Increased Linear Bone Growth by GH in the Absence of SOCS2 Is Independent of IGF-1

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Growth hormone (GH) signaling is essential for postnatal linear bone growth, but the relative importance of GHs actions on the liver and/or growth plate cartilage remains unclear. The importance of liver derived insulin like-growth factor-1 (IGF-1) for endochondral growth has recently been challenged. Here, we investigate linear growth in Suppressor of Cytokine Signaling-2 (SOCS2) knockout mice, which have enhanced growth despite normal systemic GH/IGF-1 levels. Wild-type embryonic ex vivo metatarsals failed to exhibit increased linear growth in response to GH, but displayed increased Socs2 transcript levels (P < 0.01). In the absence of SOCS2, GH treatment enhanced metatarsal linear growth over a 12 day period. Despite this increase, IGF-1 transcript and protein levels were not increased in response to GH. In accordance with these data, IGF-1 levels were unchanged in GH-challenged postnatal Socs2−/− conditioned medium despite metatarsals showing enhanced linear growth. Growth-plate Igf1 mRNA levels were not elevated in juvenile Socs2−/− mice. GH did however elevate IGF-binding protein 3 levels in conditioned medium from GH challenged metatarsals and this was more apparent in Socs2−/− metatarsals. GH did not enhance the growth of Socs2−/− metatarsals when the IGF receptor was inhibited, suggesting that IGF receptor mediated mechanisms are required. IGF-2 may be responsible as IGF-2 promoted metatarsal growth and Igf2 expression was elevated in Socs2−/− (but not WT) metatarsals in response to GH. These studies emphasise the critical importance of SOCS2 in regulating GHs ability to promote bone growth. Also, GH appears to act directly on the metatarsals of Socs2−/− mice, promoting growth via a mechanism that is independent of IGF-1.

The anabolic role of growth hormone (GH) in long bones is well accepted. The relative contributions of GH acting on the liver (increasing growth promoting endocrine factors) or directly (on growth plate cartilage) however remain unclear (Lupu et al., 2001). It is likely that both these modes of GH action function in a highly coordinated manner to regulate growth plate function and linear bone growth. GH increases insulin like growth factor-1 (IGF-1) production in a number of tissues. Specifically, GH acting on the liver results in an increase in circulating IGF-1 which functions in an endocrine manner (Sjogren et al., 1999; Yakar et al., 1999). GH induced IGF-1 production within the growth plate cartilage functions in an autocrine/paracrine manner. Furthermore, GH may also act on the growth plate via IGF-1 independent mechanisms (Gevers et al., 2002a; Wang et al., 2004).

As expected, studies with Snell (dw/dw) and Ames (df/df) hypophysectomy dwarf mice reveal a reduction in body weight and growth retardation. Mice with global inactivated GHR (ghr), GH- releasing hormone receptor (ghrhr), IGF-1 (Igf1), IGF-1 receptor (IGF-1R; Igf1r), and insulin receptor substrate-1 (Irs-1) have a similar phenotype (Sinha et al., 1975; Smeets and Vanbuuloflers, 1983a,b; Li et al., 1990; Sornson et al., 1996; Wang et al., 2004)

The importance of circulating IGF-1 on linear growth was challenged by two independent studies. Liver-specific IGF-1 deficient (LID) mice have a significant reduction of circulating IGF-1, but body weight and bone length were essentially normal (Sjogren, 1999; Yakar et al., 1999). Furthermore, a phenotypic comparison of LID, acid labile subunit knockout (ALSKO), insulin like growth factor binding protein (IGFBP)-3 knockout (BP3KO), and triply deficient LID/ALSKO/BP3KO mice indicated that while all had decreased serum IGF-1 levels this did not predict linear growth potential (Yakar et al., 2009). Compared to WT mice, ALSKO mice (60% reduction in serum IGF-1) were 8% shorter, whereas BP3KO mice (40% reduction in serum IGF-1) were 5% longer, and LID mice (80% reduction in serum IGF-1) were of equal length. Most strikingly, despite virtually undetectable circulating IGF-1 (2.5% of WT IGF-1 levels), the triply deficient LID/ALSKO/BP3KO mice exhibited only a modest 6% decrease in body length, comparable to that of the ALSKO mice that had much greater serum IGF-1 levels (Yakar et al., 2009). However, interpretation of some of these observations is complicated by a marked increase in circulating GH to supraphysiological levels (Yakar et al., 2002) and models with a physiological level of circulating GH maybe more informative.
Some function has been attributed to circulating IGF-1, and it is possible that a threshold concentration of circulating IGF-1 is required for normal linear growth (Lupu et al., 2001). The data from the LID/ALSKO/BP3KO mice however are at odds with this concept (Yakar et al., 2002; Yakar et al., 2009). When hepatic IGF-1 production was achieved in mice lacking Igf1 gene expression in all other tissues, it was found that circulating IGF-1 contributes to 30% of the adult body size and sustains postnatal development (Stratanopoulos et al., 2008). Similarly, in Igf1 null mice with hepatic over expression of the rat Igf1, serum IGF-1 production supported normal body growth during and after puberty, despite absence of tissue IGF-1 (Wu et al., 2009; Ellis et al., 2010).

Alternative strategies to delineate between circulating and local IGF-1 effects on linear bone growth have involved the targeted deletion of Igf1l in epiphyseal chondrocytes. These mice had a 40% reduction in cartilage IGF-1 expression and normal circulating IGF-1 levels (Govoni et al., 2007). Linear growth was reduced by 27% between 2 and 4 weeks of age, highlighting that local chondrocyte-produced IGF-1 is an important regulator of longitudinal growth. While highly informative, these studies fail to address the possibility of a GH action on the growth plate, which is independent of IGF-1. Double ghrlr1 IGF-1 KO mice have a more severe phenotype than ghrl or Igf1 KO’s alone suggesting that there are GH actions on linear bone growth that are independent of IGF-1 (Lupu et al., 2001).

A role for GH acting directly on growth plate cartilage is also suggested by data from suppressor of cytokine signaling 2 (SOCS2) null mice (Metcalfe et al., 2000; MacRae et al., 2009). SOCS2 is expressed by epiphyseal chondrocytes, and is a recognised negative regulator of GH signaling via inhibition of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Hilton, 1999; Greenalgh et al., 2002; Rico-Bautista et al., 2006; Flores-Morales et al., 2006; Pass et al., 2009). Intriguingly, these mice are characterized by increased growth, including increased long bone length and mass, without elevated circulating levels of IGF-1 or GH (Metcalfe et al., 2000; Greenalgh et al., 2005; MacRae et al., 2009; Dobie et al., 2014). It can therefore be assumed that GH actions directly on growth plate cartilage (IGF-1 dependent or independent) are driving this increased linear bone growth.

The ex vivo metatarsal culture model has been exploited in many studies as a method for analysing endochondral bone (IGF-1 dependent or independent) are driving this increased linear bone growth. The middle three metatarsals were isolated from 17-day-old WT and Socs2-/- embryos (E17) or 3-day-old (PN3) WT and Socs2-/- pups. At both developmental ages the growth plate contains both proliferating and hypertrophic chondrocytes but the primary ossification center, while newly formed in E17 metatarsals, is almost completely developed in PN3 bones (van Loon et al 1995; Idris et al., 2009). Each bone was cultured individually in 1 well of a 24-well plate (Costar, High Wycombe, UK) in 300 μl αMEM medium (without ribonucleosides) or 300 μl DMEM F12 medium supplemented with 0.2% BSA (Fraction V), 5 μg/ml L-asparagine, 1 mM β-glycerophosphate, 0.05 mg/ml gentamicin, 1.25 μg/ml fungizone (Invitrogen, Paisley, UK) as previously described (Chagin et al., 2010; Pass et al., 2012). The periosteum and the primary ossification center was not removed prior to explant culture. Recombinant human (rH) GH and rHIGF-1 (Bachem, Montigny-le-Bretonneux, France) were added as used previously (Mushtaq et al., 2004a; Mushtaq et al., 2006a). Each bone was cultured for 5 days in αMEM supplemented at the same concentration as rHIGF-1 (100 ng/ml). One micrometer NVP-AEW541 (IGF-1 receptor kinase inhibitor) (Garcia-Echeverria et al., 2004; Gan et al., 2010) was added 16 h prior to the addition of GH. Bones were incubated in a humidified atmosphere (37°C, 5% CO2), for up to 13 days. Bone lengths were measured from articular surface to articular surface through the middle of the metatarsals using a Nikon eclipse TE300 microscope with a digital camera attached, using Image Tool (Image Tool Version 3.00, San Antonio, TX). For RNA extraction 3–4 bones were pooled in 100 μl Trizol reagent (Invitrogen) at days 7 and 12 of culture and RNA extracted according to the manufacturer’s instructions (RNeasy Mini Kit, Qiagen). Conditioned medium was collected at days 5, 7, and 12 and stored at −80°C.

**Materials and Methods**

**Mice**

Socs2-/- mice were generated as previously described (MacRae et al., 2009). For genotyping, tail or ear biopsied DNA was analyzed by PCR for Socs2 (WT) or the neocassette (Socs2+/-) Primer sequences can be found in Supp. Table S1 (Eurofins MWG Operon, London, UK) (Pass et al., 2012). All animal experiments were approved by Roslin Institute’s Animal Users Committee, and the animals were maintained in accordance with Home Office (UK) guidelines for the care and use of laboratory animals.

**Growth analysis and growth plate dynamics**

Six-week-old male WT and Socs2-/- mice received a subcutaneous injection of 10 mg/kg calcein (Sigma, Poole, UK) in sodium bicarbonate solution 2 days prior to sacrifice. Tibiae, fixed overnight in 4% paraformaldehyde (PFA) were embedded in methylmethacrylate and sections (5 μm) were cut and processed using standard procedures (Idris et al., 2009). The longitudinal bone growth rate was measured as previously described (Owen et al., 2009; Pass et al., 2012). In brief, the distance between the original growth plate mineralization front and the final fluorescing mineralization front within the metaphysis was measured at 10 different points along the width of the section using image analysis software and a Leica DMBR fluorescent microscope. Measurements were divided by the number of days between injection and sacrifice (2 days) to give a bone formation rate per day. Four sections per tibia were measured from 6 mice per group.

**Embryonic and postnatal metatarsal organ culture**

The middle three metatarsals were isolated from 17-day-old WT and Socs2-/- embryos (E17) or 3-day-old (PN3) WT and Socs2-/- pups. At both developmental ages the growth plate contains both proliferating and hypertrophic chondrocytes but the primary ossification center, while newly formed in E17 metatarsals, is almost completely developed in PN3 bones (van Loon et al. 1995; Idris et al., 2009). Each bone was cultured individually in 1 well of a 24-well plate (Costar, High Wycombe, UK) in 300 μl αMEM medium (without ribonucleosides) or 300 μl DMEM F12 medium supplemented with 0.2% BSA (Fraction V), 5 μg/ml L-asparagine, 1 mM β-glycerophosphate, 0.05 mg/ml gentamicin, 1.25 μg/ml fungizone (Invitrogen, Paisley, UK) as previously described (Chagin et al., 2010; Pass et al., 2012). The periosteum and the primary ossification center was not removed prior to explant culture. Recombinant human (rH) GH and rHIGF-1 (Bachem, Montigny-le-Bretonneux, France) were added as used previously (Mushtaq et al., 2004a; MacRae et al., 2006a; Pass et al., 2012). Recombinant mouse (rm)IGF-2 (R&D systems, Minneapolis, MN) was added at the same concentration as rHIGF-1 (100 ng/ml). One micrometer NVP-AEW541 (IGF-1 receptor kinase inhibitor) (Garcia-Echeverria et al., 2004; Gan et al., 2010) was added 16 h prior to the addition of GH. Bones were incubated in a humidified atmosphere (37°C, 5% CO2), for up to 13 days. Bone lengths were measured from articular surface to articular surface through the middle of the metatarsals using a Nikon eclipse TE300 microscope with a digital camera attached, using Image Tool (Image Tool Version 3.00, San Antonio, TX). For RNA extraction 3–4 bones were pooled in 100 μl Trizol reagent (Invitrogen) at days 7 and 12 of culture and RNA extracted according to the manufacturer’s instructions (RNeasy Mini Kit, Qiagen). Conditioned medium was collected at days 5, 7, and 12 and stored at −80°C.

**Growth plate micro dissection and RNA extraction**

Tibiae were dissected from 7-week-old male WT (n = 4) and Socs2-/- mice (n = 3). Bones were briefly immersed in 4% aqueous (wt./vol.) polyvinylalcohol (PVA; Grade GO4/140, Wacker Chemicals, Walton-on-Thames, UK), chilled by precipitate immersion in n-hexane (BDH, Poole, UK; grade low in aromatic hydrocarbons) and stored at −80°C (25,26). Using optimal cutting
Tibiae were dissected from 6-week-old male WT (n = 6) and Socs2-/− mice (n = 6) and fixed in 70% ethanol for 48 h at 4°C before decalcification in 10% EDTA (pH 7.4) for approximately 4 weeks at 4°C with regular changes. Tissues were finally dehydrated and embedded in paraffin wax, using standard procedures, and sectioned at 5 μm. Immunohistochemical analysis, sections were dewaxed in xylene and rehydrated before incubation in 7% hydrogen peroxide for 30 min to block endogenous peroxidase activity, followed by blocking with normal goat serum. Primary antibodies against IGF-1 (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany), IGFBP3 (1:1000, Abcam, Cambridge, MA), and Igf2 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany) were diluted in 1% bovine serum albumin (BSA) PBS and incubated overnight at 4°C. Sections were then washed in PBS and incubated with secondary antibodies. Detection was performed using the Vectorstain ABC kit (Vector Laboratories, Peterborough, UK) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin and mounted in DePeX.

Conditioned medium analysis

Total IGF-1 and IGFBP3 levels in conditioned medium from metatarsals cultured ± GH were assessed at days 5, 7, and 12 by ELISA (Quantikine, R&D Systems, Minneapolis, MN). IGF-2 levels in conditioned medium were assessed at day 12 (Ray Biotech, Inc) according to the manufacturer’s instructions. IGF-1 assays included a step to dissociate the potentially interfering binding proteins from the ligand.

Statistical analysis

Direct comparison between two sets of data was done by Student’s t-test or a suitable non-parametric test if the data was not normally distributed. Time course experiments were analyzed with a repeated measures 2-way ANOVA for which suitable post-tests for multiple comparisons were conducted. Analysis was carried out using SigmaPlot v11.0 (Systat Software Inc, London, UK). Data are presented as mean ± standard error of the mean (SEM). P values <0.05 were considered significant.

Results

SOCS2 regulation of GH induced growth in metatarsals

WT metatarsals extracted at E17 did not increase their length in response to GH over the 12 day culture period (Fig. 1A). Transcription analysis at day 7, revealed a 17-fold increase in Socs2 mRNA expression in the GH-treated group in comparison to the control group (P < 0.01) (Fig. 1B). Socs1 and Socs3 mRNA expressions were however not enhanced in response to GH (Fig. 1B). Socs2+/− metatarsals growth significantly increased in response to GH from day 7 to day 12 (P < 0.05) (Fig. 1C), similar to previous studies (Pass et al., 2012). IGF-1 stimulated longitudinal growth in WT and Socs2+/− metatarsals as expected. The level of stimulation in the presence of IGF-1 was comparable between WT and Socs2+/− metatarsals, indicating that SOCS2 does not regulate IGF-1 induced growth in this model (Fig. 1A and C). Assessment of WT and Socs2−/− metatarsals revealed a similar GH response to that noted with the embryonic metatarsal with only the Socs2−/− bones showing enhanced growth in response to GH (P < 0.01) (Supp. Fig. 1A and C). Also, Socs2−/− but not Socs1−/− or Socs3−/− mice showed enhanced growth in response to GH (P < 0.01) in WT metatarsals (Supp. Fig. 1B). Taken together, these data indicate that SOCS2 is a major regulator of GH promotion of longitudinal growth in the metatarsal organ culture model.

Increased growth of Socs2−/− metatarsals in response to GH is not associated with an increase in chondrocyte IGF-1

We next aimed to determine if the growth promoting effects of GH on Socs2−/− metatarsals were direct or mediated by IGFs. Following 7 days of GH treatment, WT metatarsals showed no increase in longitudinal growth compared to untreated samples, whereas Socs2−/− metatarsal growth increased by 15% (P < 0.01) (Fig. 2A and B). Expression levels of IGF1, IGFBP3, and Igf2 at this time point were unaltered in response to GH in WT and Socs2−/− metatarsals (Fig. 2C). Protein analysis of day 5 conditioned medium revealed increased IGFBP3 protein levels in GH-treated Socs2−/− (Control 8.2 ± 1.35, GH 23.5 ± 5.76; P < 0.01), but not in corresponding WT conditioned medium (Tables 1 and 2). ELISA analysis of day 7 conditioned medium similarly found no increase in IGF-1 or IGFBP3 protein levels in GH treated WT metatarsals (Table 1). While IGF-1 remained at basal level in GH-stimulated Socs2−/− conditioned medium at day 7, IGFBP3 levels increased significantly (Control 5.2 ± 0.94, GH 22.6 ± 3.34; P < 0.01) (Table 2).

At day 12, Socs2−/− metatarsal growth increased by 23% (P < 0.01) in response to GH, while WT metatarsal growth remained at control level (Fig. 2A and B). Similar to the day 7 data; expression levels of IGF1, IGFBP3, and Igf2 were unaltered in response to GH in WT metatarsals (Fig. 2D). Similarly, IGF1 transcript levels remained at basal level in GH-treated Socs2−/− metatarsals, whereas IGFBP3 and Igf2 transcript levels significantly increased by 3.5- (P < 0.05) and 4.6- fold (P < 0.05) respectively (Fig. 2D). A similar trend was observed when analyzing protein levels of conditioned medium (Tables 1 and...
2). GH failed to have a stimulatory effect on IGF-1 levels in WT or Soc2<sup>−/−</sup> conditioned medium. However, GH treatment did result in a 59% (P < 0.01) and 123% (P < 0.001) increase in IGFBP3 levels in WT and Soc2<sup>−/−</sup> conditioned medium, respectively. IGF-2 protein levels were also increased in Soc2<sup>−/−</sup> conditioned medium in response to GH (P < 0.001) (Table 2). No increase in IGF-2 levels was observed in corresponding WT medium (Table 1).

Experiments carried out on PN3 metatarsals produced comparable results to those observed in E17 metatarsals in terms of IGF-1 production. Despite there being an increase in growth of Soc2<sup>−/−</sup> metatarsals in response to GH, IGF-1 levels in conditioned medium at day 7 and 12 were not preferentially increased in Soc2<sup>−/−</sup> metatarsals (Supp. Tables 2 and 3). GH stimulated IGFBP3 protein levels in WT-conditioned medium, an increase that appeared to be enhanced in the absence of SOCS2 at day 12 (Supp. Tables 2 and 3). A comparable increase in IGF-2 levels in conditioned medium following GH treatment was observed in WT and Soc2<sup>−/−</sup> metatarsals (Supp. Tables 2 and 3).

These data reveal that increased longitudinal growth in response to GH observed in the Soc2<sup>−/−</sup> metatarsal model is not mediated by increased IGF-1 expression. They also highlight the possible importance of IGFBP3 and IGF-2 in this model as secondary messengers to GH in promoting longitudinal bone growth in Soc2<sup>−/−</sup> metatarsals.

Altered IGF-2 and IGFBP3 levels in the growth plates of Soc2<sup>−/−</sup> mice

Examination of growth plates from WT mice indicated that both IGF-2 and IGFBP3 protein were preferentially localized to the proliferating zone of the growth plate with little or no staining in the hypertrophic chondrocytes. The staining in the proliferating chondrocytes of the Soc2<sup>−/−</sup> growth plates of both IGF-2 and IGFBP3 appeared similar to that observed in similarly stained WT growth plates. In contrast, growth plates from Soc2<sup>−/−</sup> mice contained a greater number of hypertrophic chondrocytes that stained positively for both IGF-2 and IGFBP3 (Fig. 3). All control sections showed no staining (data not shown).

Fig. 1. SOCS2 regulation of GH-induced embryonic metatarsal growth. Graphs showing (A) WT and (C) Soc2<sup>−/−</sup> E17 metatarsal growth in response to GH or IGF-1 (100 ng/ml) over a 12 day period. Data are presented as mean ± SEM. Significance denoted by IGF-1 versus control: *P < 0.05, **P < 0.001. GH versus control: *P < 0.05, **P < 0.001, (n = 6). (B) Transcript analysis of Soc1, 2, and 3 in WT metatarsals following 7 days GH (100 ng/ml) treatment. Data represented as means ± SEM. Significance from untreated metatarsals denoted by *P < 0.05, **P < 0.01, (n = 3).
No additive effects of GH and IGF-1 action in the promotion of metatarsal linear growth

Having shown that both GH and IGF-1 stimulated longitudinal growth in Socs2−/− metatarsals, and that GH stimulation was independent of increased IGF-1 levels, we next aimed to determine if the actions of GH and IGF-1 on longitudinal growth were additive. Dual treatment with GH and IGF-1 in WT metatarsals significantly increased growth in comparison to control samples ($P < 0.001$, $P < 0.05$, respectively; Fig. 4A). This level of increase did not differ from that observed with IGF-1 treatment alone.

In Socs2−/− metatarsals, growth was significantly increased in response to both IGF-1 and GH treatment in comparison to control samples ($P < 0.01$, $P < 0.05$ respectively; Fig. 4B). There was no additional increase in metatarsal growth upon dual treatment (Fig. 4B).

The IGF-1R is critical for GH-induced growth

The cellular responses of IGF-1 and IGF-2 are both mediated through the IGF-1R. To investigate the importance of this

### Table 1. IGF-1, IGFBP3, and IGF-2 protein levels in conditioned medium from E17 WT metatarsals following 5, 7, or 12 days GH (100 ng/ml) treatment.

| Day | Treatment | IGF-1 (ng/ml) | IGFBP3 (ng/ml) | IGF-2 (ng/ml) |
|-----|-----------|---------------|----------------|---------------|
| 5   | Control   | 2.4 ± 0.48    | 9.0 ± 1.43     | nd            |
|     | GH        | 2.1 ± 0.23    | 9.7 ± 1.41     | nd            |
| 7   | Control   | 6.4 ± 0.86    | 12.2 ± 1.68    | nd            |
|     | GH        | 4.0 ± 0.70    | 19.7 ± 4.57    | nd            |
| 12  | Control   | 11.6 ± 1.16   | 25.0 ± 5.09    | 7.0 ± 1.39    |
|     | GH        | 9.5 ± 1.06    | 39.8 ± 3.69    | 8.4 ± 0.97    |

Data are presented as mean ± SEM ($n \geq 5$). Significance from day matched control samples denoted by $^*P < 0.05$. nd = no data.
receptor in mediating the effects of GH on longitudinal bone growth, we assessed the growth of Sox2\(^{-/-}\) metatarsals in the presence of GH and an IGF-1R inhibitor (NVP-AEW541). Initially, WT metatarsals were treated with varying concentrations of NVP-AEW541 for 5 days before the inhibitor was removed from the medium (Fig. 4C). All concentrations of NVP-AEW541 (1–4 μM) inhibited metatarsal growth confirming that endogenous IGF signaling is important for metatarsal growth. One micrometer of NVP-AEW541 was selected for future experiments as on the removal of the inhibitor, metatarsals showed increased growth suggesting that the inhibitor at this concentration (cf 3 and 4 μM) had no lasting toxic effects on growth potential. Sox2\(^{-/-}\) metatarsals treated with the NVP-AEW541 showed significantly decreased growth compared to control samples \((P < 0.05, \text{Fig. } 4D)\). GH treatment was unable to stimulate growth in the presence of the IGF-1R inhibitor, suggesting that the effects of GH on longitudinal bone growth are mediated through the IGF-1R.

**IGF-2 stimulation of embryonic Sox2\(^{-/-}\) metatarsals**

As GH-induced growth in Sox2\(^{-/-}\) metatarsals was associated with an increase in IGF-2 levels (Table 2), we next determined if IGF-2 could stimulate longitudinal growth. IGF-2 treatment significantly increased longitudinal growth of Sox2\(^{-/-}\) metatarsals from day 5 to 12 \((P < 0.05; \text{Fig. } 4E)\). This enhanced growth by IGF-2 was however, significantly less than that stimulated by equimolar IGF-1, indicating that IGF-1 is the more potent of the two IGF ligands in promoting linear bone growth (Fig. 4E). Similar results were observed in WT metatarsals (data not shown).

**Igfl mRNA levels in the Sox2\(^{-/-}\) growth plate**

Calcine labeling of 6-week-old Sox2\(^{-/-}\) mice revealed that tibial growth over a 2 day period of Sox2\(^{-/-}\) mice (44.5 ± 1.8 μm) was increased in comparison to WT mice (36.8 ± 1.57 μm) \((21%, P < 0.05)\) (Fig. 5A). Igf1 levels were also assessed in the growth plates of WT and Sox2\(^{-/-}\) mice and found not to be significantly altered from expression levels of WT mice. Therefore, despite increased bone growth rates in Sox2\(^{-/-}\) mice there was no elevation in growth plate Igf1 transcript levels (Fig. 5B).

**Discussion**

It is widely understood that GH action on growth plate chondrocytes is an important regulator of linear growth (Ahmed and Farquharson, 2010). The intimate relationship between GH and IGF-1 does however make it difficult to attribute specific actions to GH, or those resulting through IGF-1. Tibial growth is reduced in both ghr and Igf1 null mice. This reduction in growth becomes more severe in the double ghr/Igf1 mutants, suggesting that GH and IGF-1 have independent functions (Lupu et al., 2001). In agreement with this, mice deficient of GH show a much higher reduction in longitudinal bone growth than Igf1 null mice (Sims et al., 2000; Mohan et al., 2003; Wang et al., 2004). The present study demonstrates that despite no elevation in Igf1 gene and protein expression levels in Sox2\(^{-/-}\) metatarsals following GH treatment, GH enhances linear bone growth. This indicates that the growth promoting effects of GH are not accompanied by a rise in Igf1 expression by chondrocytes or cells of the perichondrium, which previous studies have indicated is a major source of Igf1 in the growing bone (Parker et al., 2007). This was supported by the observation of normal Igf1 expression levels in the growth plates of Sox2\(^{-/-}\) tibia which are characterized by wider growth plates and longer bones (MacRae et al., 2009; Pass et al., 2012).

In addition to their normal GH and IGF-1 serum concentrations, Sox2\(^{-/-}\) mice also have normal carbohydrate metabolism compared with ALSKO, BP3KO, LID, and IGF-1 null mice (Rico-Bautista et al., 2005; Yakar et al., 2009). Thus, the Sox2\(^{-/-}\) mouse may represent an important model to study local autocrine/paracrine pathways that promote linear bone growth without leading to insulin resistance and altered carbohydrate metabolism.

Several studies have used the fetal mouse metatarsal culture methods to investigate the anabolic effects of IGF-1 on linear bone growth. Increased growth of metatarsals in response to IGF-1 is coupled with an increase in proliferation and an increase in hypertrophic zone size (Mushtaq et al., 2004; MacRae et al., 2006, 2007; Chagin et al., 2010). The effects of

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**Table 2. IGF-1, IGFBP3, and IGF-2 protein levels in conditioned medium from E17 Sox2\(^{-/-}\) metatarsals following 5, 7, or 12 days GH (100 ng/ml) treatment**

| Day | Treatment | IGF-1 (ng/ml) | IGFBP3 (ng/ml) | IGF-2 (ng/ml) |
|-----|-----------|---------------|----------------|---------------|
| 5   | Control   | 2.7 ± 0.59    | 8.2 ± 1.35     | nd            |
|     | GH        | 2.6 ± 0.26    | 23.5 ± 5.76*   | nd            |
| 7   | Control   | 3.3 ± 0.39    | 5.2 ± 0.94     | nd            |
|     | GH        | 2.8 ± 0.28    | 22.6 ± 3.4*    | nd            |
| 12  | Control   | 9.9 ± 0.90    | 22.2 ± 3.51    | 1.7 ± 0.34    |
|     | GH        | 7.0 ± 0.39    | 49.6 ± 1.89*   | 17.1 ± 1.72*  |

Data are presented as mean ± SEM \((n > 5)\). Significance from control day matched control samples denoted by *P < 0.01; **P < 0.001.

**Fig. 3. Immunohistochemical localization of IGF-2 and IGFBP3 to the proliferating and hypertrophic chondrocytes of WT and Sox2\(^{-/-}\) growth plates. In contrast to WT growth plates, hypertrophic chondrocytes within the Sox2\(^{-/-}\) growth plate expressed high levels of both IGF-2 and IGFBP3 (arrows). Scale bars = 50 μm.**
Fig. 4. Effect of IGF-1R inhibitor, IGF-2, and combined IGF-1 + GH on metatarsal growth. Graphs showing (A) WT and (B) Soxs2−/− E17 metatarsal growth in response to GH or IGF-1 (100 ng/ml) or GH + IGF-1 over a 13–14 day period. Data are presented as mean ± SEM. Significance denoted by GH versus control aP < 0.05, bP < 0.01, cP < 0.001. IGF-1 versus control dP < 0.05, eP < 0.01, fP < 0.001. GH + IGF-1 versus control gP < 0.05, hP < 0.01, iP < 0.001, (n ≥ 6). (C) Graph showing WT E17 metatarsal growth in response to NVP-AEW481 (1–4 μM) for 5 days with 4 days recovery. Data are presented as mean ± SEM. (n ≥ 6). (D) Graph showing Soxs2−/− E17 metatarsal growth in response to GH (100 ng/ml), NVP-AEW481 (1 μM) or GH + NVP-AEW481. Data are presented as mean ± SEM. Significance of GH versus control denoted by aP < 0.05, bP < 0.01, (n ≥ 6). (E) Graph showing Soxs2−/− E17 metatarsal growth in response to IGF-1 or IGF-2 (100 ng/ml). Data are presented as mean ± SEM. Significance denoted by IGF-1 versus control aP < 0.01, bP < 0.001. IGF-2 versus control cP < 0.05, dP < 0.01, (n ≥ 6).
GH on metatarsal growth are however less clear, with GH being shown to either stimulate or have no effect on growth (Scheven and Hamilton, 1991; Mushtaq et al., 2004; Pass et al., 2012). In chondrocytes, GH is able to stimulate SOCS2 expression (Pass et al., 2012) and we have now further shown that SOCS2 is a negative regulator of GH’s capability to promote longitudinal bone growth in the metatarsal organ culture model. This observation is in agreement with previous reports where mice missing the Socs2 gene have increased linear bone growth due to increased signaling through the GHR (Metcalf et al., 2000; Greenhalgh et al., 2005; MacRae et al., 2009). Although, it is well recognized that GH signaling is also modulated by CIS and SOCS1 and 3 we have reported previously that GH does not stimulate chondrocyte SOCS1 or 3 protein levels (Pass et al., 2009, 2012). Furthermore, Socs1−/− and Socs3−/− mice are perinatal and embryonic lethal, respectively and little data exists on their ability to regulate bone growth (Greenhalgh and Alexander, 2004). While further studies are required to investigate GH ability to stimulate chondrocyte CIS expression it is of interest to note that Cis−/− mice exhibit no obvious growth phenotype (Greenhalgh and Alexander, 2004).

It is widely established that the prenatal growth promoting effects of IGF-1 are GH independent, and that GH has little or no role in promoting growth in embryonic development (Waters and Kaye, 2002). Therefore, these key studies were also completed using postnatal metatarsals, which may be more sensitive to the growth promoting actions of GH. No evidence for this was however observed in our experiments and older postnatal bones may have to be studied to see a GH response. However, this was not possible as our pilot studies (data not shown) indicated that 3-day-old postnatal metatarsals was the oldest age we could study to get measurable growth. Even at postnatal day 3, bone growth was much less than embryonic metatarsals and this is in accord with previous observations (Macrae et al. and 2006; Chagin et al., 2010). It is therefore tantalizing to speculate that variable SOCS2 expression in normal animals may “fine tune” the chondrocytes ability to respond to GH. Such a regulatory mechanism may permit postnatal bones at a specific developmental age to respond to GH and further studies are required to investigate this.

Socs2−/− mice have increased linear bone growth consistent with increased signaling through the GHR (Metcalf et al., 2000;...
Greenhalgh et al., 2005; Lorentzon et al., 2005; MacRae et al., 2009; Pass et al., 2012). As Socs2−/− mice are devoid of higher circulating IGF-1 levels, it is likely that the increased linear bone growth and structural alterations within Socs2−/− growth plate carilage (Lorentzon et al., 2005; MacRae et al., 2009). The broad distribution of both the GHR and IGF-1R within the growth plate suggests GH/IGF-1 may play a role in chondrocyte proliferation, differentiation, and hypertrophy (Lupu et al., 2001; Gevers et al., 2002b; Wang et al., 2011). The germinal zone of the growth plates are significantly enlarged in IGF-1−/− null mice, supporting the idea that GH stimulates longitudinal bone growth independent of IGF-1 (by stimulating the differentiation of growth plate precursor cells), and via an IGF-1 dependent mechanism (by inducing IGF-1 expression in differentiating chondrocytes which then in-turn stimulate chlonal expansion) (Isaksson et al., 1987; Wang et al., 2004). Although IGF-1 is expressed in all maturational growth plate zones, it is understood to act mainly on the hypertrophic zone. IGF-1−/− mice have attenuation of chondrocyte hypertrophy with no changes in proliferation (Wang et al., 1999). Addition of IGF-1 to metatarsals leads to an increase in hypertrophic zone lengths as well as increased size of individual chondrocytes (Mushtaq et al., 2004). These mechanistic observations are important as a large part of growth of cultured metatarsals is due to hypertrophic differentiation but it is recognized these observation may not translate directly to the in vivo situation where matrix production, proliferation and hypertrophy all contribute to linear bone growth (Wilsman et al., 1996).

The GH signaling mechanisms responsible for increasing linear bone growth have been recently clarified in studies where GH was found to stimulate the phosphorylation of STATs 1, 3, and 5 in chondrocyte cultures (Pass et al., 2012). The activation of chondrocyte STAT signaling was both increased and prolonged in chondrocytes from Socs2−/− mice in comparison to cells from WT mice (Pass et al., 2012). This explains the observed GH stimulation of linear growth of Socs2−/− embryonic metatarsals and the proliferation of chondrocytes within (Pass et al., 2012).

Both IGF-1 and IGF-2 are important regulators of skeletal growth. While IGF-1 is important for both fetal and postnatal growth, IGF-2 is thought to function only in the former. IGF-2 is highly expressed in a variety of tissues in the embryo, and is dramatically down-regulated shortly after birth (Lui et al., 2008). Disruption of the Igf2 gene results in progeny that are 60% smaller than WT littermates, however postnatal growth is comparable to WT (Dechiara et al., 1990). Although it is widely understood that IGF-2 actions are independent of GH regulation, GH has been shown to regulate IGF2 transcription in the human liver (von Horn et al., 2002). In this present study, both during fetal and early postnatal linear growth, IGF-2 levels are regulated by GH. It is conceivable that the increase in linear growth observed in Socs2−/− metatarsals are a result of elevated IGF-2 levels. IGF-2 can promote the expansion of fetal hypertrophic chondrocytes in cultured limb explants, possibly through activation of the PI3K and TGF-β pathways and downstream elevation of transcription factors (Hamamura et al., 2008; Chen et al., 2010). Both IGF-1 and IGF-2 bind to the IGF-1R, and the cellular responses of both ligands are mediated through this receptor. As inhibition of this receptor abrogates the growth promoting effects of GH, it stands to reason that IGFs are responsible for mediating the increase in linear growth. As there is no increase in the expression of IGF-1 in response to GH, it is possible that IGF-2 and not IGF-1 is responsible for the GH promoted linear growth of the Socs2−/− metatarsals. In agreement with this, IGF-2 was able to stimulate longitudinal bone growth in metatarsals from WT and Socs2−/− mice.

Confirmation of increased expression of IGF-2 and IGFBP3 by the hypertrophic chondrocytes of growth plates in response to unrestricted GH signaling was revealed by immunohistochemical analysis of Socs2−/− growth plates. The presence of IGF-2 and IGFBP3 in hypertrophic chondrocytes is consistent with GHR expression in these cells of the growth plate and our previous observation of increased numbers of phosphorylated STAT-5–positive hypertrophic chondrocytes of Socs2−/− growth plates (Gevers et al., 2002b; Pass et al., 2012). The staining in the proliferating chondrocytes of the Socs2−/− growth plates appeared similar to that observed in the WT growth plates however it was not possible, because of the nonquantitative nature of immunohistochemistry, to compare levels of IGF-2 and IGFBP3 in the proliferating chondrocytes. These data suggest that SOCS2 can influence hypertrophic chondrocyte IGF-2 and IGFBP3 availability and thereby modulate longitudinal bone growth.

The growth rate of tibia from ghrl/igf1 null mice are almost identical to the sum of growth deficit observed in the single ghrl and igf1 mutants suggesting that GH and IGF-1 have independent roles in promoting linear growth (Lupu et al., 2001). Local administration of GH–IGF-1 however failed to have an additive effect in hypophysectomised animals (Isgaard et al., 1986). In agreement with this, we show that in Socs2−/− metatarsals, dual treatment with GH and IGF-1 does not promote longitudinal growth beyond individual stimulation. This suggests that GH is promoting linear growth through a common pathway with IGF-1. It is however plausible that the increase in IGF-2 levels observed in Socs2−/− metatarsals observed in this study are competing with the exogenous IGF-1. As IGF-1 exhibits a higher binding affinity to the IGF-1R than IGF-2 it is likely that the growth promoting effects of IGF-2 would be masked (Danielsen et al., 1990; Germainlee et al., 1992; Oh et al., 1993a).

In the present study GH stimulation also causes significant increase in IGFBP3 levels although, like IGF-1, the source of this IGFBP3 may be both chondrocytes and/or the surrounding perichondrium (Parker et al., 2007). The increase is more apparent in Socs2−/− metatarsals, where growth is promoted. IGFBP3 is produced in a number of tissues and is regulated mainly by GH, but also to some degree by IGF-1 (Kiepe et al., 2005). It is the principal carrier of IGFs in serum, where it functions to control tissue IGF concentrations and reduce bioavailability (Clemmons, 1998). Depending on incubation time and dose, it can inhibit or potentiate the actions of IGFs (Demellow and Baxter, 1988; Stewart et al., 1993; Bagi et al., 1994). IGFBP3 may have pro-apoptotic functions on chondrocytes, independent of IGFs, which is likely mediated through its own cell surface bound receptor (Oh et al., 1993b; Spagnoli et al., 2002). The complex relationship between IGF’s and IGFBP3 make it difficult to deduce what its role may be in this system. As there appears to be a correlation between increased IGFBP3 and increased growth in response to GH, it is unlikely that the effects of IGFBP3 are inhibitory in this model.

In conclusion, using the murine metatarsal model, this study underscores the critical role of SOCS2 in controlling GH anabolic effects on linear bone growth. This study also provides compelling evidence to support the notion that in the absence of SOCS2, GH can regulate linear growth via local mechanisms that are not accompanied by a rise in local or circulating IGF-1.

Acknowledgments

The authors would like to thank Elaine Seawright for her contributions to the experiments. The authors would also like to thank Darren Smith and Alex Robertson of the Small Animal Unit. This project was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) UK and Ipsen (UK) through a CASE studentship award (RD), and Institute
Strategic Programme Grant Funding from the BBSRC (CF, VEM), BBSRC Institute Career Path Fellowship funding from the BBSRC (VEM).

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