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Increased bone mass, altered trabecular architecture and modified growth plate organization in the growing skeleton of SOCS2 deficient mice

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- SOCS2
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Abstract

Suppressor of Cytokine Signaling-2 (SOCS2) negatively regulates the signal transduction of several cytokines. Socs2<sup>−/−</sup> mice show increased longitudinal skeletal growth associated with deregulated GH/IGF-1 signaling. The present study examined the role of SOCS2 in endochondral ossification and trabecular and cortical bone formation, and investigated whether pro-inflammatory cytokines associated with pediatric chronic inflammatory disorders mediate their effects through SOCS2.

7-week-old Socs2<sup>−/−</sup> mice were heavier (27%; P<0.001) and longer (6%; P<0.001) than wild-type mice. Socs2<sup>−/−</sup> tibiae were longer (8%; P<0.001) and broader (18%; P<0.001) than that of wild-type mice, and the Socs2<sup>−/−</sup> mice had wider growth plates (24%; P<0.001) with wider proliferative and hypertrophic zones (10% (P<0.05) and 14% (P<0.001) respectively).

Socs2<sup>−/−</sup> mice showed increased total cross-sectional bone area (16%; P<0.001), coupled to increased total tissue area (17%; P<0.05) compared to tibia from wild-type mice. Socs2<sup>−/−</sup> mice showed increased percent bone volume (101%; P<0.001), trabecular number (82%; P<0.001) and trabecular thickness (11%; P<0.001), with associated decreases in trabecular separation (19%; P<0.001). TNFα exposure to growth plate chondrocytes for 48h increased SOCS2 protein expression. Growth of metatarsals from 1-day-old Socs2<sup>−/−</sup> and Socs2<sup>+/−</sup> mice, as well as expression of Aggrecan, Collagen Type II and Collagen Type X, were inhibited by TNFα, with no effect of genotype.

Our data indicate that physiological levels of SOCS2 negatively regulate bone formation and endochondral growth. Our results further suggest that pro-inflammatory cytokines
mediate their inhibitory effects on longitudinal bone growth through a mechanism that is independent of SOCS2.
Introduction

Suppressor of Cytokine Signaling (SOCS) proteins are transcribed in response to cytokine stimulation and utilise various receptor-mediated mechanisms to down-regulate cytokine signal transduction pathways (Leroith and Nissley 2005; Rico-Bautista et al. 2006). The molecular mechanisms of SOCS action involve inhibiting Janus kinase (JAK) activity, blocking signal transducers and activators of transcription (STAT) binding sites on cytokine receptors or promoting the degradation of specific signaling proteins. SOCS proteins thereby form part of a classical negative feedback circuit to avoid the detrimental consequences of excessive stimulation. (Krebs and Hilton 2001).

It has been demonstrated that SOCS2, one of eight SOCS family members (Yoshimura et al. 2007), is also an important regulator of somatic growth through its regulation of GH/IGF-I signaling. SOCS2 is able to bind the GH receptor through tyrosine residues Y595 and Y487 (Greenhalgh et al. 2005), and since Y595 is a known binding site for STAT5, SOCS2 may therefore block access of STAT5 to the activated GH receptor (Smit et al. 1996), although this has recently been questioned (Uyttendaele et al. 2007). SOCS2 can also inhibit JAK2 tyrosine phosphorylation (Ram and Waxman 1999), suggesting that SOCS2 may inhibit GH signaling by directly binding to JAK2. SOCS2 may bind to the IGF-I receptor (Dey et al. 1998) and inhibit IGF-I induced STAT3 activation (Zong et al. 2000).
Socs2 null mice display an increased longitudinal skeletal growth and a proportionate augmentation of most visceral organs (Metcalf et al. 2000). A similar phenotype is also observed in the high-growth (hg) mutation, which is caused by the deletion of the Socs2 locus (Horvat and Medrano 2001). These effects are associated with deregulated GH/IGF-I signaling, supporting the concept that SOCS2 is an important negative regulator of the GH/IGF-I signaling pathway. Indeed, SOCS2 may be a potential target for the development of novel treatment strategies for growth disturbances observed in children with chronic inflammatory disorders such as Juvenile Idiopathic Arthritis and Inflammatory Bowel Disease. Systemic concentrations of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα) are often elevated in children with these disorders and may inhibit longitudinal growth (MacRae et al. 2006a,b) by directly inhibiting growth plate chondrocyte dynamics (Martensson et al. 2004; MacRae et al. 2006c).

Interestingly, reduced bone mineral density (BMD) has been reported in Socs2 null mice by both dual x-ray absorptiometry (DXA) analyses and peripheral quantitative computerized tomography (pQCT) (Lorentzon et al. 2005). These results are not consistent with the expected augmented GH/IGF-I signaling observed in the Socs2 null mice and therefore warrant re-examination. In addition, it is unknown if cortical and trabecular bone volume are increased in the Socs2 null mice which would be consistent with increased GH/IGF-I signaling. To address these issues we have, in this study, exploited the capabilities of microCT which offers high resolution analyses of trabecular bone architecture and cortical bone geometry. In contrast, the increased longitudinal
bone growth observed in Soxs2 null mice (Metcalf et al. 2000; Lorentzon et al. 2005) is indeed consistent with increased signaling through the GH/IGF-I axis and indicates that SOCS2 protein has a functional role in growth plate chondrocyte dynamics. Nevertheless, previous histological analyses failed to disclose any obvious abnormalities of the epiphyseal growth plate of the tibia and femur (Metcalf et al. 2000). Subtle changes would only be revealed by a comprehensive histomorphometric analysis, however, and these may have been missed. Therefore, in addition to clarifying the function of SOCS2 in structural bone development, we considered it essential to understand more fully the effects of Soxs2 deficiency on growth plate structure and bone growth. Such an approach will also improve our understanding of inflammatory growth disorders associated with abnormal GH/IGF-I signaling.

In the present study, we have generated Soxs2 null (Soxs2\(^{-/-}\)) mice in order to perform a detailed examination of the role of SOCS2 in endochondral ossification and trabecular and cortical bone formation. We have also investigated whether the pro-inflammatory cytokines that are associated with pediatric chronic inflammatory disorders, such as TNF\(\alpha\), mediate their negative effects on bone growth through SOCS2.

**Material and Methods**

*Generation of the targeting construct and mutant mice*

A BAC clone, 520L19, containing the Soxs2 locus has been isolated previously (Horvat & Medrano, 1998) and used to subclone the plasmid with 7517 bp-long XbaI genomic fragment (positions 94841854 to 94849370 bp on chromosome 10; mouse genome
Ensembl release 45 - Jun 2007; http://www.ensembl.org/Mus_musculus/). This fragment contains exons 2 and 3 of the Socs2 transcript (Ensembl ID ENSMUST00000020215). This construct was partially digested with Kpnl and the reporter-selection marker cassette from plasmid pGT1.8Ires\(\beta\)geo\(^{(32)}\) was inserted into the Kpnl site within exon 2 (Figure 1A). The targeting construct was therefore bicistronic, combining the picornaviral internal ribosome-entry site (IRES) with the lacZ-neoR (\(\beta\)-geo) fusion gene (Mountford et al. 1994). The Kpnl site was chosen for insertion of 70 nucleotides upstream of the translation start site to ensure that the polyadenylation site within the \(\beta\)-geo cassette would prematurely terminate transcription and cause lack of Socs2 function. 300\(\mu\)g of the NotI-linearised construct was electroporated into \(10^7\) HM1 embryonic stem (ES) cells from strain 129Sv (Magin et al. 1992) and after 48 h in culture selected in 160\(\mu\)g/ml G418 (Geneticin, Life Technologies, MD, USA) to obtain drug resistant ES cell colonies. Positive targeted events were identified in the ES clones by Southern analysis of BamHI digested genomic DNA (see Figure 1) using an external hybridisation probe, downstream of the 3' homology arm (EcoRI to XhoI fragment, Figure 1A), to detect clones with the expected fragment size of the wild type and targeted alleles. Targeted ES clones were identified by the presence of an appropriate doublet, a \(\sim\)24-kb wild type band and a \(\sim\)22-kb targeted fragment, which was shorter due to an additional BamHI site between the neo and LacZ genes within the \(\beta\)-geo cassette (Fig. 1A and 1B). Identical Southern blot screening was also used to identify homozygous individuals in the F\(_2\) generation for phenotypic analysis.
From several positively targeted ES clones, two karyotypically normal G418 resistant clones were selected for injection into C57BL/6J blastocysts to generate chimeras (Bradley et al. 1987), which in turn were back-crossed with C57BL/6J mice. Germline transmission was confirmed in agouti coat-coloured offspring and potential founder animals identified using DNA isolated from tail biopsies and Southern blot analysis (as above). Subsequent generations were tested by tail biopsy followed by DNA isolation and analysis using PCR. Primers for Socs2 wild-type allele (Forward 5’CAA CTT TAG TGT CTT GGA TCT; Reverse 5’TGT TTG ACT GAG CTC GCG C3’) and neo (5’CGG CCG CTT GGG TGG AGA GGC3’; Reverse 5’TCG GGC ATG CGC GCC TTG AGC3’) were used. The neo gene was present only in heterozygotes and homozygotes for targeted Socs2 allele, while the Socs2 wild type allele was present only in wild type homozygote and heterozygote animals.

**Growth analysis**

At weekly intervals, from 3 to 7 weeks of age, six female wild-type and six female Socs2−/− mice were weighed. Following euthanasia at 7 weeks of age, body length (crown – rump) measurements were recorded.

**Micro-computed tomography**

Tibiae were dissected from 7 week old female wild-type and Socs2−/− mice (n=5), fixed in 10% neutral buffered formalin for 48h, rinsed in tap water for 3h and stored in 70% ethanol. The tibiae were briefly rehydrated in physiological saline (0.09%) prior to being scanned using a micro-computed tomography (µCT) system (Skyscan 1172 X-Ray...
Microtomograph, Aartselaar, Belgium) to evaluate trabecular architecture and cortical bone geometry. High-resolution scans with an isotropic voxel size of 5µm were acquired (scan time approximately 1h 30mins for each bone), then reconstructed using NRecon (Skyscan, Belgium) and analyzed using CTAn (Skyscan, Belgium).

In trabecular bone, bone volume (BV/TV), trabecular number (Tb.N; /mm), trabecular thickness (Tb.Th; mm), trabecular separation (Tb.Sp), structure model index (SMI) and trabecular pattern factor (TPF) were evaluated. For each bone, a 5% region (of total bone length) of the metaphysis was analyzed, extending from 10% distal to the proximal head of the tibia. Bone mineral density (BMD g/cm³) of the trabecular bone was also calculated after appropriate calibration of the Skyscan CT analyser with known density calcium hydroxyapatite phantoms.

In cortical bone, tissue area, periosteal perimeter, bone area, endosteal perimeter, cortical thickness, mean moment of inertia (MMI polar) (more difficult to bend) and eccentricity (deviation from a circular shape) were evaluated in a 0.5mm segment of the mid-diaphysis, 0.25mm either side of the mid-diaphysis of the bone. Bone mineral density (BMD g/cm³) of the cortical bone was determined as described above.

**Analysis of tibiae**

To measure tibia dimensions, tibiae were dissected from 7 week-old female wild-type and Socs2−/− mice (n=6). Tibia length and width were measured using DigiMax digital vernier callipers (R. S. Components Ltd, Corby, Northants, UK), after which the bones
were processed through to wax as previously described (Mushtaq et al. 2004). The size of the growth plate, and the proliferating and hypertrophic zones of seven wild-type and nine $Socs2^{-/-}$ tibiae were determined using image analysis software (Nikon, Kingston upon Thames, Surrey, UK) on Toluidine blue stained paraffin sections. The width of the growth plate and the two distinct maturational zones was determined at 10 different points along the breadth of the growth plate in two sections from each bone.

Serum analysis

Immediately following euthanasia, blood samples from six 7-week-old female wild-type and five female $Socs2^{-/-}$ mice were obtained by cardiac puncture and serum samples were prepared. A solid phase immunofixed enzyme activity assay was used to determine osteoclast-derived tartrate-resistant acid phosphatase form 5b (TRAP 5b) in the serum (Mouse TRAP™ assay; Immunodiagnostic Systems, Boldon, Tyne and Wear, UK). A sandwich ELISA kit was used to measure serum levels of osteocalcin (Mouse Osteocalcin EIA Kit; Immunodiagnostic Systems). An immunoenzymometric assay was used to determine serum IGF-I concentrations (Rat/Mouse IGF-I ELISA; Immunodiagnostic Systems).

ATDC5 chondrocyte cell culture

The ATDC5 chondrocyte cell line was sourced from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi and colleagues (Atsumi et al. 1990). Cells were cultured as described previously (MacRae et al. 2006c) and samples collected on day 6 and day 13. At these time points, the cells express established markers of
chondrogenesis (collagen type II and aggrecan) and the differentiated phenotype (collagen type X), respectively (Mushtaq et al. 2002).

Isolation of Primary Murine Chondrocytes

All animal experiments were approved by Roslin Institute’s Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals. Primary chondrocyte cultures were prepared from male and female fetal (E14-E19) and neonatal (1 and 9-day-old) mouse chondrocostal growth plates using an isolation procedure and culture system previously described (Lefebvre et al. 1994). Briefly, the rib cage and sternum were dissected, rinsed in PBS, incubated at 37°C for 30min in 2mg/ml pronase (Sigma, Poole, Dorset, UK) in PBS, and then in 3mg/ml collagenase type 2 (Worthington Biochemical Corporation, Lakewood, New Jersey, US) in DMEM containing 4.5g/L glucose and L-Glutamine (Invitrogen, Paisley, Strathclyde, UK) for 30min. Soft tissues were detached from the cartilage by repeated gentle pipetting. The denser cartilage was subsequently allowed to sediment, and the soft tissues aspirated; the cartilage was then further digested with collagenase for 3h. Undigested bony parts were discarded and the remaining cell suspension was aspirated repeatedly with a Pasteur pipette, filtered through a 45µM cell sieve, and rinsed in DMEM.

Cell culture

Chondrocytes from 1-day-old female neonatal mice were seeded in 6-well plates at 100,000 cells per well in DMEM containing 4.5g/L glucose and L-Glutamine,
supplemented with 10% FCS (Invitrogen), 50µg/ml gentamycin (Invitrogen) and 50µg/ml L-ascorbic acid phosphate (Wako Pure Chemicals Ltd, Neuss, North Rhine-Westphalia, Germany). To study the effects of pro-inflammatory cytokines, chondrocytes were exposed to TNFα (10ng/ml) (Autogen Bioclear, Calne, Wiltshire, UK) for 48h before RNA or protein extraction.

**Organ culture**

The middle three metatarsals were aseptically dissected from male and female 19 day-old fetal and 1 day-old neonatal wild-type and *Socs2*−/− mice. Bones were cultured and metatarsal lengths were measured as described previously (MacRae et al. 2006c, 2007). TNFα was added at a final concentration of 10ng/ml for the duration of the experiment (8 days). All results are expressed as a percentage change from harvesting length, which was regarded as baseline.

**Western blotting analysis**

Cells were lysed in RIPA buffer (20mM Tris-HCl, 135mM NaCl, 10% glycerol, 1% IGEPAL, 0.1% SDS, 0.5% deoxycholic acid, 2mM EDTA) containing “Complete” protease inhibitor cocktail according to manufacturer's instructions (Roche, East Sussex, UK). Immunoblotting was undertaken as described previously (MacRae et al. 2006d). Nitrocellulose membranes were probed overnight at 4°C with anti-SOCS2 primary antibody (1:500 dilution in 5% milk) (Abcam Plc, Cambridge, UK), washed in TBST and incubated with anti-rabbit IgG-peroxidase (Cell Signaling Technology, Beverly, MA, USA) for 1h (1:1000 dilution in 5% milk). Membranes were then washed in ‘stripping
buffer’ (Pierce, Rockford, IL, USA) and re-probed for 1h for β-actin expression (1:5000 dilution in 5% milk; anti β-actin clone AC15; Sigma). After washing, membranes were incubated with anti-mouse IgG-peroxidase for 1h (Sigma). Semi-quantitative assessment of band intensity was achieved using Quantity One image analysis software (Bio-Rad Labs Inc., Hercules, CA, USA).

**Immunohistochemistry**

Tibiae were dissected from 3 week-old female Swiss mice that had been euthanized, fixed in 100% ethanol, decalcified in 10% EDTA (pH 8.0) for 4 days at 4°C, dehydrated and embedded in paraffin wax before sectioning at 5µm. For histological analysis, sections were de-waxed and de-masked for 10min in 0.1% trypsin. Endogenous peroxidises and non-specific antibody binding were blocked before overnight incubation at 4°C with 0.5µg IgG/ml anti-SOCS2 antibody. The sections were then washed in PBS, incubated with goat anti-rabbit IgG peroxidise (1:100 dilution; DAKO, Glostrup, Denmark) for 60min, and incubated with DAB substrate reagent (0.06% DAB, 0.1% H2O2 in PBS) for 5min. The sections were finally dehydrated, counterstained and mounted in DePeX. Control sections were incubated with non-immune rabbit IgG (0.5µg IgG/ml) in place of the primary SOCS2 antibody.

**Analysis of Socs2 expression using semi-quantitative RT-PCR**

RNA was extracted from chondrocytes using RNeasy total RNA (Qiagen Ltd, Crawley, West Sussex, UK), according to the manufacturer’s instructions. For each sample, total RNA content was assessed by absorbance at 260nm and purity by A260/A280 ratios.
RNA was reverse transcribed and the PCR reaction undertaken as described previously (Farquharson et al. 1999; Houston et al. 1999). For the PCR reaction, primers for 18S rRNA gene (20 cycles) (Ambion, Huntingdon, Cambs, UK, sequence unknown) and Socs2 (35 cycles) (SuperArray Bioscience, Maryland, USA; sequence unknown) were used.

*Analysis of chondrogenic gene expression using quantitative RT-PCR*

RNA was extracted, quantified and reverse transcribed as described above. RT-qPCR was performed using the Stratagene Mx3000P real-time QPCR system (Stratagene, CA, USA). Primers were designed to span at least one intron. Primers for *collagen type II, Col2a1* (Accession number NM031163) (Forward 5’CGG TCC TAC GGT GTC AGG3; Reverse 5’GCA GAG GAC ATT CCC AGT GT3’), *collagen type X, Col10a1* (Accession number NM009925) (Forward 5’CAT AAA GGG CCC ACT TGC TA3’, Reverse 5’CAG GAA TGC CTT GTT CTC CT3’) and *Gapdh* (Accession number NM008084) (Forward 5’TGAGGCCGGTGCTGAGTATGTCG3’, Reverse 5’CCA CAG TCT TCT GGG TGG CAG TG3’) were used. Primers for *aggrecan* (Accession number NM007424) were obtained from SuperArray Bioscience (sequence unknown).

*Statistical analysis*

General Linear Model analysis incorporating ANOVA and the Students t-test were used to assess the data. All data are expressed as the mean +/- S.E.M. Statistical analysis was performed using Minitab 14. $P<0.05$ was considered to be significant.
Results

Growth of $\text{Socs2}^{+/}$ mice

Initial studies addressed whether the $\text{Socs2}^{+/}$ mice displayed an increased growth phenotype resembling that previously reported for $\text{Socs2}$ null mice (Metcalf et al. 2000). We found that the weight of the $\text{Socs2}^{+/}$ mice was indistinguishable from wild-type mice prior to weaning (Fig. 2), but the $\text{Socs2}^{+/}$ mice were significantly heavier (15%; $P<0.01$) than their wild-type counterparts by 4 weeks of age; a difference that became more pronounced during the 7-week study period. More detailed analysis of 7 week-old female mice indicated that the $\text{Socs2}^{+/}$ mice were significantly heavier (27%; $P<0.001$) and longer (6%; $P<0.001$) than wild-type mice (Table 1). In addition, the tibiae of $\text{Socs2}^{+/}$ mice were longer (8%; $P<0.001$) and broader (18%; $P<0.001$) than that of wild-type mice (Table 1), and furthermore, the $\text{Socs2}^{+/}$ mice had wider growth plates (24%; $P<0.001$) with significantly wider proliferative and hypertrophic zones (10% ($P<0.05$) and 14% ($P<0.001$) respectively) (Table 1). Our data confirm previous studies that a deletion of the $\text{Socs2}$ gene causes increased growth and bone length, but also extends these findings to disclose structural changes to the growth plate which may account for the increased bone growth.

Bone architecture in $\text{Socs2}^{+/}$ mice

The reduced BMD observed in $\text{Socs2}$ null mice by Lorentzon and colleagues (Lorentzon et al. 2005), is not consistent with the enhanced GH/IGF-I signaling observed in the $\text{Socs2}$ null mice. Therefore, we completed a comprehensive high resolution (5µm) µCT
analysis of the tibia to more fully examine the effects of SoCS2 deficiency on bone phenotype. Examination of the mid-diaphyseal cortical bone of tibiae from SoCS2−/− mice showed increased total cross-sectional bone area (16%; P<0.001), coupled to increased total tissue area (17%; P<0.05) compared to tibia from wild-type mice (Table 2). Tibiae from SoCS2−/− mice also showed an increased mean moment of inertia (39%; P<0.001). No difference in periosteal and endosteal perimeter, eccentricity, BMD or cortical cross-sectional thickness was observed between genotypes (Table 2).

Increased bone volume in SoCS2−/− mice was apparent in the trabecular compartment of the tibia, where SoCS2−/− mice showed increased percent trabecular bone volume (101%; P<0.001; Fig. 3), trabecular number (82%; P<0.001) and trabecular thickness (11%; P<0.001), with associated decreases in trabecular separation (19%; P<0.001) (Table 2). The structure model index (SMI), which quantifies the characteristic form of a 3D structure in terms of amounts of plates and rods composing the structure (Hildebrand and Ruegsegger 1997), was also significantly lower in tibia from SoCS2−/− mice (22%; P<0.001). This indicates that the trabeculae in SoCS2−/− mice appear to be more 'plate-like' and more connected, which is consistent with greater 'strength' (Table 2). Trabecular BMD measured by µCT was unchanged in the SoCS2−/− mice. These results indicate that SoCS2−/− mice have increased bone mass in both the cortical and trabecular compartments of the tibia, and that changes in bone architectural organization in SoCS2−/− mice are likely to be consistent with a greater resistance to bending.
Biochemical markers of bone formation and resorption

Serum concentrations of TRAP5b, a marker of bone resorption, were significantly increased in the Socs2\(^{-/-}\) mice compared with wild-type mice (110%; P<0.001) (Table 3). Similarly, a trend for elevated concentration of osteocalcin, a marker of bone formation, was also observed in the Socs2\(^{-/-}\) mice (35%; NS) (Table 3). Serum IGF-I concentrations were not significantly different between the Socs2\(^{-/-}\) and wild-type mice (Table 3). These results suggest a tendency for increased bone turnover in Socs2\(^{-/-}\) mice.

SOCS2 expression in the growth plate

To determine if the increased growth observed in Socs2\(^{-/-}\) mice was likely to be due to direct effects at the growth plate, it was first necessary to confirm that chondrocytes do indeed express Socs2 mRNA and protein. Primary chondrocytes derived from both fetal (E14-E18) and neonatal (1 and 9-day-old) cartilage were found to express Socs2 mRNA (Fig. 4a), whereas terminally-differentiated ATDC5 cells showed decreased SOCS2 protein levels compared to ATDC5 cells in the chondrogenic phase (Fig. 4b). Immunohistochemical analysis of sections of tibia revealed SOCS2 labeling in both proliferating and hypertrophic chondrocytes of the growth plate (Fig. 5). Consistent with changes in the levels of SOCS2 expression levels observed during ATDC5 cell differentiation (Fig. 4b), more intense SOCS2 labeling was observed in cells of the less mature proliferative zone than the hypertrophic zone of the growth plate (Fig. 4a). These studies confirm that growth plate chondrocytes express SOCS2, highlighting the likelihood that the increased bone growth and observed structural differences within the
epiphysis observed in $Socs2^{-/-}$ mice is a direct consequence of altered SOCS2 signaling in chondrocytes of the growth plate.

*Effect of pro-inflammatory cytokine exposure on chondrocyte gene expression*

Increased SOCS2 protein expression in response to cytokine stimulation is known to be involved in the suppression of cytokine signal transduction pathways in various cell types. If increased SOCS2 expression is responsible for mediating the growth inhibitory effects of pro-inflammatory at the growth plate, it is evident that SOCS2 expression in chondrocytes should also be increased following cytokine exposure. This was indeed the case; we found that exposure to TNFα (10ng/ml) for 48h increased SOCS2 protein expression in primary 1 day-old murine chondrocytes (Fig.4c). Having established that TNFα increased chondrocyte SOCS2 expression, we next determined whether cytokine-induced changes in chondrocyte ‘marker’ genes differed in chondrocytes derived from wild-type and $Socs2^{-/-}$ mice. RT-qPCR studies revealed that exposure to TNFα significantly reduced, by comparable amounts, the expression of *aggrecan*, *collagen type II* and *collagen type X* mRNA in both wild-type and $Socs2^{-/-}$ mice (Fig. 6). This lack of effect of *Socs2* genotype on the cytokine-induced diminution in chondrocyte marker genes indicates that chondrocytes from 1 day-old wild-type and $Socs2^{-/-}$ mice are equally susceptible to the adverse effects of pro-inflammatory cytokines and, therefore, that SOCS2 is unlikely to mediate these effects.
Growth of cultured wild-type and Socs2<sup>−/−</sup> murine metatarsals and their response to pro-inflammatory cytokine exposure.

We next aimed to determine if the growth trajectories of metatarsals from wild-type and Socs2<sup>−/−</sup> mice differed under basal conditions or in response to pro-inflammatory cytokines. Our findings show that neither the length of freshly dissected metatarsals from 19 day-old fetal and 1 day-old neonatal Socs2<sup>−/−</sup> mice, nor their growth over the ensuing 8 days in culture, differed significantly from age-matched wild-type control metatarsals (Figs 7a and 7b). Growth of wild-type and Socs2<sup>−/−</sup> fetal metatarsals was also significantly inhibited, by comparable amounts, following exposure to TNFα (26% and 27% respectively; both p<0.001) (Fig.7a). Similarly, TNFα reduced growth to a similar extent in metatarsals derived from 1 day-old neonatal Socs2<sup>−/−</sup> and wild-type mice (33% and 47% respectively; both p<0.001) (Fig. 7b). This indicates that the Socs2 genotype does not modify either the in vitro growth of fetal and neonatal metatarsals or their reduced growth following cytokine exposure.

Discussion

Our study has confirmed and extended recent reports that SOCS2 is an important regulator of post-natal skeletal growth. It is also the first study to report that the SOCS2 protein is a negative regulator of bone turnover, mass and architecture.

In the present study, wild-type and Socs2<sup>−/−</sup> mice showed comparable metatarsal lengths at E19 and 1 day of age and their growth curves did not significantly diverge until 4 weeks of age. At this and later ages, the Socs2<sup>−/−</sup> mice had greater body weights and lengths, and
had longer and broader tibiae compared to the wild-type mice. The wider widths of the proliferative and hypertrophic zones within growth plate of the Socs2<sup>−/−</sup> mice are consistent with increased tibia length. This, together with our observation that SOCS2 protein is present at high levels within the proliferative chondrocytes of the growth plate, suggests that the structural differences in the epiphysis of the Socs2<sup>−/−</sup> mice are a direct consequence of altered chondrocyte SOCS2 signaling. Interestingly, elevated levels of SOCS2 expression were also observed during the chondrogenic, as compared to terminal differentiation phase of ATDC5 growth and this supports our immunohistochemical data. From observations in other cell types, it is possible that SOCS2 may be linked to the transition from the proliferative to the differentiated chondrocyte phenotype. SOCS2 induces the differentiation of C2C12 mesenchymal cells into myoblasts or osteoblasts (Ouyang et al. 2006) as well as the differentiation of neuronal progenitor cells into neurons (Goldshmit et al. 2004; Wang et al. 2004).

Socs2 null mice have been shown to exhibit several features of deregulated GH/IGF-I signaling, including collagen accumulation in the dermis, affected production of major urinary protein, and increased local production of IGF-I in several organs including the heart, lungs and spleen (Metcalf et al. 2000). We, and others (Metcalf et al. 2000; Lorentzon et al. 2005), however have observed no increase in serum IGF-I concentrations in the Socs2<sup>−/−</sup> mice. As the liver is recognised as the major source of circulating IGF-I (Yakar et al. 1999; Sjögren et al. 1999) this observation is consistent with normal IGF-I mRNA expression in the liver of Socs2<sup>−/−</sup> mice (Metcalf et al. 2000). Interestingly, mice with the high-growth (hg) mutation, which is caused by the deletion of the Socs2 locus
(Horvat and Medrano 2001), show a 30-50% increment in postnatal growth as well as elevated circulating levels of IGF-I (Medrano et al. 1991). The reasons for the discrepancy in serum IGF-I levels between the Soxs2−/− and high-growth mouse strains are unclear but may involve their different genetic backgrounds. Alternatively, as the high-growth mutant strain contains a large deletion of 500-kb in chromosome 10, it is possible that a deletion of other genes or DNA segments flanking Soxs2 locus may be responsible for the elevated circulating IGF-I concentrations in the high-growth mutant.

Various studies, encompassing independent targeting or spontaneous null mutations have shown that disruption of the IGF-I gene results in severe intrauterine growth retardation (Liu et al. 1993; Powell-Braxton et al. 1993; Lupu et al. 2001) whereas ablation of GH signaling does not inhibit growth until approximately post-natal day 14 (Lupu et al. 2001). It is therefore more likely that relief of GH inhibition and not IGF-I signaling by SOCS2 is central to the increased post-natal body growth observed in the Soxs2−/− mice. This is consistent with the knowledge that GH is the major regulator of post-natal growth in mice, where peak GH activity occurs between postnatal days 20-40 (Wang et al. 2004). Additionally, the increased growth of Soxs2−/− mice was not observed when they were crossed with either mice lacking the STAT5b gene or those with a point mutation in the GH-releasing hormone receptor (Ghrhrlit/lit mice) (Greenhalgh et al. 2002, 2005). This provides further confirmation that the Soxs2−/− overgrowth phenotype is dependent on aberrant GH signaling.
In this present study, we found that the tibia of \textit{Socs2}\textsuperscript{−/−} mice was characterized by an increase in both cortical and trabecular bone mass. This was particularly evident in trabecular bone and these changes in architectural organization are likely to engender greater resistance to bending (or strength) in both compartments. Such changes have not previously been reported in \textit{Socs2}\textsuperscript{−/−} mice and are consistent with an inhibitory role for SOCS2 in the growth of osseous tissues and the enhanced GH/IGF-I signaling seen in \textit{Socs2}\textsuperscript{−/−} mice. The increased bone mass of the \textit{Socs2}\textsuperscript{−/−} mice is associated with a commensurate increase in total mineral content, as volumetric BMD measured by µCT in both cortical and trabecular bone was similar to wild-type levels. This data contrasts with previous reports where a reduction in both cortical thickness and cortical and trabecular volumetric BMD was reported (Lorentzon et al. 2005). These latter results are difficult to reconcile with the known anabolic effects of enhanced GH/IGF-I signaling on the skeleton (Ohlsson et al. 1998; Andreassen and Oxlund, 2002; Kasukawa et al. 2004) but may be due to the different ages of mice examined, as well as differences in the resolution of the analyses employed. DXA-based measures of BMD are derived from the total bone mass within the projected bone area, and may therefore conceal distinct effects in trabecular and cortical bone. In contrast, both pQCT and µCT estimate volumetric BMD, distinguish between cortical and trabecular bone, and provide accurate estimates of cortical dimensions, but µCT allows a greater spatial resolution down to 5-10 microns to be achieved. Increased GH/IGF-I signaling is also consistent with increases in serum indices of bone resorption (TRAP5b) and formation (osteocalcin). It is possible that these indices reflect higher basal rates of bone turnover in \textit{Socs2}\textsuperscript{−/−} mice. We propose that the altered tibial bone structure found in the \textit{Socs2}\textsuperscript{−/−} mice are a consistent and a
direct consequence of increased GH/IGF-I signaling. It remains to be determined, however, whether these altered bone parameters are a consequence only of the faster rates of longitudinal growth evident in SoCS2−/− mice, or represent a tissue autonomous role for SOCS2 in the maintenance of bone mass.

Previous reports indicate that the induction and/or enhancement of SOCS2 expression by a wide number of pro-inflammatory cytokines in several biological systems, including pancreatic cells (Santangelo et al. 2005), leukocytes (Dogusan et al. 2000) and the liver (Shen et al. 2000) is linked to the behavior of these systems in chronic inflammatory states. We have now shown that SOCS2 expression by chondrocytes increases following exposure to TNFα, a pro-inflammatory cytokine associated with pediatric chronic inflammatory disorders (MacRae et al. 2006a,b). This is the first evidence, indicating a likely role for SOCS2 in mediating cytokine effects on the dynamics of the growth plate.

Abnormal growth patterns are commonly observed in children suffering from chronic inflammatory diseases. These disorders are associated with the increased production of pro-inflammatory cytokines, which inhibit growth plate chondrocyte dynamics (MacRae et al. 2006a,b). To examine whether pro-inflammatory cytokines inhibit bone growth by elevating local SOCS2 to impair the GH/IGF-1 axis, we cultured metatarsals and growth plate chondrocytes derived from SoCS2−/− and wild-type mice and examined their response to pro-inflammatory cytokines. However, our studies revealed no effect of genotype on either bone growth or chondrogenic markers following TNFα exposure. It is possible that due to low levels of GH signaling, the in vivo role of SOCS2 is not essential or is
redundant in pre-weaning mice. It therefore remains possible that distinct biological systems exhibit differing time courses in the development of SOCS2-mediated regulation of cytokine-induced events. This would help to explain why TNFα produced similar effects regardless of the Socs2 gene context. Our data indicate that in pre-weaning mice, pro-inflammatory cytokines exert their inhibitory effects on the growth plate through a SOCS2-independent mechanism. It is possible that the effects of TNFα may only be revealed in metatarsals from older mice of an age experiencing active GH signaling and which show gigantism in Socs2 null background. Alternatively, TNFα may mediate their inhibitory effects on bone growth through other SOCS proteins.

In conclusion, the increased bone growth and bone mass observed in the Socs2+/− mice support the hypothesis that physiological levels of SOCS2 inhibit signaling through the GH/IGF-I axis and therefore negatively regulate bone formation and endochondral growth. Our data further suggest that pro-inflammatory cytokines mediate their inhibitory effects on longitudinal bone growth through mechanisms that are independent of SOCS2.

References


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Figure Legends

I. Targeting and screening strategy for construction of SoCs2−/− mice. (A) The wild type (+) allele and targeting construct (TG) are shown. An insert (~ 7.8 kb) containing IRES-β-geo cassette has been inserted into the KpnI site within the exon 2 of the SoCs2 transcript Ensembl ID ENSMUST00000020215 (coding region shown in black). Numbers above restrictions sites are positions within the mouse genome from the Ensembl release 45 - Jun 2007 [http://www.ensembl.org]. (B) Southern analysis showing a screen for targeted ES clones (left panel) and F2 mice (right panel). Southern probe (3’-SoCs2 probe) outside the homology arms (see Fig. 1a) was used on BamHI-digested genomic DNA to detect the wild type band (~ 24 kb, upper arrow) and a smaller targeted allele-band (~22 kb, lower arrow) due to introduction of additional BamHI site within the IRES-β-geo cassette. Left panel shows two targeted ES cell clones (+/-) and original wild type ES cell line (HM1, +/+); HG is high-growth mice DNA known to contain a deletion of this Soc2 locus; M is a DNA size marker (10 kb ladder). The right panel shows a screen of F2 mice of homozygous wild type (+/+), heterozygous (+/-) and homozygous knockout (−/−) genotype; HG DNA again serves as a negative control.

Figure II: Body weight (g) growth curves for female wild-type and SoCs2−/− mice (mean ±SEM, n=6).

Figure III: Three dimensional μCT imaging of the metaphyseal trabecular compartment of the tibia in 7-week-old female (a) wild-type and (b) SoCs2−/− mice.
Figure IV: (a) Socs2 mRNA expression in wild-type fetal (E14-E18) and neonatal (1 and 9-day-old) primary murine chondrocytes. (b) SOCS2 protein expression in ATDC5 cells during chondrogenesis (C) and terminal differentiation (TD). (c) SOCS2 protein expression in primary costochondral chondrocyte cultures following exposure to TNFα (10ng/ml) for 48h.

Figure V: Immunohistochemical localization of SOCS2 protein expression in a 3-week-old mouse tibia (a) Strong staining within proliferating chondrocytes (arrow heads) whereas weaker staining was observed in the hypertrophic cells (arrows). (b) Osteoblasts (arrows) within the metaphysis close to the chondro-osseous junction stained positive for SOCS2, whereas those in control sections (c) were negative. Scale bars = 50 µm.

Figure VI: Quantitative RT-PCR analysis of chondrogenic marker expression in primary costochondral chondrocytes of neonatal female wild-type and Socs2−/− 1-day-old mice, following exposure to TNFα (10ng/ml) for 48h compared to control. Chondrogenic markers are aggrecan (a) wild-type and (b) Socs2−/−; collagen II (c) wild-type and (d) Socs2−/−; and collagen X (e) wild-type and (f) Socs2−/−. Data are presented as mean ± SEM, (n=3). Significantly different from control, * P<0.05

Figure VII: Percentage (%) change in length of (a) fetal and (b) postnatal wild-type and Socs2−/− murine metatarsals treated with control medium or TNFα (10ng/ml) over an 8 day period (all 10ng/ml) Data are presented as mean ± SEM, (n=6).