Biochemical and Biophysical Research Communications 446 (2014) 1108–1113

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

New insights into the location and form of sclerostin

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ARTICLE INFO

Article history:
Received 12 March 2014
Available online 22 March 2014

Keywords:
Sclerostin
Cartilage
Bone
Wnt

ABSTRACT

Sclerostin is widely reported to be a monomeric osteocyte specific protein. In this study we have investigated whether sclerostin is produced in different forms and in which cell and tissue types they are produced.

We have demonstrated that recombinant sclerostin is composed of monomers and dimers, and that these, and other forms, notably 46 and 70 kDa forms, are found widely throughout the musculo-skeletal system. We have shown that 'dimeric' sclerostin is highly resistant to reduction, implying the presence of highly stable, non-reducible covalent bonds. We have also demonstrated that the form of sclerostin is not associated with the mineralisation state of the tissue or cell. Sclerostin was secreted by bone explants as high molecular weight forms that were reducible to the dimeric form. This dimeric form was detected in sera and in non-skeletal soft tissues specifically kidney, live, heart and lung.

We therefore hypothesise: (a) sclerostin exists in multiple forms not associated with the mineralised state of the cell/tissue and (b) circulating sclerostin is dimeric, as is the sclerostin found in non-musculoskeletal soft tissues. These observations may have significant implications for the therapeutic modulation of sclerostin.

1. Introduction

The discoveries that alterations around the region of the SOST gene were significantly associated with high bone mass disorders [1,2], coupled with the phenotypic characterisation of SOST knockout mice [3], have classified the protein produce of SOST, sclerostin, as a potent negative regulator of bone formation. Sclerostin has important potential therapeutic uses in osteoporosis and oncology and many publications have described measuring sclerostin levels in serum and plasma and correlating these levels with disease states [4–6].

Sclerostin is a glycoprotein similar to the DAN family of bone morphogenetic protein (BMP) antagonists [2]. Initial descriptions from expression system experiments described sclerostin as having a molecular weight (MW) in the region of 27/28 kDa [7,8], to be secreted in a monomeric form [7] and have two sites of potential glycosylation [2].

SOST gene distribution is widespread in the body, with high levels of SOST detected in kidney, lung, heart, aorta and bone [1,2]. However, no data currently exists on the presence of sclerostin in these tissues. In the musculo-skeletal system there are conflicting reports as to the localisation of SOST and sclerostin. Many publications state that sclerostin is an osteocyte-specific protein [9–12]. However, other reports describe sclerostin in osteoblasts, terminally differentiated hypertrophic chondrocytes [13], cementocytes [13] and osteoclasts [7,14].

Sclerostin inhibits bone formation [15], although the mechanism by which this occurs sclerostin is not fully understood. Sclerostin has actions as both a BMP antagonist [8] and Wnt signalling antagonist via LRP5/6 binding leading to inhibition of the canonical beta-catenin pathway [14,16].

Despite much work having been done on sclerostin, close examination of the literature does reveal a number of unanswered questions; there is poor correlation between mRNA levels and protein expression in many experimental systems [17,18], poor agreement as to which cell types contain sclerostin and observations that sclerostin in different parts of the same tissue responds differently to challenge [19]. In addition, serum sclerostin levels have recently been found to be associated with diabetes, adiposity and kidney function, independently of bone turnover status [20]. Answering these questions is important. Sclerostin has been elevated to a 'magic bullet' molecule; anti-sclerostin neutralising antibodies, having been demonstrated to increase bone mass in animal models [21,22], are in use as a clinical therapy for osteoporosis [10,23,24]. Many of the assumptions as to the effects of inhibiting

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http://dx.doi.org/10.1016/j.bbrc.2014.03.079
0006-291X/

sclerostin are based on the dogma that the protein is only found in osteocytes, it is secreted into the blood as a monomer and that the resultant serum levels solely reflect bone turnover.

The aims of this study were to investigate the possibility that sclerostin exists in forms other than the previously described monomer and to investigate the distribution of these forms in musculo-skeletal tissues and cells.

2. Materials and methods

2.1. Antibodies

Polyclonal anti-human sclerostin antibodies ab63097 (Abcam) and AF1406 (R&D systems), polyclonal anti-human LRP5/6 antibody Ab51910 (Abcam) and a monoclonal anti-GAPDH-peroxidase (G9295, Abcam).

2.2. Recombinant proteins

Recombinant sclerostin from R&D systems (1406-ST-025/CF), recombinant SOSTDCI from Novus biologicals (NBP2-23453).

2.3. Ethics statement

Tissue samples from human donors were collected under ethical consent obtained from the Cambridge 2 Research Ethics Board. Written consent was obtained for all specimens used. Tissue samples from animal donors were collected under ethical consent obtained from the Department of Veterinary Medicine, University of Cambridge.

2.4. Tissue and serum samples

Human tissue lysates were purchased from Abcam. Human orthopaedic and blood samples were from human donors.

2.5. Cell lines used

Three immortalised chondrocyte cell lines, ATDC5 (Sigma), TC28a2 (gift from Dr. Mary Goldring) and HCS2/8 (gift from Professor Takigawa [25]), and one osteosarcoma cell line (MG63). ML0-Y4 cells (gift from Dr. Bonewald [26]) and HeLa cells (ECACC) were also used.

2.6. Tissue and cell culture

Human bone and cartilage explants were harvested from donated tissue and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with additives at 37 °C, 5% CO_2. Chondrocytes and osteoblasts were isolated using sequential enzymatic digestion. Cell lines were cultured as above plus 10% fetal bovine serum (FBS) (Gibco, UK). ML0-Y4 cells were cultured in alpha MEM. For mineralisation studies 10 mM β-glycerophosphate, 10 nM dexamethasone and 50 μg/ml L-ascorbate-2-phosphate was added. Mineralisation was detected by measuring 2% Alizarin red (Sigma).

2.7. Immunofluorescence

Immunofluorescence was performed on monolayer chondrocytes using anti-sclerostin and anti-LRP5/6 antibodies. Cells were washed, permeabilised using 0.1% Triton X in PBS and incubated in primary antibody at concentrations of 1:200 anti-sclerostin and 1:500 anti-LRP-5/6. Secondary fluorescent antibodies were used at concentrations of 1:250.

2.8. Protein extraction and Western blotting

Proteins were extracted from snap frozen tissues, cell pellets and conditioned media. Frozen tissues were pulverised using a micro dismembrator (Braun GE) and protein extracted with Cell Extraction Buffer (Invitrogen) containing Protease Inhibitor Cocktail (Sigma). Total protein was quantified using a BCA protein quantification assay (Pierce) and normalised for loading on to Western blots. Protein extracts plus SDS sample buffer was incubated at 60 °C for 30 min. Samples were reduced with Tris (2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma) Samples were separated on 4–20% Tris–glycine gels (Novex, Invitrogen) and Western blotting performed using PVDF membranes (Immobilon, Millipore) and ECL prime (GE Healthcare). Secondary antibodies were supplied by Sigma. Blots were stripped using Restore Western Blot stripping Buffer (Pierce) and reprobed using a monoclonal GAPDH-peroxidase antibody as a loading control.

2.9. De-glycosylation

Cell suspensions were deglycosylated by treating with 10^{-3} units/ml Chondroitinase ABC, 10^{-3} units/ml Keratanase I and 10^{-4} units/ml Keratanase II alone or in combination in 0.1 M Tris Acetate buffer pH 6.5 for 4–24 h at 37 °C. The resultant protein products were analysed by Western blotting.

2.10. PCR

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen), including an on-column gDNA elimination treatment. RNA concentration was quantified using the Nanodrop-2000 Spectrophotometer. RNA was reverse-transcribed in triplicate plus a negative control with 1 μg RNA/reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

Quantitative PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) with thermocycling conditions of 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 62 °C for 30 s, using the Stratagene MX3000P and Ct values recorded using MxPro Software. Expression of human and mouse SOST was analysed and 18S used as a housekeeping gene, using commercial QuantiTect primers (Qiagen). PCR products were confirmed by visualising on a 1.2% agarose gel.

3. Results

3.1. Sclerostin occurs in multiple forms

Western blotting identified sclerostin in all explants and cell types studied (Fig. 1 A and B). Both antibodies used recognised identical multiple bands in the Western blots under reduced conditions. In explants and cell types sclerostin appeared consistently at Mw of approximately 47, 54 and 70 kDa. In addition, in cartilage small amounts of sclerostin were detected at 27 kDa, with higher amounts in ATDC5 (Fig. 1C) and HCS2/8 cell lines. In order to investigate whether the higher MW forms of sclerostin were due to glycosylation, a series of de-glycosylation experiments were performed on cells and but were not found to alter the form of the sclerostin (data not shown).

In order to verify the specificity of the antibodies that produced this suprising result, the antibodies were absorbed with recombinant sclerostin prior to blotting, this removed the Western blot signal. Potential cross-reactivity with the sclerostin homologue, SOSTDCI, was also investigated by Western blotting both sclerostin

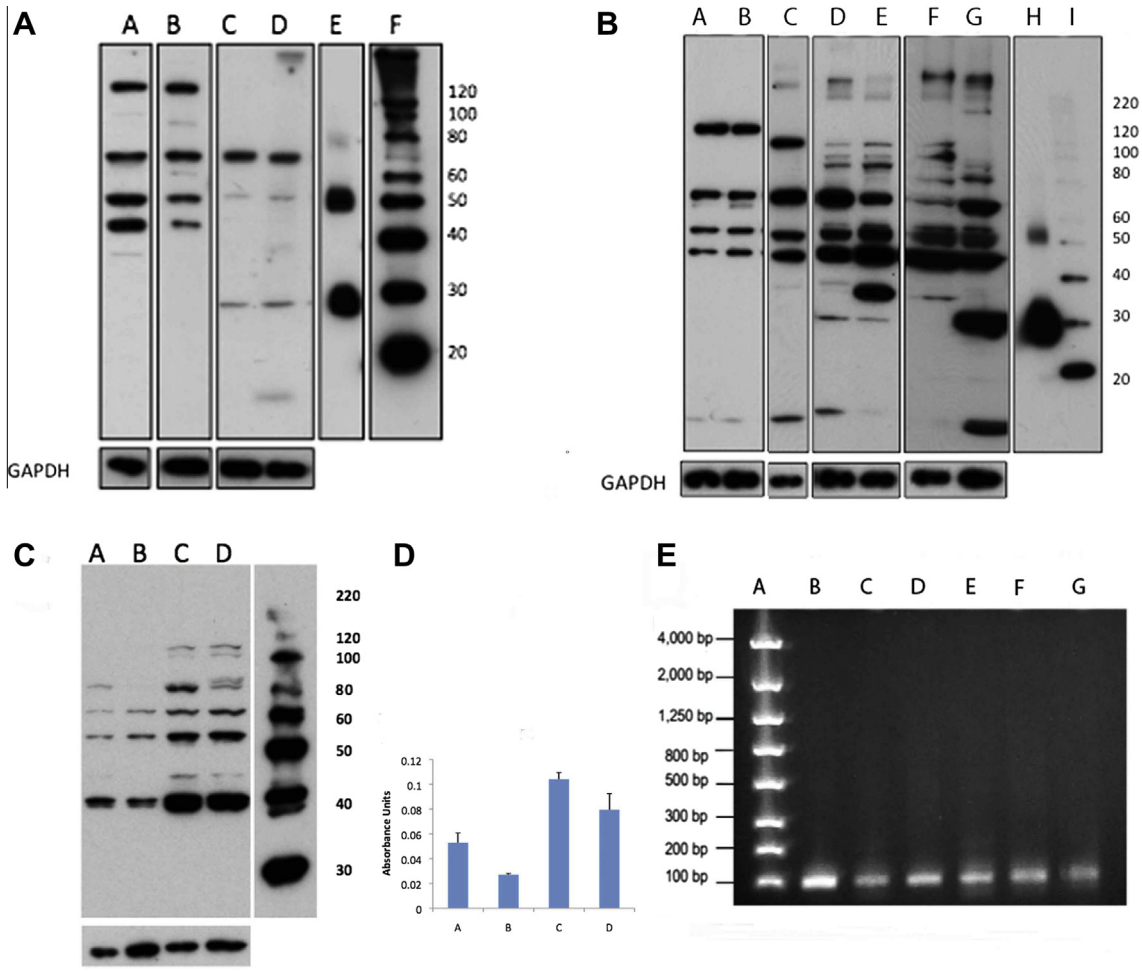

Fig. 1. Sclerostin is present in tissues and cells of musculo-skeletal lineage. (A) Western blots of bone and cartilage. Lanes A and B Trabecular bone, C and D articular cartilage, E recombinant sclerostin, F Mw marker. Sclerostin is present in bone and cartilage. In bone sclerostin is detected at approximately 46, 54 and 70 kDa plus a higher Mw form, in cartilage there is 70 kDa sclerostin and less 54 and 27 kDa sclerostin. Recombinant sclerostin is detected as a 27 kDa monomer and a 54 kDa 'dimer' with a very weak signal from an 81 kDa putative trimer. GAPDH blots included to show relative protein levels. (B) Western blots of cells. Lanes A, B, primary chondrocytes, C MG63 cells, D primary osteoblasts, E HeLa cells, F TC28a2 cells, G HCS2/8 cells line, H Recombinant sclerostin, I Mw marker. In all cell lines the 46, 54 and 70 kDa forms of sclerostin are present. 27 kDa sclerostin is only detected in significant amounts in HCS2/8. (C) Western blot of ATDC5 cells under normal and mineralising conditions of culture. Lanes A, B normal culture, lanes C, D mineralised cells. There is no difference in the forms of sclerostin produced in mineralised ATDC5, however the amount of sclerostin produced is increased. (D) Alizarin Red levels in samples A, D, C and D from blot (C). There is increased Alizarin red in the ATDC5 cells cultured under mineralising conditions (C and D). All blots performed using Abcam polyclonal anti-human sclerostin antibody under reducing conditions. (E) SOST PCR products from bone and cells. Lane A Base-pair size markers, B Bone, C primary osteoblasts, D primary chondrocytes, E TC28a2 cells, F Hela cells, G ATDC5 cells. The SOST gene product is identical in all samples.

antibodies against recombinant SOSTDC1. No significant cross-reactivity with SOSTDCI protein was detected with either antibody.

3.2. Recombinant sclerostin occurs in multiple forms

Western blotting of recombinant sclerostin was performed. These blots showed that recombinant protein is composed of a monomer of MW of approximately 27 kDa and a dimer of approximately 54 kDa (Fig. 1A, lane E). In addition very small amounts of apparent trimer (MW approximately 81 kDa under non-reducing conditions) were present. Sclerostin monomers and dimers were detected with both primary antibodies. The 'trimeric' form of the recombinant protein was reducible but the dimeric form was only partially reducible (Fig. 1B, lane H).

3.3. All forms of sclerostin are upregulated by mineralisation

Primary osteoblasts, chondrocytes and ATC5 cells were cultured under mineralising conditions. In the presence of increased mineral, as measured by alizarin red, there was an increase in the total sclerostin produced by all three cell types (Figs. 1C and D). However the form expressed by the cells i.e., the 47, 54 and 70kDa bands remained unchanged (Fig. 1C).

3.4. PCR detects SOST in all explants and cell lines

PCR analysis confirmed that bone, cartilage and cell lines shown in Figs. 1A and B contained SOST transcripts, whether or not they contained the 27 kDa protein (Fig. 1E).

3.5. Immunofluorescence of cells

Immunofluorescence of cells that contained (HCS2/8) or did not contain (chondrocytes) the 27 kDa protein did not show a difference in the staining pattern of sclerostin (Fig. 2). Sclerostin was localised within the cells, often in vesicles and co-localised with the receptor LRP6 in both cell types.

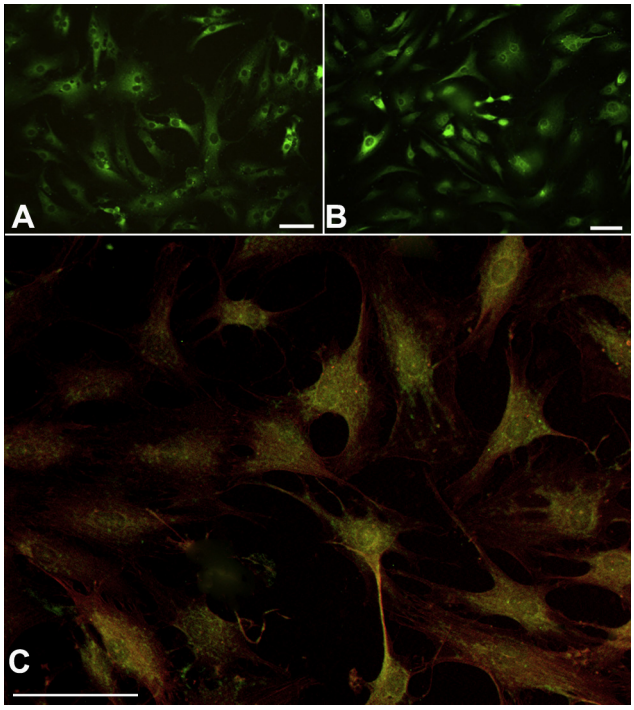

Fig. 2. Immunofluorescence of sclerostin in primary chondrocytes using R and D polyclonal anti-human sclerostin antibody. (A) Sclerostin (green) is present within the cells with a strong pericellular staining detected, very similar to that seen in B. (B) Immunofluorescence of LRP-5/6 (green). (C) Higher magnification showing immunofluorescence of sclerostin, packaged in vesicles (green) with a phalloidin (red) counterstain. Scale bars A, B 25 μm, C 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Sclerostin secretion by bone explants and osteocyte-like cell line MLO-Y4

In order to investigate whether sclerostin is a secreted protein we investigated sclerostin production and secretion in the MLO-Y4 cell line and bone explants (Fig. 3). Detection of secreted protein was initially hampered by high levels of sclerostin in bovine serum (Fig. 3A, lane D). Subsequent experiments were performed either in serum free media or, where serum was necessary for monolayer cultures, cells were washed repeatedly prior to protein extraction.

Western blotting revealed that bone explants secreted sclerostin at a Mr of approximately 54 kDa (Fig. 3A). MLO-Y4 were shown to produce monomeric sclerostin as well as the 47, 54 and 70 kDa forms when grown in either serum free or serum containing media (Fig. 3A), however When cells were washed and switched to serum free media for 24 h, only 70 kDa and higher MW forms were detected in the media. That the serum contained sclerostin epitopes was confirmed by a Western blot of media containing FCS that had not been incubated with cells Major serum proteins close to this MW, serum albumin, transferrin and alpha-1 trypsin inhibitor showed no binding with either sclerostin antibody (data not shown) thus eliminating the possibility of antibody cross-reaction with serum proteins.

3.7. Sclerostin protein is present in non-musculoskeletal tissues and serum as a 54 kDa protein

In human tissue lysates sclerostin was detected in the liver, heart, kidney and lung. Sclerostin levels were highest in the kidney and heart. In liver, kidney and heart the predominant species was the 54 kDa form of sclerostin under reducing conditions (Fig. 3B). In serum the predominant species was also the 54 kDa under reducing conditions (Fig. 3C)

4. Discussion

The results presented in this paper describe novel findings relating to sclerostin.

The major finding in this study is that sclerostin is rarely detected as the expected 27 kDa protein. We have demonstrated that recombinant sclerostin forms higher MW weight complexes, at molecular weights equivalent to dimer and trimer production, and that tissues and cells routinely produce protein with higher MW than expected, of approximately 47, 54 and 70 kDa. Our evidence that these higher MW forms are sclerostin is (i) two different polyclonal antibodies, reported as 'sclerostin specific' in the literature, recognise the higher MW forms, as well as the 27 kDa form. (ii) Absorption of the antibodies with sclerostin prior to Western blotting removed the signals from all MW bands, (iii) these antibodies did not significantly detect the Sclerostin homologue SOSTDC1, (iv) PCR demonstrated that, even in cells with no 27 kDa protein, sclerostin RNA is present, (v) IF demonstrated that sclerostin is localised as described in the literature and co-localises with its receptor LRP5/6 in cells with and without 27 kDa protein present and (vi) recombinant sclerostin is capable of spontaneous multimerisation; specifically that it forms higher Mw proteins with sizes equivalent to dimers (and small amounts of trimers) in addition to retaining the monomeric form. All of this evidence, taken together, is strongly supportive of the concept that sclerostin does not exist solely as a monomeric protein.

With regard to the underlying biochemistry behind different forms of sclerostin, our experiments did not show that glycosylation generated the higher Mw proteins. The reducibility of trimeric recombinant sclerostin implies the presence of inter-molecular disulphide bonds, likely via exposed cysteine molecules at positions 70 and 124 [27]. Other DAN family members form highly stable hetero/homodimers before binding to their receptors [1], indeed dimerization is considered to be critical for their regulation and function [28]. Previously it has been reported that sclerostin, along with another cysteine knot protein, Sostdc1, is unusual amongst the DAN family in being described solely as a monomer [29]. This monomeric state was attributed to the lack of 'spare' cysteine residues to form disulphide bonds [7,30]. However, more recent experiments have shown that, under certain conditions, SOSTDC1 can form dimers [28]. The molecular basis for the formation of sclerostin dimers is not known, however it may be due to the formation of highly stable non-reducible covalent bonds, as reported in other DAN family members [31], possible due to extensive β-sheet strand contacts producing a long stretch of backbone hydrogen bonds, as has been shown in Protein related to Dan and Cerberus (PRDC) [32].

The results presented in this paper clearly demonstrate that sclerostin protein is more widespread in the body than currently reported. We have demonstrated that sclerostin is found in bone and cartilage and in the cells derived from these tissues. It is interesting to note that only in some samples of cartilage, MLO-Y4 cells and two immortalised chondrocyte cell lines was the expected monomeric sclerostin detected [7,8]. Significantly, we failed to detect any monomeric sclerostin in protein extracts of human bone, bone explants in culture or their culture supernatants.

The anti-sclerostin antibodies used in this study also detected sclerostin in liver, heart, kidney and lung. In these tissues, the predominant forms of sclerostin were of high MW that reduced to a size consistent with that of a sclerostin dimer, i.e., 54 kDa, the same size as that detected in the serum. These results suggest that the 54 kDa form of sclerostin is the secreted form of the protein.

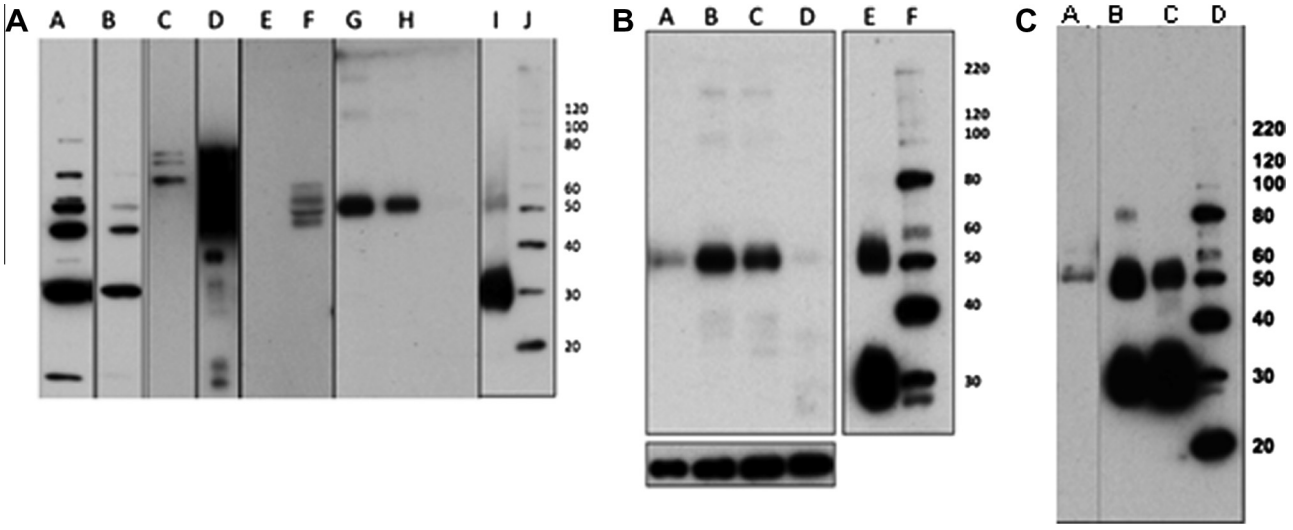

Fig. 3. Sclerostin in tissue lysates, MLO-Y4, serum and secreted from bone. (A) Sclerostin from MLO-Y4 cells and bone Western Blot of MLOY4 cells, conditioned media and bone explants. A: cells + serum-containing media. MLO-Y4 cells produce 27, 46, 54 and 70 kDa forms of sclerostin. B: cells + serum-free media. The absence of media does not alter the form of sclerostin produced, C: conditioned media, serum-free cell supernatant. 70 kDa sclerostin and higher forms are detected, D: conditioned media + serum. Serum contains significant detectable sclerostin, E: media, serum free, no cells. No sclerostin present, F: media + serum, no cells. (diluted 1:100), G, H: conditioned media from bone explants, I Recombinant sclerostin, J Mw marker. All blots performed using R&D polyclonal anti-human sclerostin antibody under reducing conditions. (B) Western Blot of human tissue lysates. A Liver, B kidney, C heart, D lung, E recombinant sclerostin, F Mw marker. All lanes reduced. Sclerostin is present in the tissues in the 54 kDa form. (C) Western blot of hyman serum (Lane A), B recombinant sclerostin, C recombinant sclerostin, D Mw markers. Lanes A and C reduced. Sclerostin is present in the serum in the 54K Da form.

These results are in contrast to many publications that continue to state that only osteocytes produce sclerostin [33,34] and may have important consequence for the use of anti-sclerostin antibodies as targeted therapy [35,36]. Our findings here are not unexpected as previous studies investigating the presence of SOST mRNA have shown SOST is widespread in the body, with high levels of transcripts in heart, aorta, kidney and bone [1,2].

Throughout our experiments we sought to assign a function to the different forms of sclerostin. We did not find an association with the mineralised state or mineralisation potential of the cell line or tissue from which sclerostin was obtained. However, in osteoblasts, chondrocytes and ATDC5 cells, mineralisation increased the amount of all forms of sclerostin produced by these cells. Significant dimeric protein was found in unmineralised tissue (e.g., liver, heart and cartilage) and mineralised tissues (e.g., bone), with monomeric sclerostin only occasionally detected in cartilage. However, some recent work also supports our observations that the controlling mechanisms determining the presence or absence of sclerostin in a cell is more complicated than whether or not a tissue is mineralised [37].

Having identified that sclerostin is expressed in a range of tissues and cells we next investigated to what extent sclerostin was being exported into the culture media as it is described in the literature as a secreted protein. The evidence that sclerostin is only a secreted protein is based primarily on the fact that it contains a signal sequence, that over-expression insect cells lines did not retain the protein and that sclerostin is assayed in serum [5,38]. The results presented in this paper demonstrate that, in bone explants sclerostin was indeed secreted but as high MW forms, that reduced to dimers, the identical form to that detected in serum, suggesting that sclerostin could have a paracrine (or possible endocrine) action on osteoblasts, but not in the previously described monomeric form.

In conclusion, our novel results have demonstrated that sclerostin is a multimeric protein, widespread in tissues and cells and that it is secreted predominantly in the dimeric form from bone. Given that 'neutralising antibodies' that exert their bone effect via inhibition of binding of sclerostin to LRP5/6 it is tempting to speculate that the paracrine action in bone is mediated via forms other than the circulating dimeric form, which may be involved in BMP inhibition in remote tissues. Further work is ongoing in our laboratory to further characterise the underlying biochemistry and behaviour of these different forms of sclerostin.

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