-3 protein epsilon	29,326	40

IDHC_MOUSE	Isocitrate dehydrogenase [NADP] cytoplasmic	47,044	39
ROA2_MOUSE	Heterogeneous nuclear ribonucleoproteins A2/B1	37,437	38
TIMP1_MOUSE	Metalloproteinase inhibitor 1	23,298	37
EF1A1_MOUSE	Elongation factor 1-alpha 1	50,424	36
CATL1_MOUSE	Cathepsin L1	38,093	36
ANXA5_MOUSE	Annexin A5	35,787	35
FSTL1_MOUSE	Follistatin-related protein 1	35,672	34
ROAA_MOUSE	Heterogeneous nuclear ribonucleoprotein A/B	30,926	33
MDHC_MOUSE	Malate dehydrogenase, cytoplasmic	36,659	29
TPM1_MOUSE	Tropomyosin alpha-1 chain	32,718	27
FINC_MOUSE	Fibronectin	276,017	27
TSP1_MOUSE	Thrombospondin-1	133,555	26
GELS_MOUSE	Gelsolin	86,287	24
MYH9_MOUSE	Myosin-9	227,429	22
IBP7_MOUSE	Insulin-like growth factor -binding protein 7	29,977	21
COPD_MOUSE	Coatmer subunit delta	57,649	21
DOC11_MOUSE	Dedicator of cytokinesis protein 11	240,242	21
MDHM_MOUSE	Malate dehydrogenase, mitochondrial	36,045	21
ACTB_MOUSE	Actin, cytoplasmic 1	42,052	20
ANXA4_MOUSE	Annexin A4	36,178	19
BIG3_MOUSE	Brefeldin A-inhibited guanine nucleotide-exchange protein 3	243,245	17
PHAG1_MOUSE	Phosphoprotein associated with glycosphingolipid-enriched microdomains 1	47,147	16

Table 2. List of the proteins secreted by C2C12 cells differentiated with DMEM-5 µg/ml insulin and identified by MASCOT. Proteins have been ordered based on the MASCOT protein score.

Molecular function



Biological process

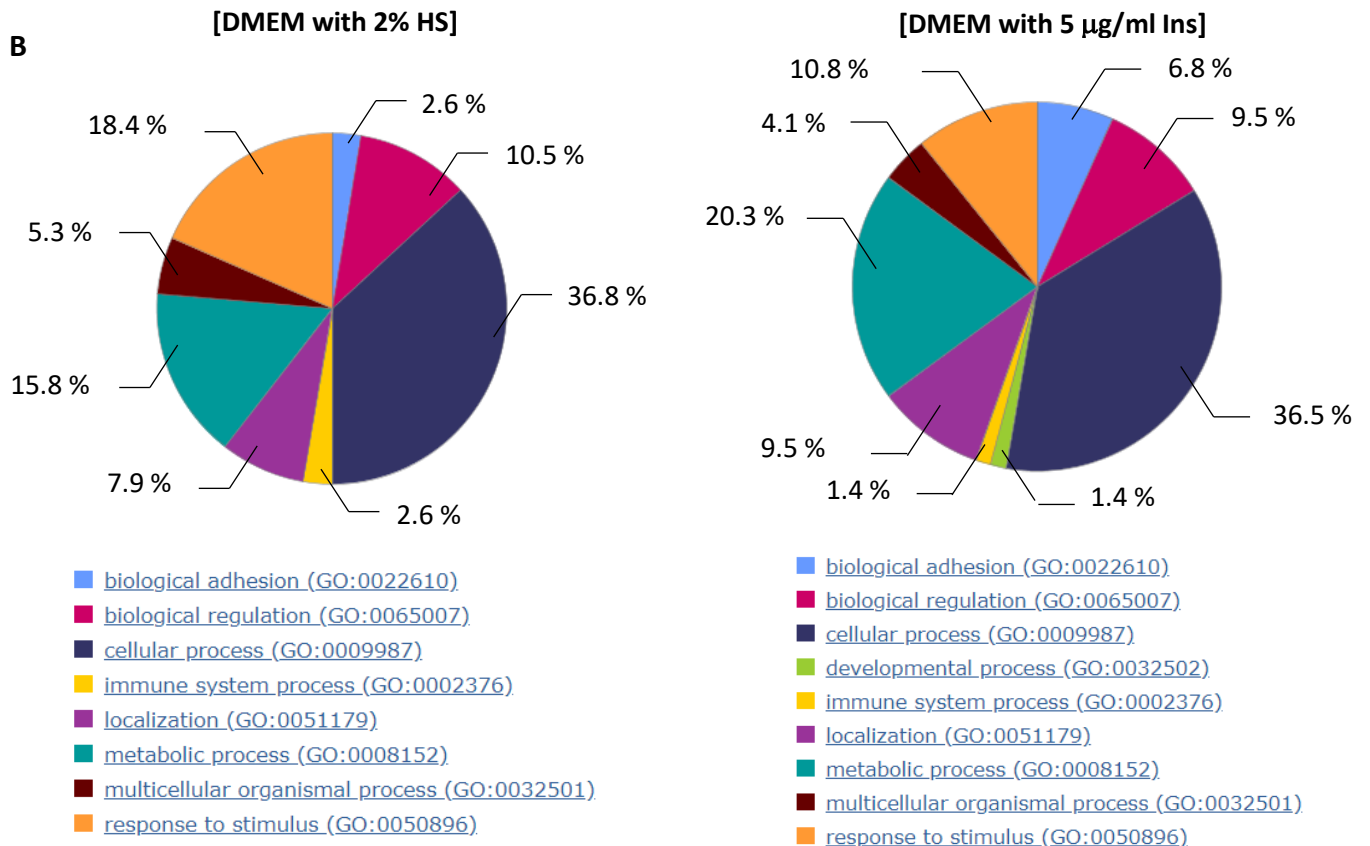


Figure 18. Enrichment analysis of the hits found by means of mass spectrometry (LCMSQE). Myogenic proteins were clustered based on two PANTHER ontology classes: (A) molecular function and (B) biological process.

Being particularly interested in finding secretory proteins putatively responsible for the alterations seen in osteogenic differentiation, we sought to identify proteins that could somehow interact with the modulation of bone homeostasis, both at the systemic and cellular level. To do so, we analyzed all the hits found by LCMSQE analysis by means of proteomic and interatomic databases and we validated the results with literature. A series of proteins were identified as belonging to the Gene Ontology (GO) annotations: skeletal system development (GO:0001501), response to cytokines (GO:0034097) and response to mechanical stimuli (GO:0009612).

Under the skeletal system development annotation, we found collagen type I alpha 2 (Col1a2) and collagen type III alpha 1 (Col3a1), detectable in both protocols; C-type lectin domain family 3 member b (Clec3b) was present only in the Horse serum protocol and collagen type I alpha 1 (Col1a1) and galectin-3 were found only in the Insulin protocol.

Under the response to cytokine annotation, we found collagen type III alpha 1 (Col3a1) and secreted acidic cysteine rich glycoprotein (SPARC) in both protocols, while two tissues inhibitor of metalloproteinase (TIMP) proteins were found, respectively TIMP1 in the Insulin protocol and TIMP2 in the Horse serum one.

Finally, under the response to mechanical stimuli annotation we found collagen type III alpha 1 (Col3a1) and Decorin (Dcn) secreted with both protocols.

In conclusion, we provide evidence that several proteins secreted by C2C12 muscle cells along differentiation, with two different protocols, act on bone remodeling and homeostasis in various ways. However, further investigations are required to establish their impact as single factors and/or as a combination of myokines on osteogenic differentiation.

In addition, we sought to identify Sclerostin in conditioned media by LCMSQE. To get a Sclerostin-enriched preparation, we excised the Coomassie-stained band at the predicted Sclerostin molecular weight and we also immunoprecipitated the protein (data not shown). However, we could never identify this protein by Mass Spectrometry. This may be explained by glycosylation of sclerostin that may interfere with its detection⁷⁸.

4. *In vivo* characterization of Sclerostin expression

To corroborate the production of Sclerostin by muscle cells *in vitro*, we moved to an *in vivo* context by analyzing the presence of Sclerostin in muscles of mice of different ages (young, 6 weeks; adult, 5 months; old, 18 months). For each age we isolated: i) a muscle composed of fast or glycolytic fibers (Gastrocnemius, GA); ii) a muscle composed of slow or oxidative fibers (Soleus, SO), which are subjected to phasic movement and important muscular work; iii) a muscle of the back (Trapezius, TR), less loaded according to the phasic activity of the limbs, to observe eventual relations between muscular work and sclerostin production. Also, a muscle of the upper limb (Triceps Brachii, TB) was isolated, to compare it with the muscles of the lower limb (Fig. 19).

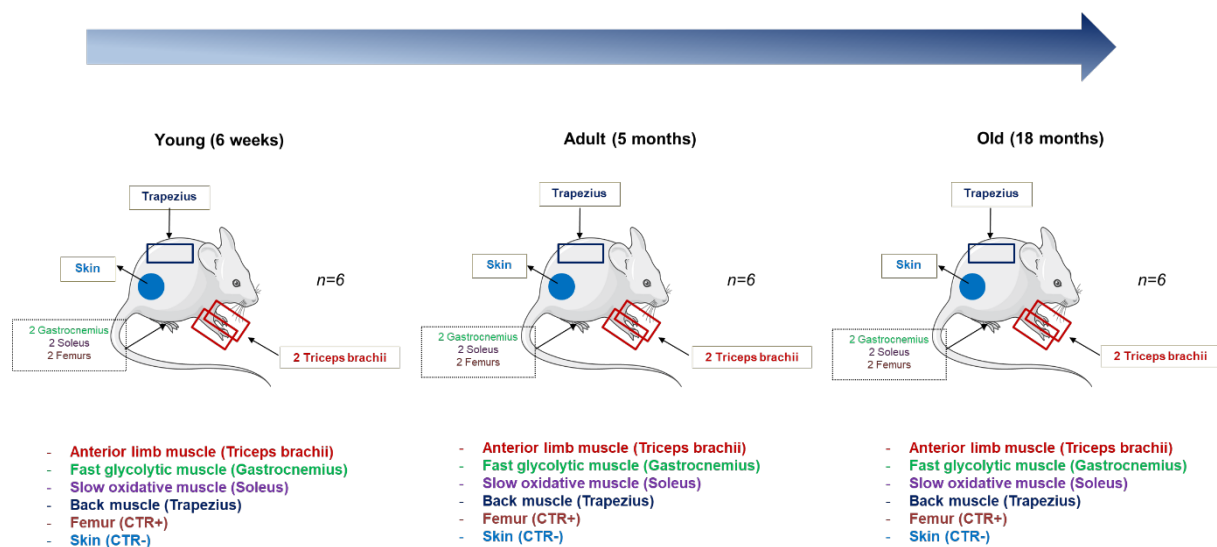


Figure 19. Graphical scheme of the experiment.

4.1 Molecular characterization of Sclerostin expression

First, in order to analyze the presence and the eventual amount of Sclerostin in muscles with different metabolic profiles, we performed Western Blot and ELISA assays. Two animals per age group were probed ($n=2$) and the same muscle was employed for both assays, except for the Soleus muscle, which was only tested with ELISA due to its small size (Fig. 20 A, B). The Sclerostin concentration yielded by ELISA (pg/ml) was normalized on the muscle weight (pg/mg). The results obtained with Western Blot show that Sclerostin is synthesized by all the muscles analyzed, with the back muscle (Trapezius) having a higher amount of the protein than the others in all the three groups

(Fig. 20 A). The ELISA assay showed a similar trend; Soleus values were comparable to the other muscles and the Sclerostin concentration in Trapezius was higher than the other fibers only in adult and old groups, though without reaching statistical significance ($p>0.05$).

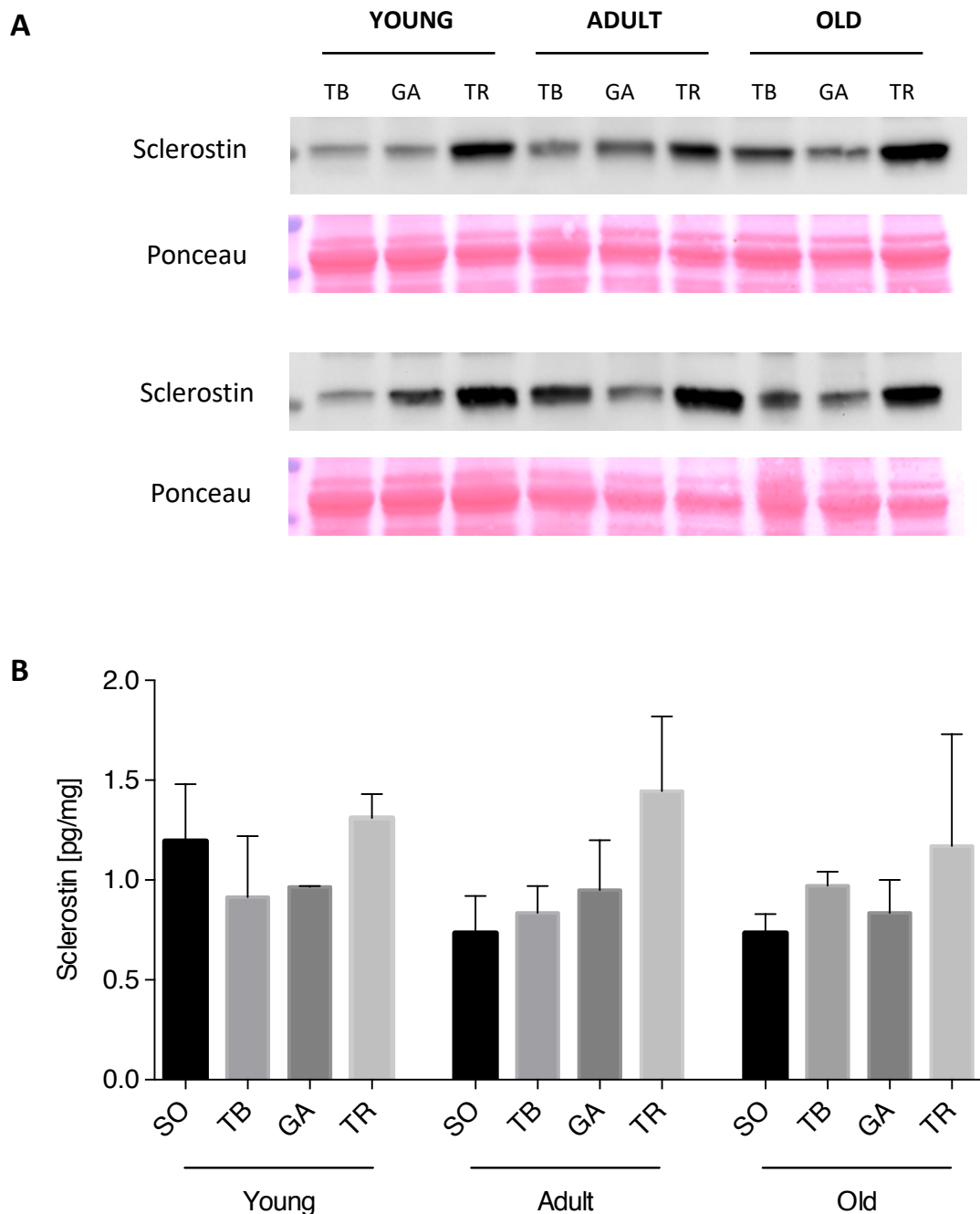


Figure 20. A) Western Blot for mouse Sclerostin of $n=2$. B) Normalized Sclerostin concentration (pg/mg) in muscles of differently aged mice. SO, Soleus; TB, Triceps brachii; GA, Gastrocnemius; TR, Trapezius. Values are expressed as mean $\pm$ SEM. One-way ANOVA was performed within each age group with Fisher's LSD test.

4.2 Immunohistochemical characterization of Sclerostin expression

Next, we analyzed Sclerostin expression by immunohistochemistry to detect its localization in muscle fibers (Fig. 21). Femur and skin were chosen as positive and negative controls, respectively. In femurs, Sclerostin appeared with a clear DAB-specific staining limited to some lacunae, coherently with the fact that some osteocytes are positive for Sclerostin and others negative; in skin there was no specific staining.

The femur C- was negative, meaning that the anti-sclerostin antibody is selective and specific.

All the muscles analyzed were negative, even if Sclerostin was detected with Western Blot and ELISA assays. This apparent discrepancy can be explained from a technical point of view (see below – Section Discussion).

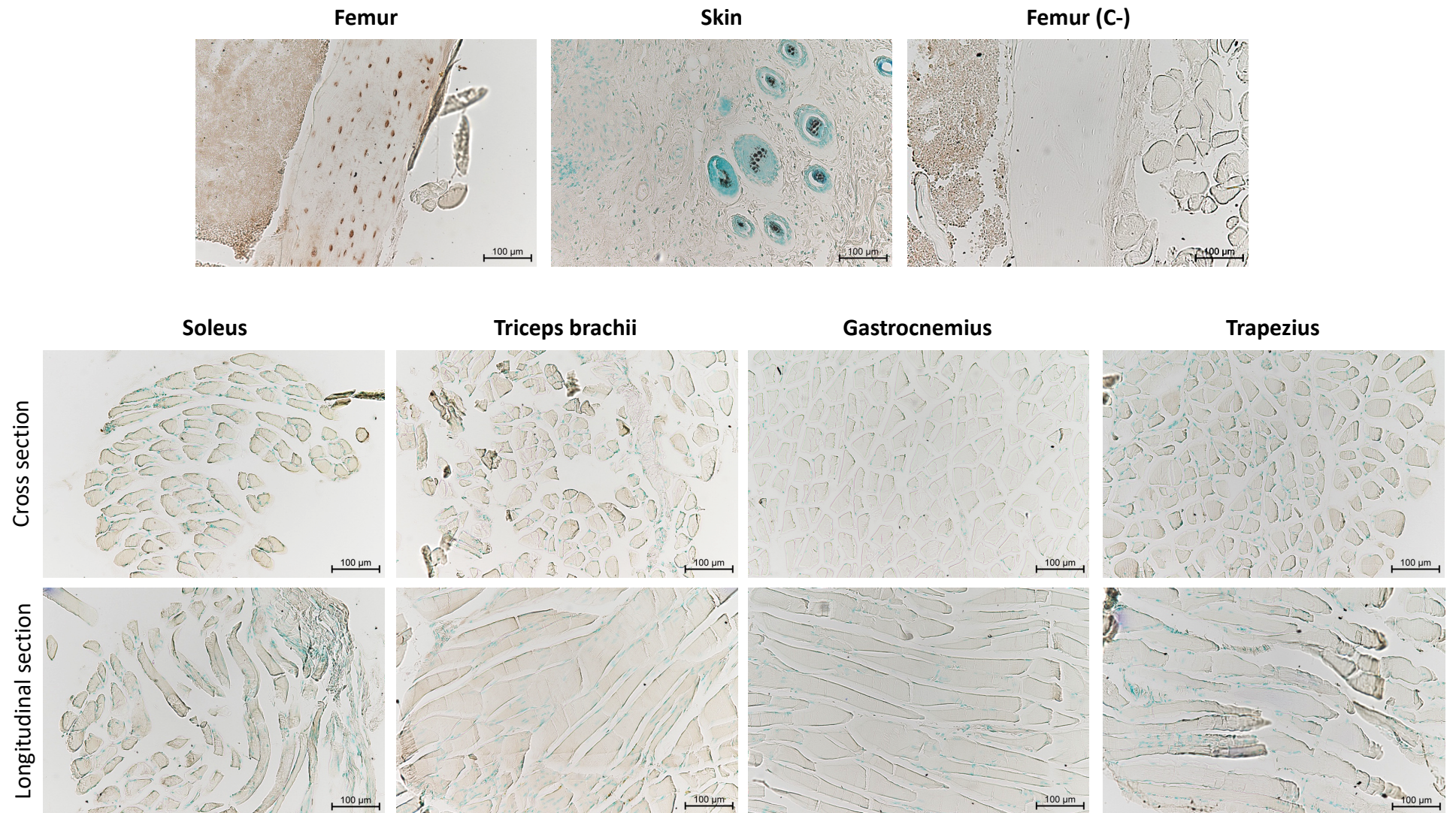


Figure 21. Representative immunohistological images of femur (positive control), skin (negative control), femur stained without primary antibody (femur C-), transversal and sagittal sections of muscles (soleus, triceps brachii, gastrocnemius, trapezius). The brown areas are DAB-stained.

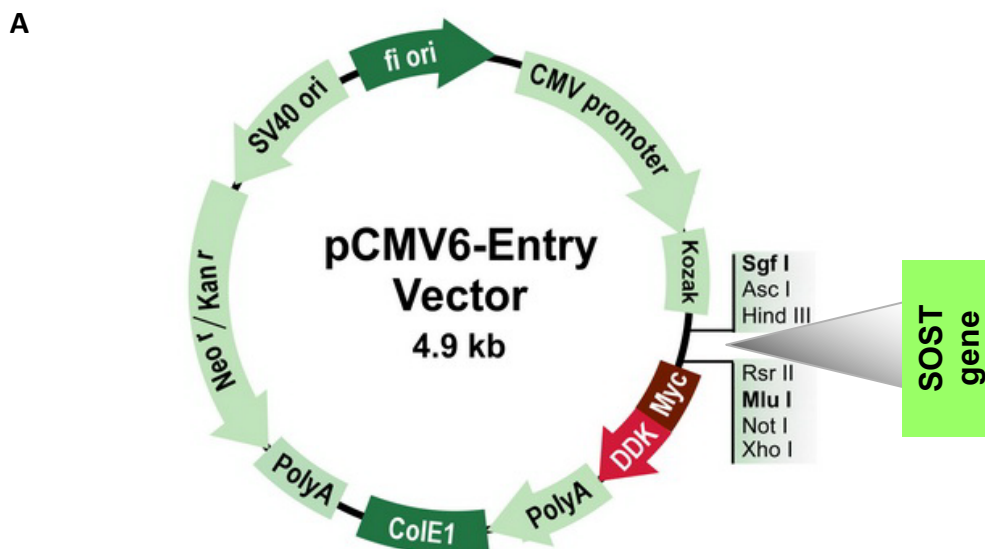
5. *In vivo* muscle-specific overexpression of Sclerostin

We performed an overexpression of Sclerostin in muscles of young mice (11 days of age) via electroporation (ET) of a plasmid containing the SOST gene (Fig. 22 A) in order to investigate the effects of a muscle-specific overproduction of the protein.

This study was based on 3 experimental groups: Control (CTR, 3 mice sacrificed at 25 days of age without electroporation); EMPTY-VECTOR (EV-ET, 3 mice electroporated with pCMV6 vector) and SCLEROSTIN-VECTOR (SOST-ET, 3 mice electroporated with pCMV6 vector in which the SOST gene was cloned) (Fig. 22 B).

The electroporation was first performed at 11 days of age and repeated 6 days later, since the exogenous DNA is stable for nearly 7 days; the animals were sacrifice at 25 days of age. The muscles selected were: quadriceps femoris (QF), gastrocnemius (GA) and tibialis anterior (TA) of both limbs, which allowed to target the skeletal segments of femur and tibia with the paracrine secretion of Sclerostin.

This experiment was aimed to: i) monitor SOST overexpression in the electroporated muscles and Sclerostin secretion into the circulation, ii) verify the degree of muscular damage induced by ET, iii) observe whether and to what extent the whole skeleton and the bones adjacent to the electroporated muscles (femur and tibia) are affected by the increase of Sclerostin.



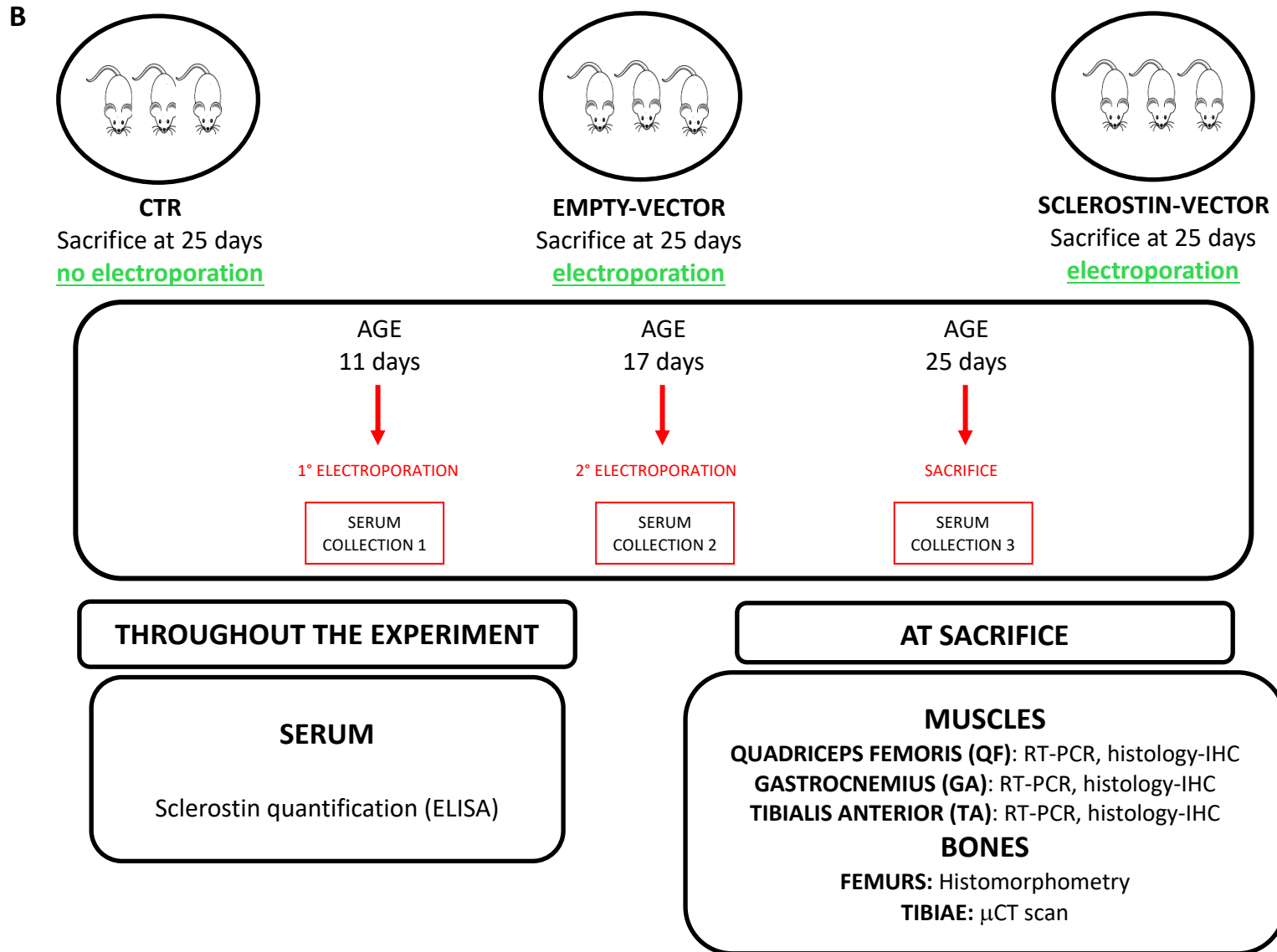


Fig 22. A) Genetic structure of the mammalian vector used to target the SOST gene. B) Graphical scheme of the experiment.

5.1 Analysis of SOST expression in muscles and in blood serum

First, in order to evaluate the efficiency of electroporation, we performed quantitative reverse transcription-PCR (qRT-PCR) analysis to detect both the myc-tagged mouse gene SOST, index of exogenous SOST, and the mouse SOST gene, whose expression includes the endogenous and exogenous ones (Fig. 23 A). Results showed a higher level of myc-tagged SOST in all muscles electroporated, compared to the ones injected with the empty vector; this comparison reached statistical significance ($p < 0.05$) in Tibialis Anterior (TA) and Gastrocnemius (GA) muscles. This result was confirmed by the analysis of SOST expression, which reached significance ($p < 0.01$) only in GA. Next, we evaluated the quantity of circulating Sclerostin with an ELISA assay in animal sera before the first electroporation, as a starting level and at the moment of sacrifice (Fig. 23 B). The physiological concentration of circulating Sclerostin was significantly higher ($p < 0.05$) in SOST-electroporated animals (mean $\pm$ SD: 360.7 ± 18.05) compared to the EV-electroporated (mean $\pm$ SD: 273.3 ± 30.3), probably due to experimental variability. At sacrifice, the circulating Sclerostin was higher in SOST-ET (mean $\pm$ SD: 385.5 ± 68.5) compared to EV-ET (mean $\pm$ SD: 320.7 ± 22.3) animals, though not significant. Taken together, these data confirm that the plasmid electroporation was successful, since it yielded an increase in SOST transcript expression in the muscles targeted as well as an increase in the circulating protein.

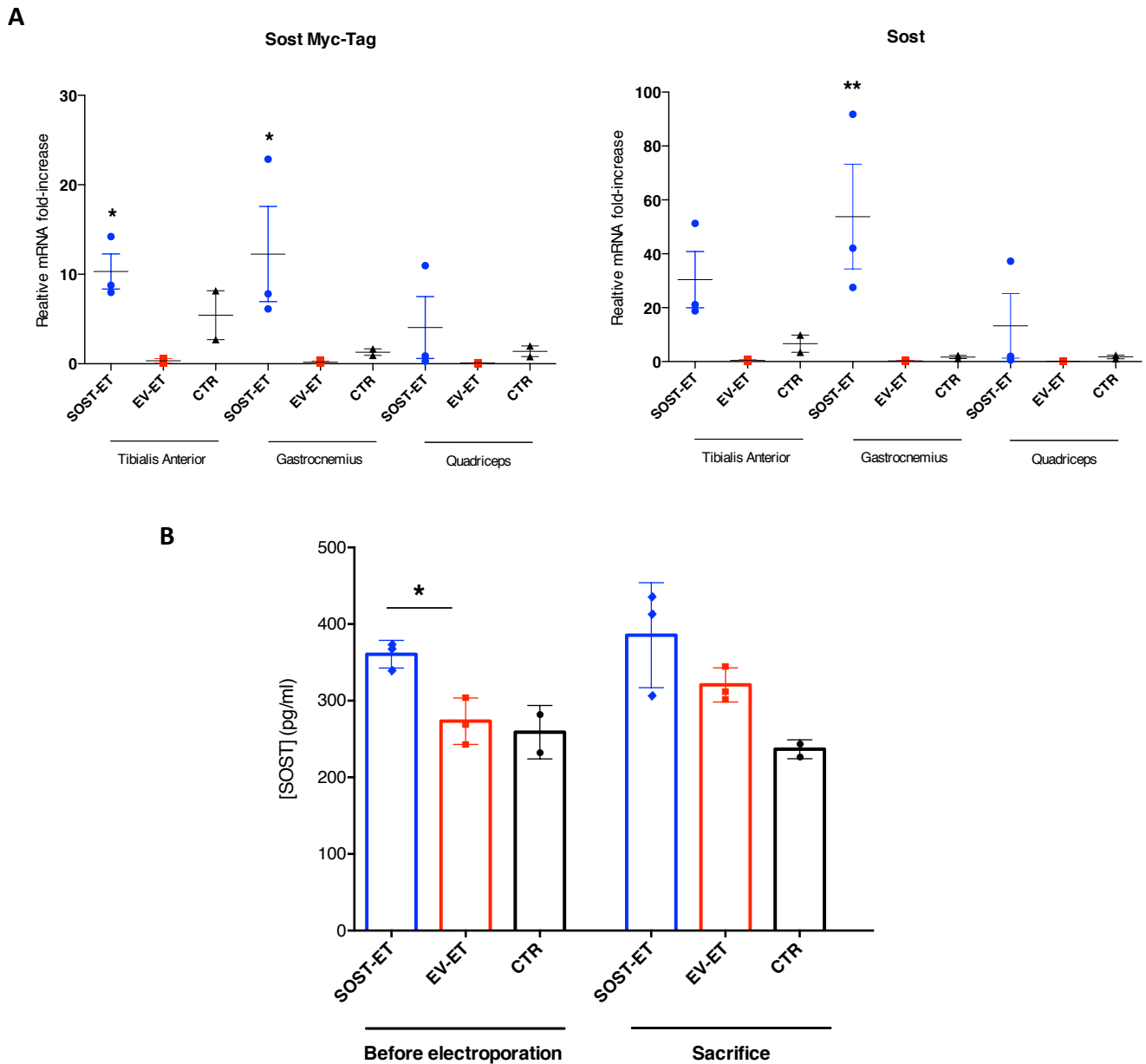


Figure 23. A) RT-PCR for mouse Myc-tagged SOST and endogenous SOST. B) Serum Sclerostin levels before SOST overexpression and at sacrifice.

5.2 Local reactions to muscle electroporation

The damage induced by the electroporation was assessed by clinical observation, histological and immunohistochemical examination of muscle sections. Clinical symptoms were not observed in any of the mice during the observation period of two weeks. At sacrifice, the electroporated muscles were stained with Hematoxylin and Eosin (H&E) and with anti-CD34 and anti-LCA antibodies (Fig. 24).

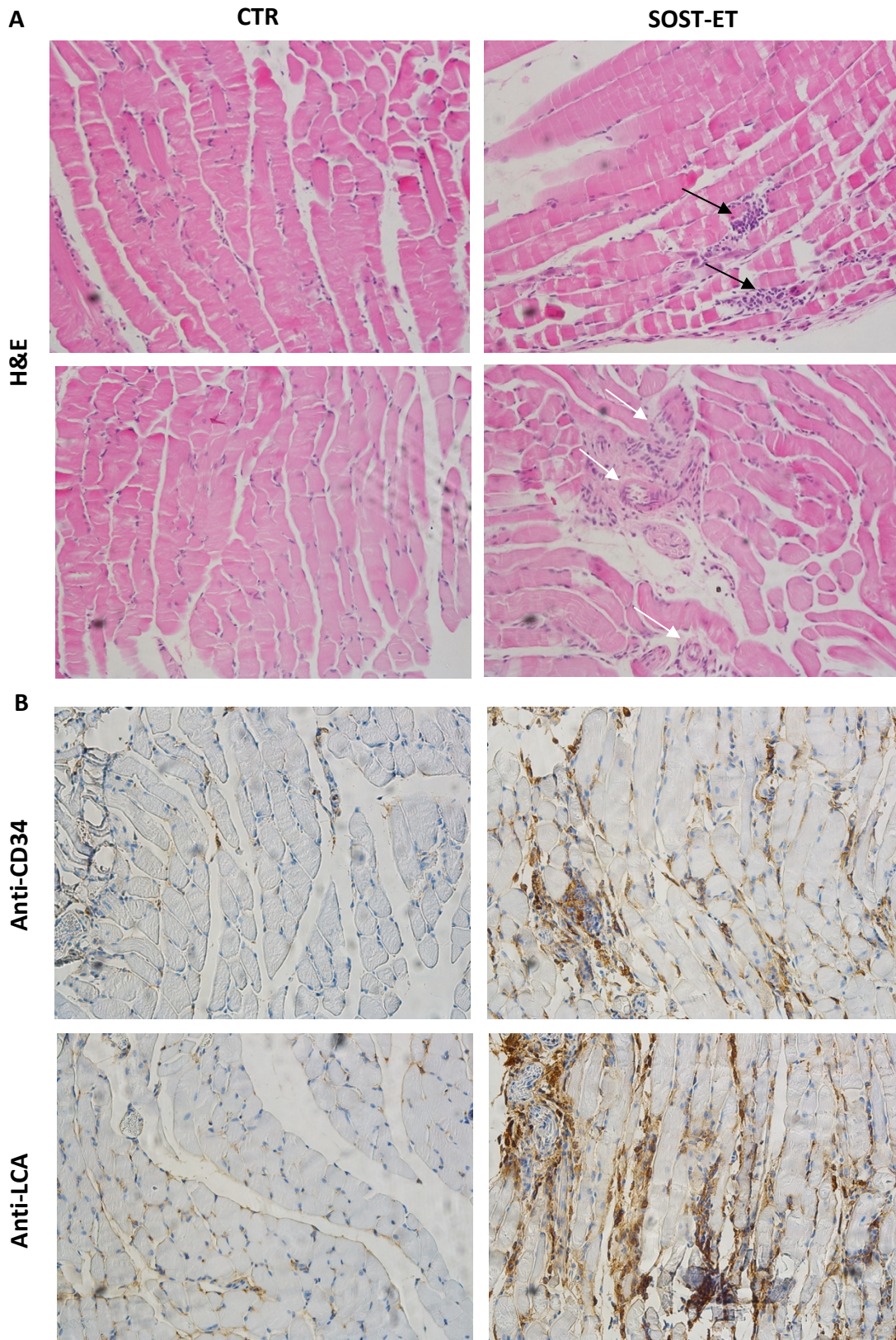


Figure 24. The photographs show representative histological sections of the tibialis anterior of C57BL/6 mice. Magnification 20X. A) Sections of CTR and SOST-ET mice stained with H&E. The black arrows indicate inflammatory infiltrate and white arrows indicate vascularization sites. B) Sections stained with anti-CD34 and anti-LCA antibodies.

Microscopic examination of H&E stained fibers in SOST-electroporated mice revealed the presence of inflammatory infiltrates (Fig. 24A, black arrows), whereas control samples present fibers correctly arranged with subsarcolemmal (i.e., peripheral) nuclei. The inflammatory infiltrates indicate infiltration by mast cells, eosinophils and mononuclear cells in plasmid-injected muscles. Moreover, the immunohistochemical localization of human leukocyte common antigen (LCA) (Fig. 24B), a major membrane glycoprotein restricted to leukocytes, showed an evident recall of granulocytes.

Despite the induction of inflammation, newly formed vessels were visible in electroporated mice (Fig. 24A, white arrows), suggesting the induction of muscle regeneration. Indeed, neovascularization was confirmed by the staining with anti-CD34, detecting a transmembrane phosphoglycoprotein expressed almost ubiquitously by hematopoietic cells⁷⁹. Electroporated muscles present positively-stained regions at the level of muscle mass and the surrounding connective tissue (Fig. 24 B).

Taken together, these data suggest that the electroporation procedure induced inflammatory reactions, however counterbalanced by the onset of regenerative processes.

5.3 Analysis of SOST overexpression effects on the skeleton

5.3.1 X-ray scans

In order to evaluate the effects of a transient muscular overexpression of Sclerostin, we analyzed the bone responses at the systemic level of SOST-electroporated, EV-electroporated and control littermate mice using X-ray scans. The radiographic images were jointly observed by two experts who were blind to other experimental information. The following parameters were evaluated: i) skeletal malformations of individual bone segments, ii) changes in bone density, iii) focal structural alterations of bone segments, iv) alterations of the juxta-osseous soft tissues (calcifications or masses). From the radiographic comparison of the skeleton of the CTR group (non-electroporated) and of the electroporated groups (EV- or SOST-electroporated), it emerged that no growth anomalies were found following the electroporation treatment (Fig. 25). Furthermore, there are no

appreciable differences, at least at the macroscopic skeletal level, inducible to the electroporation treatment of the vector for Sclerostin.

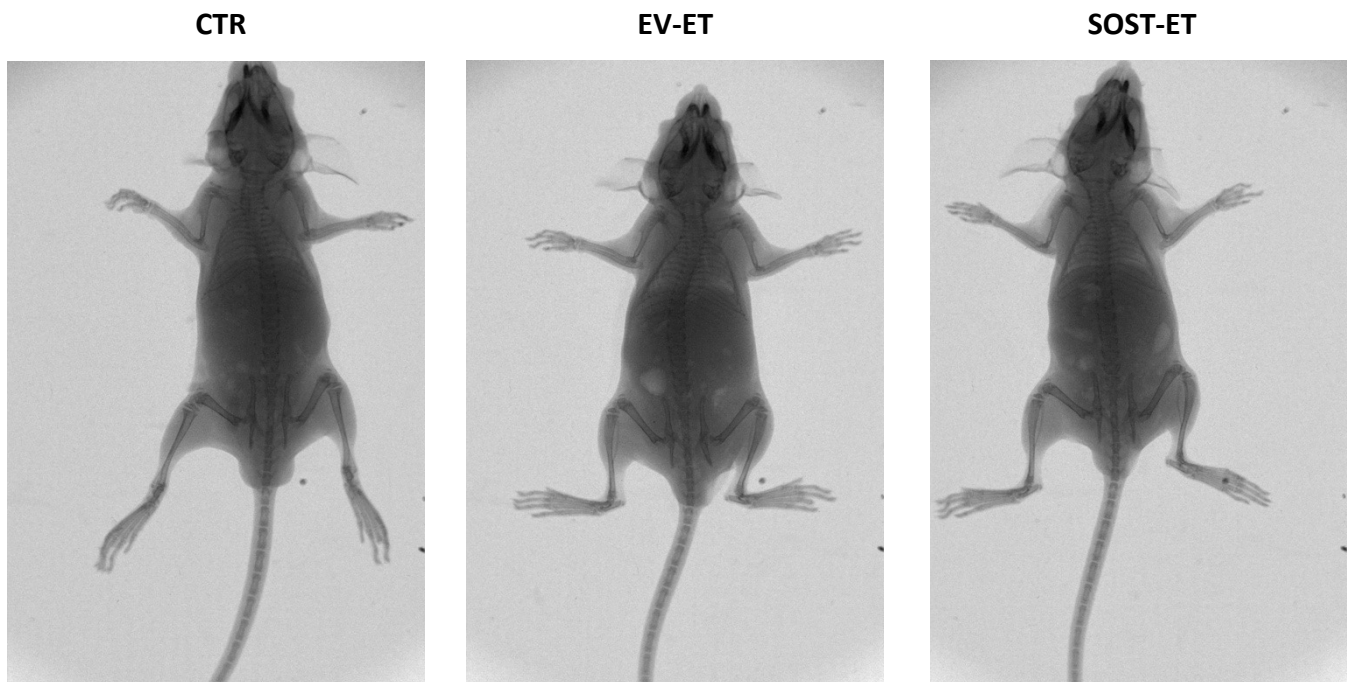


Figure 25. The photographs show representative x-ray scans of the skeleton of each mice group.

5.3.2 Histology and histomorphometric analysis of femurs

Histological observations of bone sections of femurs from EV-electroporated (EV-ET), SOST-electroporated (SOST-ET) and control (CTR) animal groups are shown in Fig. 28. SOST-ET animals were compared to the EV-ET littermates and CTR mice were taken as a reference.

As it is expected, the bone amount was clearly lower in SOST-ET, with respect to EV-ET and CTR animals. Comparing the histological sections, it emerged that bone mass appears more abundant in EV-ET with respect to SOST-ET sections, as confirmed by the histomorphometric analysis (see below).

The results of static histomorphometry of bone mass are shown in Fig. 29. It is to be noted that the mean values of BV/TV, Tb.N and Tb.Sp reported in **A** histograms are recorded from the trabecular bone of femur, whereas the values of Ct-B.Ar and diaphyseal thickness reported in **B** are recorded from femur cortical bone.

The results obtained in trabecular bone of the femur distal metaphysis show that the mean values of BV/TV and Tb.N in the SOST-ET group were statistically lower with respect to the EV-ET group. Moreover, in the SOST-ET group the Tb.Sp value was significantly higher than in the EV-ET group. BV/TV is the ratio between the trabecular bone area (BV) and the total area (TV) (i.e., the trabecular bone volume), Tb.N is the trabecular number and Tb.Sp indicates the trabecular separation. Conversely, no statistically significant differences were found in cortical bone area (Ct-B-Ar) and diaphyseal thickness at the femoral mid-diaphyseal level among the three groups. Collectively, these data suggest that the muscular overexpression of Sclerostin induced a loss of trabecular bone but it did not influence cortical bone parameters. This datum is in line with the fact that trabecular bone, compared to cortical bone, has a higher turnover rate and is more metabolically active than cortical bone⁸⁰, thus being more easily influenced.

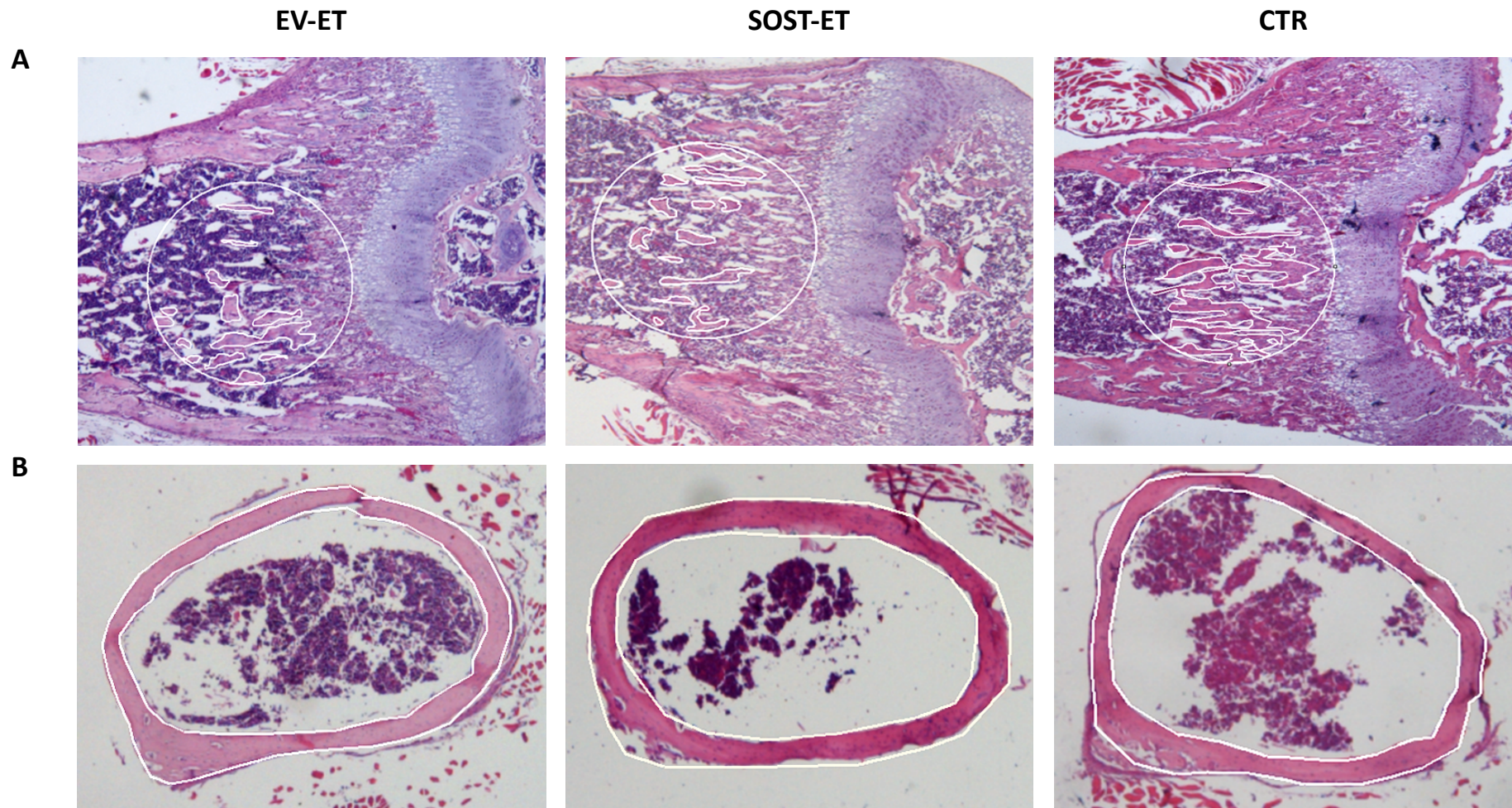


Figure 28. Exemplificative histological sections (5 μ m) of femurs of non-electroporated (CTR), Empty vector- and SOST-electroporated animals in which the histomorphometric analyses were performed. A) Sagittal section of the distal third of femur (4X magnification); B) transversal section at the mid-diaphyseal level of femur (10X magnification). The white line indicates the areas in which histomorphometric evaluations were recorded.

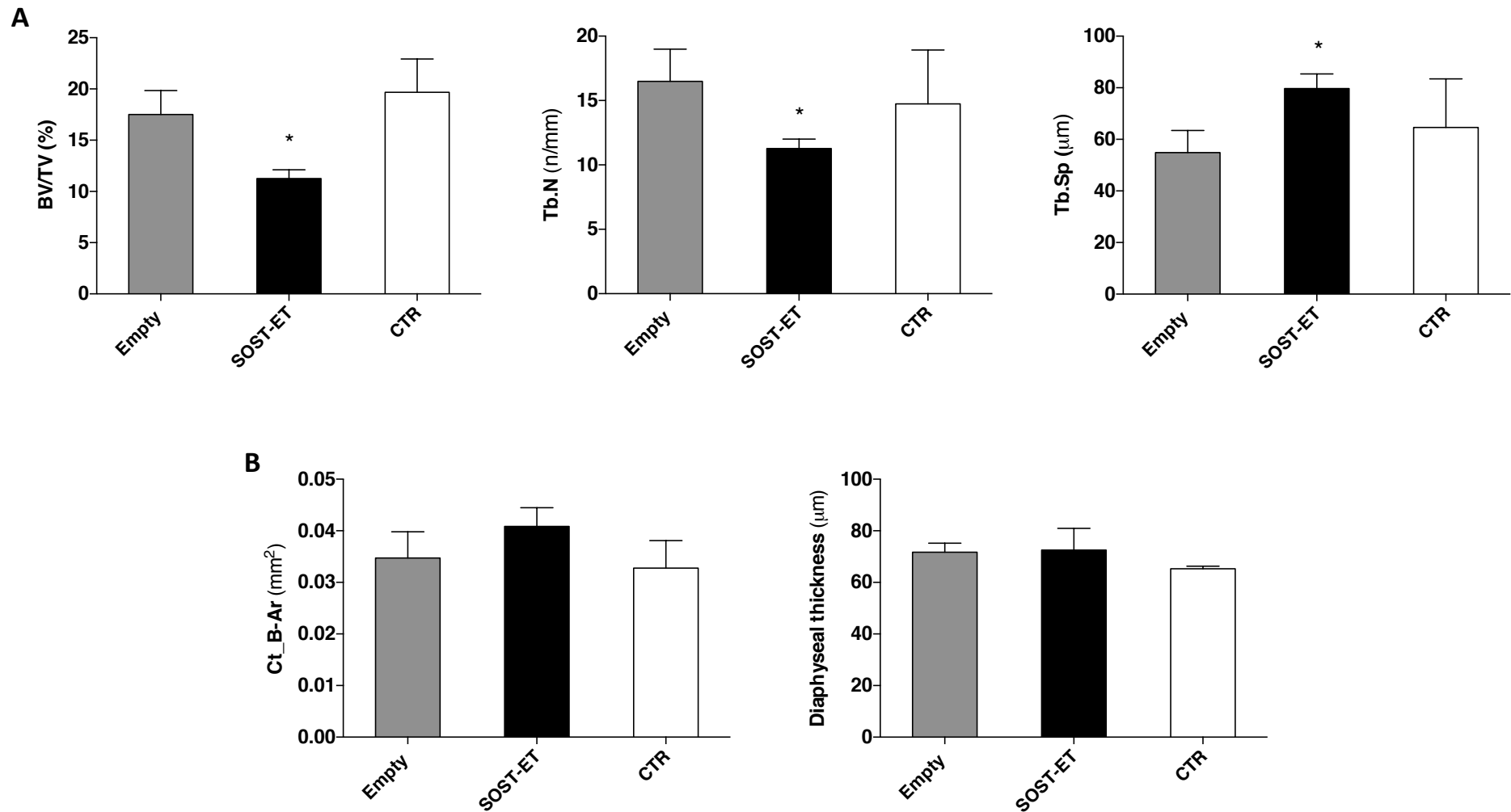


Figure 29. Mean values of histomorphometric parameters, expressed as BV/TV (%), Tb.N (n/mm), Tb.Sp (μm), Ct_B-Ar (mm²) and diaphyseal thickness (μm), in both trabecular and cortical bone of the three animal groups. A) Sagittal section of the distal third of femur; B) transversal section at the mid-diaphyseal level of femur. Values are expressed as mean ± SEM. *P<0.05 versus EV-ET (Student's t test).

5.3.3 μ CT analysis of tibiae

Concerning the transient overexpression of Sclerostin in muscles of growing mice via electroporation of a plasmid containing the SOST gene, in order to investigate the effects of the muscle-specific protein overproduction, micro-CT analyses were performed and compared to data obtained by static histomorphometry.

At the regional level (epiphysis, metaphysis, epiphyseal plate and diaphysis), no significant changes were detected in any of the features analyzed, neither in response to the EV-electroporation, nor in response to SOST- electroporation.

For each tibia, axial sections [from distal epiphysis to middle diaphysis] were acquired and then the coronal sections were extracted, on which the analysis of the epiphysis, metaphysis and epiphyseal plate were performed. The metaphysis was then further segmented through the subdivision of metaphyseal trabeculae into 16 different types (classes). Figure 26 shows exemplificative coronal sections (A), the heat maps that clarify the composition of trabecular classes (B) and the class legend (C).

Analyses were focused on two types of bone trabeculae differently arranged, with respect to the metaphyseal growing plate, in longitudinal (class-7, PERP) and parallel (class-13, PAR) mode, representing respectively more or less load-bearing trabeculae during skeletal growth. The number of metaphyseal trabeculae and the trabecular area are shown in figure 27.

Compared to the control group, the number of trabeculae of **class 7 (PERP)** increased significantly following electroporation with empty vector (+ 30%, $p = 10^{-5}$, $p < 0.0021$), and then returned to levels comparable to those of control following electroporation with sclerostin (Fig. 27 A).

Compared to the control group, the number of trabeculae of **class 13 (PAR)** increased following EV-electroporation, albeit without significance (EV-ET compared to CTR: + 16%, $p = 0.040$, threshold $p < 0.0021$), but decreased significantly following SOST-electroporation (SOST-ET compared to EV-ET: -39 %, $p = 10^{-10}$, threshold $p < 0.0014$) (Fig. 27 A).

The percentage of metaphysis (Trabecular class area) occupied by the trabeculae of **class 7 (PERP)** increased significantly (compared to the control group) following electroporation with empty vector (+ 63%, $p = 10^{-5}$, $p < 0.0021$) and further increased following electroporation with sclerostin (Fig. 27 B).

As regards the trabecular area occupied by the trabeculae of **class 13 (PAR)**, this parameter decreased significantly in EV-electroporated animals compared to controls (EV-ET compared to CTR: -83%, $p = 10^{-12}$, threshold $p < 0.0021$) and further decreased following SOST electroporation but not significantly (SOST-ET compared to EV-ET: -29%, $p = 10^{-4}$, threshold $p < 0.0014$) (Fig. 27 B).

Therefore, micro-CT analyses showed a significant decrease of class-13 trabeculae in mice that underwent electroporation of a plasmid containing the SOST gene with respect to both mice electroporated with empty vector and controls, inversely to class-7 trabeculae that showed increased values after treatment.

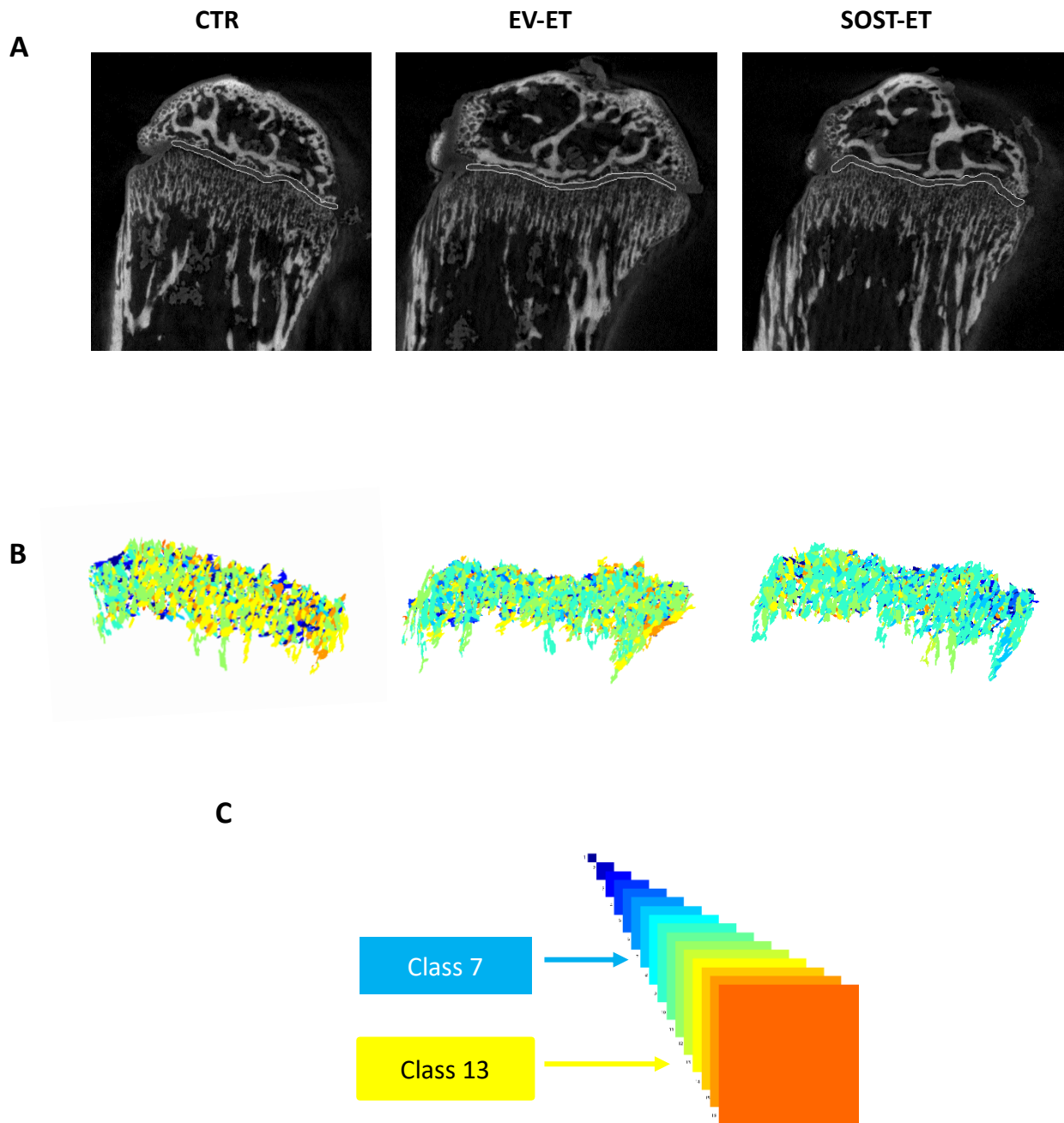


Figure 26. A) Exemplificative μ CT-scans of the metaphyseal plate. B) Heat maps that represent the space occupied by the various classes with a color code. C) Legend of the classes.

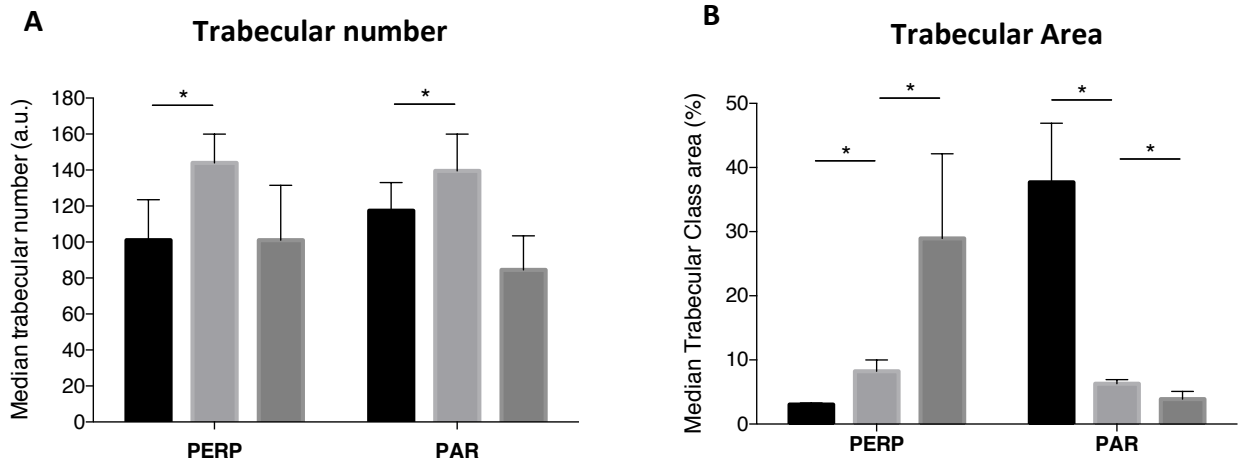


Figure 27. A) Variation in the median number [$\pm$ median absolute deviation (MAD)] of trabeculae for classes (typology) 7 and 13. B) Change in the percentage of metaphysis (median $\pm$ MAD) occupied by the trabeculae of class (typology) 7 and 13. Asterisk (*) indicates significant comparisons, according to the Wilcoxon-Mann-Whitney test with Bonferroni correction.

Discussion

Myokines are cytokines or peptides synthesized and released by myocytes in muscle tissue both at basal level and as a response to muscular contractions⁸¹. Myokines are implicated in the autocrine regulation of metabolism in muscles as well as in the para/endocrine regulation of other tissues and organs, including bone⁸². Indeed, studies have shown that bi-directional biomechanical, paracrine and endocrine signals modulate muscle and bone growth⁸³. Since the discovery of myostatin in 1997, the first myokine, secretome-based analysis of human myocyte culture medium has revealed over 600 myokines till date⁸⁴. However, the majority of these myokines are still not sufficiently characterized, yet need to be extensively investigated to gain insight into the pathogenesis of musculoskeletal diseases.

In this study, we proposed to model *in vitro* the muscle-to-bone communication with an osteogenic (2T3) and a myogenic (C2C12) cell line. After having established efficient differentiation protocols, we differentiated 2T3 osteoblastic cells in a medium conditioned by early, mid or late C2C12 muscle cells, to evaluate the effect of sequential patterns of myokines. We found that the early conditioned medium altered significantly the functional maturation of osteoblasts. Therefore, we hypothesized the presence of muscle cell-secreted factors that may exert an inhibitory action on osteogenesis.

Since Sclerostin, an inhibitor of the Wnt/ β -catenin pathway that impairs bone formation, has become an attractive therapeutic target for treating osteoporosis, we wondered if this molecule could also be produced by muscle cells. Despite being considered an osteocyte-specific protein, *SOST* mRNA expression was found in other organs, including skeletal muscle⁷⁶, but no one had ever characterized its actual synthesis by muscle in detail.

Thus, we analyzed *SOST*/Sclerostin mRNA and protein expression in C2C12 muscle cells and its secretion into the culture medium, discovering that it is produced at all these levels. Sclerostin was also detected in C57 primary muscle cells by RT-PCR, Western Blot and Immunofluorescence.

Speculating that in media conditioned by C2C12 cells there could be other secretory molecules with a potential impact on osteogenesis, a broad Mass Spectrometry analysis was carried out. Several proteins were found [collagen type I, C-type lectin domain family 3 member b (Clec3b),

galectin-3, secreted acidic cysteine rich glycoprotein (SPARC), tissues inhibitor of metalloproteinase (TIMP) proteins, decorin] that interact with bone processes in various ways, as documented in the literature (see below).

Type I collagen molecules are involved in the mechanical properties of bone. Several studies indicate that collagen plays a substantial role in its toughness (capacity to absorb energy), while the mineral content is mainly involved in determining bone stiffness⁸⁵.

Clec3b is a homotrimeric protein involved in extracellular matrix formation that has been suggested to play a role in tissue remodeling, due to its ability to stimulate plasminogen activation and it is expressed in developing tissues, such as bone and muscle. Its mRNA has been found to be upregulated along the tenogenic differentiation of the mesenchymal cell line C3H10T½, concluding that this secreted factor may exert a major impact on tendon development⁸⁶. A skeletal phenotype has also been reported for Clec3b-knockout mice⁸⁷.

Galectin-3 belongs to the family of the β -galactoside binding animal lectins and is constitutively expressed in various tissues, including the bone⁸⁸. Of great interest for bone biology, intracellular galectin-3 is a key regulator of the Wnt/ β -catenin signaling pathway⁸⁹ and it has been shown to be a marker of both chondrogenic and osteogenic cell lineages. Galectin-3 protein was found to be increased in the differentiated chondrocytes of the metaphyseal plate cartilage, where it favors chondrocyte survival and cartilage matrix mineralization. It was also shown to be highly expressed in differentiating osteoblasts and osteoclasts, in concomitance with expression of osteogenic markers and with the appearance of a mature phenotype⁹⁰. Galectin-3 is also expressed by osteocytes, though its function in these cells has not been fully elucidated. The effects of galectin-3 on bone cells were also investigated in galectin-3 null mice, further supporting its role in all stages of bone biology, from development to remodeling⁹¹.

SPARC, also referred to as osteonectin, is a calcium-binding matricellular protein and one of the most abundant non-collagenous protein expressed in mineralized tissues⁹². Studies from mice and humans have revealed that SPARC plays a critical role in regulating bone remodeling and maintaining bone mass and quality. The mechanisms by which SPARC influences bone formation, maintenance, and repair might occur through multiple pathways that include the regulation of

procollagen processing and assembly in the bone matrix, mineralization, and osteoblast/osteoclast differentiation and activity⁹².

The expression and activity of **TIMPs**, which act as endogenous inhibitors of matrix metalloproteinases (MMPs) activity⁹³, has been observed in both osteoblasts and osteocytes⁹⁴. Prideaux et al. used MLO-A5 cells to determine the temporal gene expressions of TIMPs during osteocytogenesis, observing that the expression of these inhibitors decreases with differentiation into an osteocyte-like phenotype. This is consistent with the notion that a reduced blockade upon matrix metalloproteinase activity is required during osteocytogenesis⁹⁵.

Of particular interest is **decorin**, since it's a recently discovered myokine. It belongs to the small leucine-rich repeat proteoglycans (SLRPs) class I and directly binds to myostatin in a paracrine manner, thereby inhibiting its action⁹⁶. Decorin is secreted from myotubes in response to contraction and the serum levels of decorin increase after exercise in human. Subsequently, decorin can promote the formation of bone matrix and calcium deposition to control bone morphogenesis⁹⁷.

Hence, several proteins secreted by C2C12 muscle cells along differentiation act on bone remodeling and homeostasis in various manners, paving the way for further studies on new myokines. Unfortunately, it was not possible to identify Sclerostin by Mass Spectrometry, probably because glycosylation may preclude its detection⁷⁸.

We then focused on the characterization of Sclerostin "*in vivo*", highlighting that it is widely expressed in young, adult and old mouse muscles and that its expression undergoes changes also depending on the metabolic profile of the muscle. Unfortunately, it was not possible to detect it via immunohistochemistry. The reason for this is that immunohistochemistry marks the presence and localization of a protein inside a tissue, whereas WB and ELISA detect it in a tissue homogenate where the protein of interest is enriched and highly accessible. Sclerostin is highly expressed by some osteocytes confined in little spaces (lacunae), thereby being morphologically detectable. We have demonstrated that it is present in muscle as well, but may be dispersed in small amounts in the whole tissue, thereby not being observable with immunohistological techniques.

In order to determine whether the function of muscular Sclerostin was similar to that of osseous Sclerostin, a transient SOST knock-in experiment was performed *via* plasmid electroporation in

muscles of young mice, since the skeleton is growing. This experiment has allowed to outline that muscular Sclerostin: *i)* does not have macroscopic effects on the muscles electroporated; *ii)* being secreted in the circulation, it did not provoke paracrine or endocrine effects on the skeletal system as a whole. As for the analysis of muscular sclerostin effects onto adjacent skeletal segments, the working hypothesis is that induced overexpression of sclerostin of muscular origin produces the same effect of bone sclerostin in downregulating bone formation. This hypothesis was confirmed by the static histomorphometry performed in the trabecular bone of the femur distal metaphysis, showing that the volume and number of trabeculae in the SOST-ET group were statistically lower with respect to the EV-ET group. Accordingly, in the SOST-ET group the trabecular separation was significantly higher than in the EV-ET group.

The micro-CT outcome revealed that SOST-overexpression induced a decrease in the number and extension of parallel trabeculae, while the area occupied by the perpendicular ones increased in SOST-ET animals.

Briefly, we argue that in particularly dynamic situations (such as in the growing skeleton) and despite sophisticated analyses (such as micro-CT) the effect of down-regulation on osteogenesis exerted by sclerostin of muscular origin can be masked by the effect exerted on the skeleton by the increase in body mass during somatic growth.

Taken together, these data indicate that the action of muscular sclerostin resembles the activity of the osseous one. Therefore, muscular Sclerostin seems to behave as an inhibitory factor for osteogenesis that may act synergistically with bone Sclerostin to block bone deposition.

In suggesting the *in vivo* role of muscular sclerostin on bone tissue, that could open new fields of research, the authors are however aware of the need to confirm the role of muscular sclerostin after the end of the somatic growth before proposing the validation of this protein as a therapeutic target for the treatment of bone diseases. In line with this target, further step points will be: *i)* detailed analyses of the biochemical structure of muscular Sclerostin (comparing it with the osseous one) and *ii)* investigations on the potential of anti-Sclerostin compounds to inhibit also the muscular form.

In conclusion, this study highlights the presence of a new form of Sclerostin produced at muscular level that can act in combination with bone Sclerostin in the phenomena of increased bone

fragility. This discovery is particularly relevant for the treatment of diseases such as osteoporosis, sarcopenia, and aging. Targeting pathways that centrally regulate bone and muscle or pathways that facilitate communication between the two tissues are the privileged directions for the identification of new chemical entities, which could simultaneously prevent, reduce or restore bone and muscle age-related wasting.

Acknowledgements

I would like to thank my tutor Professor Jessika Bertacchini and my co-tutor Professor Carla Palumbo for the careful supervision of this thesis work and for their support and encouragement during the project. Their positive outlooks and confidence in my research inspired me and gave me determination. Their accurate editing contributed enormously to the production of this thesis.

I wish to thank all my lab colleagues for having supported me along the three years of PhD training, in particular Doctors Marta Checchi, Emanuela Amore and Geltrude Rita Pugliese.

I am grateful to Professor Delphine B. Maurel from **BioTis (University of Bordeaux)** for hosting me in her lab and for providing scientific support to develop the *in vivo* part of the project with regards to the characterization of Sclerostin expression.

I would like to acknowledge Professor Stefano Biressi and Dr. Francesca Florio from **CIBIO (University of Trento)** for their help in the realization of *in vivo* SOST overexpression and for their valuable advice in data interpretation.

From the **University of Bologna**, a special thanks goes to Drs. Maria Pia Morigi and Matteo Bettuzzi for performing X-ray computed tomography and to Drs. Alessandro Bevilacqua and Ilaria De Santis for the rigorous work of image processing, data extraction and analysis.

From the **University of Modena and Reggio Emilia**, a sincere thanks goes to Professor Pietro Torricelli and Dr. Riccardo Bonacini for their careful evaluation of X-Ray scans; to Professor Luca Reggiani Bonetti for lending his expertise to the histological evaluation of muscular reactions induced by electroporation. Many thanks go to Drs. Tullia Maraldi and Manuela Zavatti for the histomorphometry analyses.

Finally, I wish to thank Dr. Francesco Potì from the University of Parma for helping me with qRT-PCR and related techniques.

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