Liver-Derived IGF-I Contributes to GH-Dependent Increases in Lean Mass and Bone Mineral Density in Mice with Comparable Levels of Circulating GH

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The relative contributions of circulating and locally produced IGF-I in growth remain controversial. The majority of circulating IGF-I is produced by the liver, and numerous mouse models have been developed to study the endocrine actions of IGF-I. A common drawback to these models is that the elimination of circulating IGF-I disrupts a negative feedback pathway, resulting in unregulated GH secretion. We generated a mouse with near total abrogation of circulating IGF-I by disrupting the GH signaling mediator, Janus kinase (JAK)2, in hepatocytes. We then crossed these mice, termed JAK2L, to GH-deficient little mice (Lit). Compound mutant (Lit-JAK2L) and control (Lit-Con) mice were treated with equal amounts of GH such that the only difference between the two groups was hepatic GH signaling. Both groups gained weight in response to GH but there was a reduction in the final weight of GH-treated Lit-JAK2L *vs.* Lit-Con mice. Similarly, lean mass increased in both groups, but there was a reduction in the final lean mass of Lit-JAK2L *vs.* Lit-Con mice. There was an equivalent increase in skeletal length in response to GH in Lit-Con and Lit-JAK2L mice. There was an increase in bone mineral density (BMD) in both groups, but Lit-JAK2L had lower BMD than Lit-Con mice. In addition, GH-mediated increases in spleen and kidney mass were absent in Lit-JAK2L mice. Taken together, hepatic GH-dependent production of IGF-I had a significant and nonredundant role in GH-mediated acquisition of lean mass, BMD, spleen mass, and kidney mass; however, skeletal length was dependent upon or compensated for by locally produced IGF-I. **(*Molecular Endocrinology* 25: 1223–1230, 2011)**

The original somatomedin hypothesis suggested that the postpubertal growth-stimulating effects of GH were principally mediated by the production of the effector molecule, IGF-I, in the liver and the subsequent actions of IGF-I on peripheral tissues (1). However, this idea was challenged after the surprising finding of normal growth in mice with liver-specific deletion of IGF-I (2, 3). To attempt to determine the relative endocrine and paracrine/autocrine contributions of IGF-I, there have since been numerous studies of mice with reduced production or stability/activity of circulating IGF-I (4). Each model has particular advantages, but one major confounder that is common to all such models is that the elimination of active, circulating IGF-I disrupts a negative feedback pathway and results in unregulated GH secretion from the anterior pituitary gland. Consequently, each of the previous studies compared wild-type mice with normal circulating IGF-I and normal GH levels to mice with little to no active circulating IGF-I and high levels of circulating GH. This increase in GH secretion has made it difficult to assess the relative contributions of liver- and locally derived IGF-I because of the potential for increases in peripheral IGF-I production and compensatory actions of endocrine and paracrine/autocrine IGF-I. GH has also been shown to have IGF-I-independent effects, further complicating the interpretation of previous studies (5, 6).

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Abbreviations: ALS, Acid-labile subunit; BMD, Bone mineral density; Con, control; GHBP, GH binding proteins; GHR, GH receptor; JAK, Janus kinase; Lit, Little; TG, triglyceride.

Considering the reduction in circulating IGF-I and the increase in circulating GH in the aforementioned mouse models, there is an obvious paradox: despite little endocrine IGF-I activity, the mice are not small in size, suggesting that the contribution of liver-derived IGF-I to mouse growth is insignificant. On the contrary, despite large increases in circulating GH and thus, local GH activity, the mice are not extremely large in size, which would point to a significant role of circulating IGF-I. Taken together, both hepatic and local GH signaling appear to play important roles in mouse postpubertal growth.

To study the precise relative contributions of endocrine and paracrine/autocrine IGF-I, we engineered a mouse model aimed at addressing the potential confounder of unregulated GH secretion. Little (Lit) mice, harbor a mutation in the GHRHR and are unable to secrete GH (7–9). Previously, we generated mice with hepatocyte-specific disruption of Janus kinase (JAK)2 (JAK2L), a fundamental protein in the GH-signaling pathway that is necessary for GH-stimulated IGF-I production. JAK2L mice have a more than 90% reduction in circulating IGF-I (10). We crossed Lit and JAK2L mice to generate a compound mutant mouse, referred to as Lit-JAK2L, with a near-total reduction in liver-derived IGF-I and GH secretion. We gave back equal amounts of GH to Lit-JAK2L mice and the relevant controls (Lit-Con) such that the only difference between these two groups was the lack of hepatic, JAK2-dependent, GH signaling and thus, circulating IGF-I. Comparisons of untreated and GH-treated Lit-JAK2L mice and Lit-Con mice demonstrated that hepatic GH-dependent production of circulating IGF-I has a significant and nonredundant role in GH-mediated acquisition of lean mass, bone mineral density (BMD), kidney mass, and spleen mass. In contrast, GH-mediated increases in body length are dependent upon or compensated for by locally produced IGF-I.

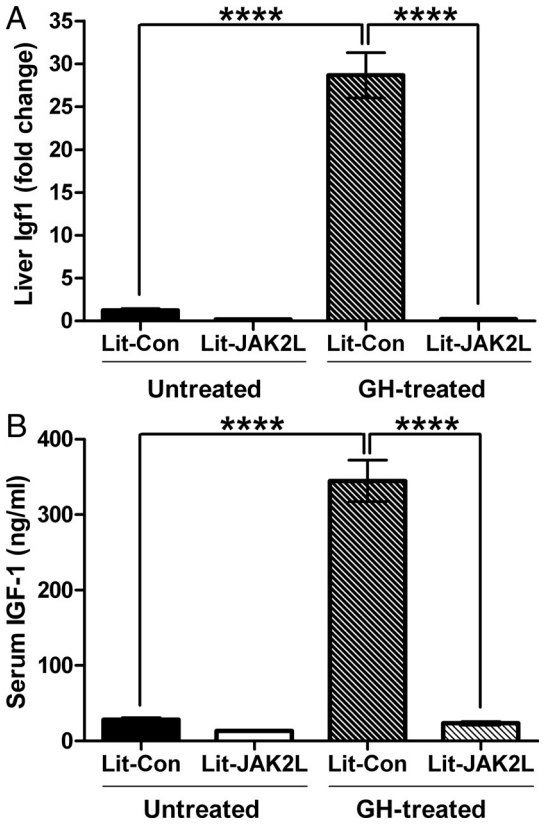

FIG. 1. Lit-JAK2L mice have a more than 90% reduction in GH-dependent IGF-I production. A, Gene expression was determined by real-time PCR and $2^{-\Delta\Delta CT}$ analysis. GH increased liver *Igf1* expression in Lit-Con mice but not in Lit-JAK2L mice (n = 7–8). B, Serum IGF-I was measured by ELISA at d 26. GH increased serum IGF-I in Lit-Con mice but not in Lit-JAK2L mice, resulting in a 93% reduction in circulating IGF-I in GH-treated Lit-JAK2L mice *vs.* GH-treated Lit-Con mice (n = 15–18). ****, $P < 0.0001$, one-way ANOVA with Bonferroni post test.

Results

Lit-JAK2L mice have a dramatic reduction in GH-induced circulating IGF-I

GH-deficient little mice and JAK2L mice were crossed to generate compound mutant mice. The progeny of this cross were viable, and the offspring were born in the expected Mendelian ratios. An equal number of males and females were assessed in each group, but no statistical sex differences were found in any measures, thereby justifying the combination of male and female animals.

We measured liver gene expression by real-time PCR (n = 7–8) and used the $2^{-\Delta\Delta CT}$ method to calculate the fold change in gene expression from the average gene expression of untreated Lit-Con mice (11). As expected, untreated Lit-JAK2L and Lit-Con mice had similarly low levels of liver *Igf1* expression ($P > 0.05$; 95% CI: −3.88 to 6.02; Fig. 1A and Table 1). *Igf1* gene expression was reduced in GH-treated Lit-JAK2L mice as compared with GH-treated Lit-Con mice ($P < 0.0001$; Fig. 1A and Table 1). The expression of IGF-binding protein acid-labile subunit (*Igfals*), which functions to stabilize circulating IGF-I along with the IGF-binding protein 3 (*Igfbp3*), was also increased in response to GH in Lit-Con mice, but not in Lit-JAK2L mice ($P < 0.0001$; Table 1). In contrast to *Igfals*, the expression of *Igfbp3* was comparable between all four groups. There were also differences in the hepatic expression of GH receptor (*Ghr*); *Ghr* was significantly increased in response to GH in Lit-Con mice, but not in Lit-JAK2L mice ($P < 0.05$; Table 1). As a control, we also measured the expression of *Igf1* in heart; in contrast to *Igf1* expression in the liver, expression of *Igf1* in GH-treated Lit-Con and Lit-JAK2L hearts was similar (1.91 ± 0.15 *vs.* 1.69 ± 0.18-fold change compared with un-

TABLE 1. Expression of GH-related genes in the liver

Gene	Untreated		GH-Treated	
	Lit-Con	Lit-JAK2L	Lit-Con	Lit-JAK2L
IGF-I *(Igf1)*	1.28 ± 0.18	0.21 ± 0.04	28.65 ± 2.65[a]	0.24 ± 0.05[b]
IGF-binding protein 3 *(Igfbp3)*	1.03 ± 0.11	1.14 ± 0.32	1.41 ± 0.13	0.80 ± 0.14
IGF-binding protein, acid-labile subunit *(Igfals)*	1.19 ± 0.20	0.19 ± 0.03	15.90 ± 2.78[a]	0.18 ± 0.03[b]
GHR *(Ghr)*	1.20 ± 0.10	1.03 ± 0.14	2.21 ± 0.43[c]	1.04 ± 0.14[d]

Mean ± SEM (n = 7–8). [a] $P < 0.0001$; [b] $P < 0.0001$; [c] Significance compared with untreated genotype-matched mice; [c] $P < 0.05$; [d] significance compared with treatment-matched Lit-Con mice, [d] $P < 0.01$.

treated Lit-Con, respectively; $P > 0.05$; 95% CI: −0.51 to 0.96).

Corresponding to the reduced liver gene expression of *Igf1* and *Igfals*, there was a 93% reduction in circulating IGF-I in GH-treated Lit-JAK2L mice as compared with GH-treated Lit-Con mice ($P < 0.001$; Fig. 1B). Untreated Lit-JAK2L and Lit-Con mice had similarly low levels of serum IGF-I, as expected ($P > 0.05$; 95% CI: −22.10 to 51.09; Fig. 1B). There were no significant differences in serum IGF-I between untreated and GH-treated Lit-JAK2L mice. Untreated Lit-JAK2L and Lit-Con mice also had comparably low levels of serum GH (4.6 ± 1.2 *vs.* 2.6 ± 0.6 ng/ml, respectively; $P > 0.05$; 95% CI: −10.01 to 5.92). Although equal amounts of GH were administered to all animals, serum GH levels were higher in Lit-JAK2L as compared with Lit-Con mice (32.6 ± 2.7 *vs.* 20.0 ± 2.6 ng/ml, respectively; $P < 0.001$).

GH-stimulated weight gain is partially dependent on liver-derived IGF-I

Because both Lit-Con and Lit-JAK2L mice have equally low levels of endogenous circulating GH, the weights of all mice were similar at baseline. To account for non-GH factors contributing to postpubertal growth, half of the mice from each group were left untreated. The remaining mice were implanted with minipumps designed to deliver a continuous, weight-based GH dose. We intentionally chose a starting dose that was higher than physiological levels because we anticipated some GH degradation in the pumps and to account for anticipated increases in mouse weight over the 26-d study period. We measured the weight of each mouse every 2–3 d for 26 d and normalized weights to individual baseline values. Both Lit-Con and Lit-JAK2L mice gained weight in response to GH ($P < 0.0001$, two-way ANOVA; Fig. 2A), but there was a difference in the amount of weight gained over time in GH-treated Lit-Con *vs.* Lit-JAK2L mice ($P < 0.0001$, two-way ANOVA; Fig. 2A). There were no differences in weight in the untreated mice over time. End weights were also normalized to individual baseline weights and compared separately; the end weight of GH-treated Lit-JAK2L mice was significantly less than that of Lit-Con mice ($P < 0.05$; Fig. 2B). We then established the average amount of postpubertal weight gain in GH-deficient mice (Lit-Con) treated with GH to be 100% and compared this group to untreated Lit-Con mice to determine the percentage of GH-independent and GH-dependent weight gain (38.5% and 61.5%, respectively). Differences in weight gain between untreated Lit-Con and Lit-JAK2L mice would have highlighted a role for additional JAK2-dependent hormones/growth factors in mouse postpubertal growth; however, GH-independent weight gain was similar in these two groups. Furthermore, by com-

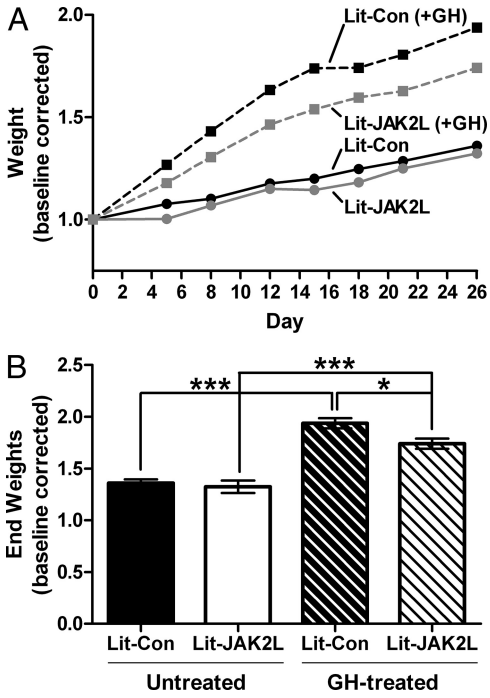

FIG. 2. Liver-derived IGF-I is necessary for a maximal GH-stimulated weight gain. A, All mice treated with GH (+GH) gained significantly more weight than untreated mice, but GH-treated Lit-Con mice gained more weight over time than GH-treated Lit-JAK2L mice (n = 15–18, $P < 0.0001$; two-way ANOVA). B, A separate comparison of final weights showed increased weights in both groups after GH treatment, but a significantly reduced final weight in GH-treated Lit-JAK2L mice *vs.* Lit-Con mice (n = 15–18; *, $P < 0.05$; ***, $P < 0.001$; one-way ANOVA with Bonferroni post test).

paring the postpubertal weight gain of GH-treated Lit-JAK2L mice to GH-treated Lit-Con mice, we assessed the relative dependence of GH-dependent weight gain on intact, hepatic GH signaling and production of IGF-I. Under these conditions, we determined that 38.5% of mouse postpubertal weight gain is independent of GH, 40.5% is attributable to local GH activity, and 21.0% of the final weight gain requires GH-induced circulating IGF-I.

Circulating IGF-I affects GH-dependent gains in lean mass

Analogous to the observed changes in body weight, lean mass increased in both GH-treated Lit-Con and Lit-JAK2L mice; however, the increase in lean mass was muted in Lit-JAK2L as compared with Lit-Con mice. Specifically, the final lean mass of GH-treated Lit-JAK2L mice was 12% lower than that of GH-treated Lit-Con mice ($P < 0.01$; Fig. 3A). The percentage of lean mass to total body weight was increased in GH-treated Lit-Con mice as compared with untreated mice (73.1 ± 0.8 *vs.* 67.0 ± 0.8%, respectively, $P < 0.001$) and in GH-treated Lit-JAK2L mice as compared with untreated mice (70.8 ± 0.8 *vs.* 66.6 ± 0.5%, respectively, $P < 0.001$). There were

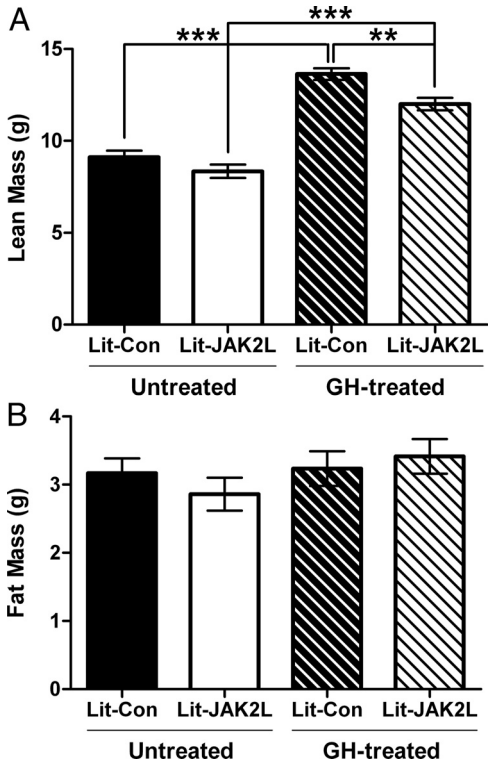

FIG. 3. Liver-derived IGF-I is necessary for a maximal GH-induced increases in lean mass. A, Lean mass and fat mass were determined by dual energy x-ray absorptiometry scanning at d 26. GH increased lean mass increased in both groups, but GH-treated Lit-Con mice had significantly 12% greater lean mass than Lit-JAK2L mice. B, Fat mass was similar between untreated Lit-Con and Lit-JAK2L mice, and GH treatment did not alter the amount of fat in either genotype (n = 15–18; **, $P <$ 0.01; ***, $P < 0.001$; one-way ANOVA with Bonferroni post test).

no significant differences in the percentage of lean mass to total body weight measured in GH-treated Lit-Con and Lit-JAK2L mice, indicating that the majority of weight gain in these animals was due to increases in lean mass.

Fat mass was similar between untreated Lit-Con and Lit-JAK2L mice, and GH treatment did not alter the amount of fat in either group; thus, total fat mass was comparable between GH-treated Lit-Con and Lit-JAK2L mice (Fig. 3B). The percentage of fat to total body weight was decreased in GH-treated Lit-Con mice as compared with untreated mice (16.9 ± 0.8 *vs.* 23.0 ± 0.9%, respectively; $P < 0.001$) reflecting the increase in lean mass with GH treatment. GH did not significantly reduce the percentage of fat to total body weight in Lit-JAK2L mice as compared with untreated mice (19.6 ± 0.8 *vs.* 22.2 ± 1.0, respectively), and this is likely due to the fact that this group gained less lean mass than Lit-Con mice over the course of the study.

Liver-derived IGF-I is important for GH-stimulated increases in bone density but not length

In addition to the metabolic influences of GH/IGF-I, we measured tip-to-tail lengths of mice at d 26 and normalized those lengths to individual baseline values. After GH-treatment, both Lit-Con and Lit-JAK2L mice had significant skeletal growth as compared with untreated mice ($P < 0.001$; Fig. 4A), but we found no differences between GH-treated Lit-Con and Lit-JAK2L tip-to-tail lengths. We then established the average increase in skeletal length in GH-deficient mice (Lit-Con) treated with GH to be 100% and compared this group with untreated Lit-Con mice to calculate the percentage of GH-independent and GH-dependent gains in skeletal length. Under these conditions, we determined that 45.3% of tip-to-tail growth is independent of GH, and the remaining 54.7% is dependent on local GH signaling. Because Lit-JAK2L mice responded to GH in a similar manner as Lit-Con mice, we concluded that circulating IGF-I does not play a major, nonredundant role in GH-mediated increases in skeletal length. We also measured femoral lengths at d 26 by dual energy x-ray absorptiometry; no baseline measurements were recorded. Femurs from GH-treated Lit-Con and Lit-JAK2L mice were significantly longer than untreated mice ($P < 0.001$; Fig. 4B), but we found no differences between GH-treated Lit-Con and Lit-JAK2L mice, indicating that liver-derived circulating IGF-I is not necessary for GH-mediated increases in femoral length.

Lit-Con and Lit-JAK2L mice had significant increases in total skeletal BMD in response to GH as compared with untreated mice ($P < 0.001$; Fig. 4C), but the response to GH was muted in Lit-JAK2L mice as compared with Lit-Con mice. Specifically, skeletal BMD was 4.1%

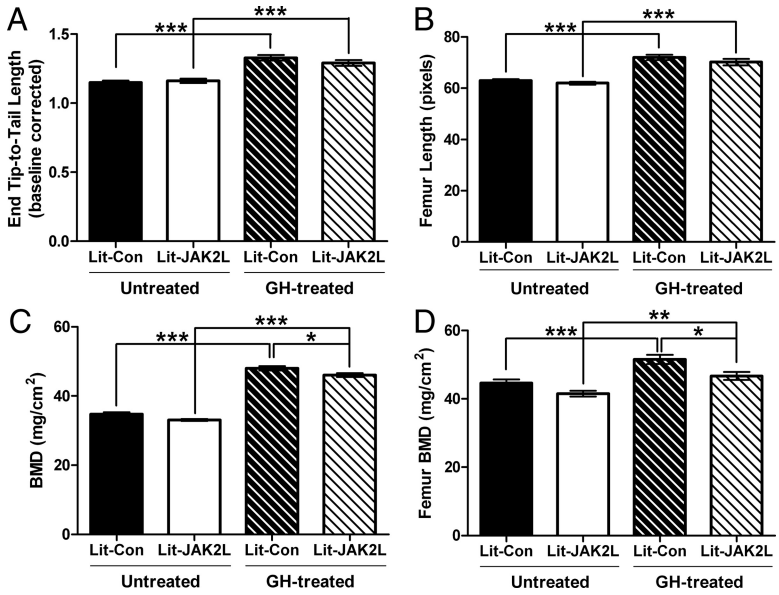

FIG. 4. Loss of liver-derived IGF-I affects GH-induced changes in BMD, but not skeletal length. All mice treated with GH (+GH) had increased tip-to-tail length (A) and femoral length (B), and no differences were found between GH-treated Lit-Con and Lit-JAK2L mice. GH increased skeletal (C) and femoral (D) bone density in both groups, but GH-treated Lit-Con mice had significantly greater density than Lit-JAK2L mice (n = 15–18; *, $P < 0.05$; ***, $P < 0.01$; one-way ANOVA with Bonferroni post test).

lower in GH-treated Lit-JAK2L mice as compared with GH-treated Lit-Con mice ($P < 0.05$; Fig. 3C). Likewise, there were significant increases in femoral BMD with GH treatment in Lit-Con and Lit-JAK2L mice as compared with untreated mice ($P < 0.01$; Fig. 4D), but again, the response to GH was muted in Lit-JAK2L mice. Specifically, femoral BMD was 9.4% lower in GH-treated Lit-JAK2L mice as compared with GH-treated Lit-Con mice ($P < 0.05$; Fig. 4D). The increased dependence of femoral *vs.* total skeletal BMD on liver-derived IGF-I is likely due to the disproportionate amount of cancellous *vs.* cortical bone in the observed areas. The femur is comprised of a greater percentage of cortical bone than the total skeleton, and circulating IGF-I has been shown to have a larger impact on cortical bone density (12).

Circulating IGF-I is essential for GH-dependent increases in spleen and kidney size

We also collected and weighed the major organs of each mouse at d 26 of the study and normalized these weights to total body weight. GH treatment did not influence heart weight; heart weights were comparable between all four groups at the time of euthanasia (Fig. 5A). Liver weights increased in response to GH treatment in both groups ($P < 0.001$; Fig. 5B), and there were no significant differences between GH-treated Lit-Con and Lit-JAK2L mice. This result indicates that GH-mediated increases in liver mass may be independent of both local and circulating IGF-I. This notion is supported by Liu and colleagues (13) who suggested that GH may increase liver mass in IGF-I null mice via direct actions on hepatocyte growth and/or differentiation. Alternatively, recent work from our laboratory has described profound fatty liver and a concomitant 2-fold increase in liver mass in JAK2L mice (10). We therefore measured the liver triglyceride (TG) content of Lit-Con and Lit-JAK2L mice. The amount of liver TG was comparable between untreated mice (Lit-Con, 15.0 ± 1.0 μg/mg; Lit-JAK2L, 18.0 ± 2.2 μg/mg). GH treatment did not significantly alter the amount of liver TG in Lit-Con mice (23.8 ± 3.2 μg/mg), but there was a significant increase in the liver TG content of GH-treated Lit-JAK2L mice (43.1 ± 7.9 μg/mg; $P < 0.01$ *vs.* untreated). We therefore interpret GH-induced increases in liver mass with caution because any changes in Lit-JAK2L liver weight in response to GH will be clouded by the additional lipid content in these livers.

Kidney weights were also increased in response to GH in Lit-Con mice ($P < 0.01$; Fig. 5C), but not in Lit-JAK2L mice. In fact, GH-treated Lit-JAK2L mice had significantly smaller kidney weights as compared with untreated Lit-JAK2L mice ($P < 0.05$). There was also an increase in spleen weight in response to GH in Lit-Con mice ($P < 0.001$; Fig. 5D) but not in Lit-JAK2L mice. The greater dependence of GH-induced kidney and spleen growth on circulating IGF-I compared with locally produced IGF-I has been demonstrated previously (14). Taken together, these data indicate that heart mass is not influenced by GH, liver mass may be influenced by GH via an IGF-I-independent mechanism, and kidney and spleen are dependent on circulating IGF-I for GH-induced growth.

Discussion

We aimed to determine the relative contributions of circulating *vs.* locally derived IGF-I on postpubertal growth in mice using a unique genetic model with disruption of both liver-specific GH signaling and GH secretion from the anterior pituitary gland. The main advantage of this study over previous mouse models with altered hepatic GH signaling/IGF-I production is that we were able to adjust the amount of circulating GH in mice with liver-specific IGF-I-deficiency and control mice in an attempt to make this parameter more comparable between the

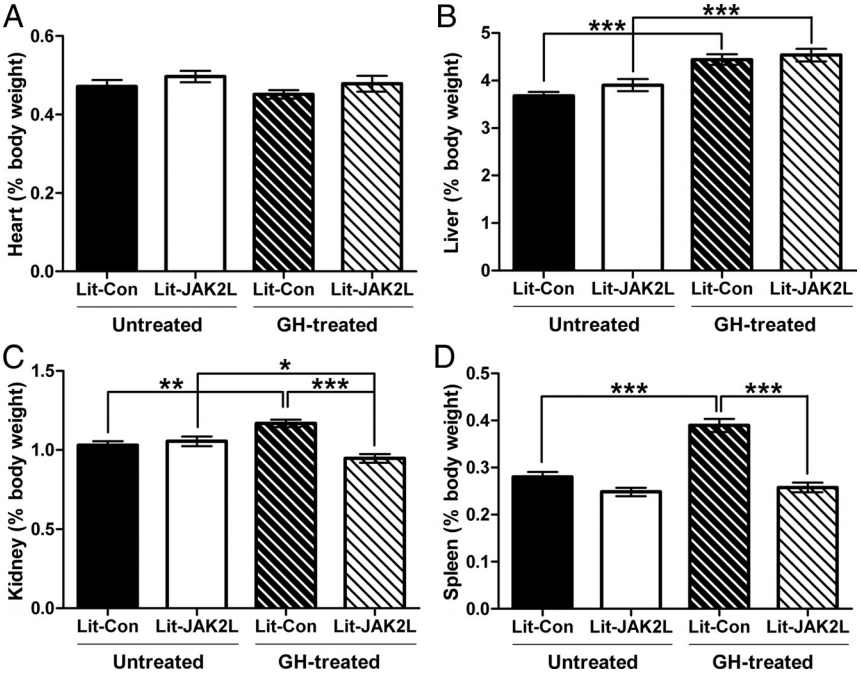

FIG. 5. Circulating IGF-I is essential for GH-induced increases in spleen and kidney weight. Organs were weighed at d 26 and normalized body weight. A, Heart weights were comparable between all four groups. B, Liver weight increased in response to GH-treatment in both groups, but there were no differences between GH-treated Lit-Con and Lit-JAK2L mice. C, GH increased kidney weight in Lit-Con mice but not in Lit-JAK2L. D, There was also an increase in spleen weight in response to GH in Lit-Con mice but not in Lit-JAK2L mice (n = 15–18; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA with Bonferroni post test).

two groups. This has not been possible in other studies because the reduction of circulating IGF-I disrupts a negative feedback pathway, resulting in the uncontrolled release of GH into the circulation. Marked differences in the levels of circulating GH confound the data interpretation of circulating *vs.* local IGF-I action due to potential increased local IGF-I production and/or IGF-I independent actions of GH. Our comparisons of GH-treated Lit-JAK2L mice and Lit-Con mice demonstrated that hepatic JAK2-dependent production of circulating IGF-I has a significant and nonredundant role in GH-mediated acquisition of lean mass, BMD, kidney mass, and spleen mass; however, circulating IGF-I is not necessary for GH-induced increases in body length.

Although we administered equivalent amounts of GH to Lit-Con and Lit-JAK2L mice, we did observe a 1.6-fold increase in serum GH in Lit-JAK2L as compared with Lit-Con mice. We can only speculate that this difference results from changes in GH binding proteins (GHBPs) or altered renal function. GHBPs have been shown to increase the half-life of circulating GH *in vivo* (15). GHBP is produced through alternative splicing of *Ghr* mRNA and proteolytic cleavage of the GHR extracellular domain (16). Although the production of GHBP and expression of *Ghr* are not definitively correlated in rodents, we did find that liver *Ghr* expression was 2.1-fold greater in GH-treated Lit-Con mice as compared with GH-treated Lit-JAK2L mice, and this could lead to differences in circulating GHBP between Lit-Con and Lit-JAK2L mice. Because the GH immunoassay we used includes a polyclonal antibody, it likely does not discriminate between free GH and GHBP-GH complexes. The role of GHBP on GH signaling is not well understood, but *in vitro* studies indicate that GHBP can inhibit the effects of GH on IGF-I production (17). Although the interpretation of how changes in GHR and/or GHBP affect levels of GH is complex and beyond the scope of this manuscript, it is important to allow that changes in GHR or GHBP levels could affect levels of active GH and GH signaling. The increased level of circulating GH in Lit-JAK2L mice might also reflect differences in renal function. Kidney size is reduced in GH-treated Lit-JAK2L mice compared with Lit-Con mice, which could relate to reduced renal clearance of GH. Overall, there was a 1.6-fold difference in GH levels between Lit-Con and Lit-JAK2L mice, and this may lead to small, but significant, differences in GH action. The fact that there was no difference in *Igf1* expression in a nonliver tissue (heart) argues that the difference in GH action is likely very small. Regardless, a 1.6-fold difference in circulating GH is substantially less than the at least 10-fold difference that occurs with uncontrolled release of GH in JAK2L mice (10).

Although the model presented in this study has distinct advantages, we recognize that JAK2 is involved in numerous signaling pathways in hepatocytes and that we are potentially disrupting cytokine signaling pathways that are distinct from GH, including IL-6 and leptin. However, untreated Lit-Con and Lit-JAK2L mice, despite very low levels of GH, have appreciable gains in weight and length over time, and we believe that this largely results from the actions of non-GH growth factors. Importantly, untreated Lit-Con and Lit-JAK2L mice are comparable in all measures, suggesting that in the absence of GH, deletion of hepatocyte JAK2 has minimal effects on postpubertal growth. Although we or others have not formally compared JAK2L mice to mice with hepatocyte-specific deletion of GHR (so-called GHRLD), the published phenotypes of both animals suggest that they are very similar (10, 18). Despite their many similarities, there are some

differences between these two models, notably in the expression of *Igfbp3*.

Whereas expression of *Igfbp3* is normal in Lit-JAK2L mice, *Igfals* expression is significantly reduced, and we have not controlled for non-IGF-I actions of ALS. Again, untreated Lit-Con and Lit-JAK2L mice are identical in measures of growth and furthermore, ALS-deficient mice and patients have only a mild growth deficiency, indicating that the loss of this factor likely has negligible effects on the growth phenotype outside of its IGF-I binding functions (19, 20). In addition, both male and female mice were given continuous administration of GH for 26 d. Males normally secrete GH in a pulsatile fashion, and we and others have shown that pulsatile *vs.* continuous GH secretion results in differences in gene expression (21, 22); therefore we cannot rule out the possibility that pulsatile administration of GH would have resulted in different conclusions. Despite these caveats, this model presents a major advancement in defining the role of liver-derived IGF-I in growth and metabolism.

A greater understanding of GH signaling and IGF-I action on growth is important for the optimization of treatments for different forms of IGF-I deficiency. Treatment regimens with varying doses and durations of *r*GH or *rh*IGF-I are currently in use, but an optimal protocol has yet to be established because the risk/reward profile often depends on the underlying cause of IGF-I deficiency and the goals of the treatment such as gain is longitudinal bone growth, BMD, and/or lean mass. Ideally, these studies will help to further elucidate the contributions of circulating *vs.* locally produced IGF-I in growth and aide in the development of improved treatments for growth disorders.

Materials and Methods

Mice

Mouse care and use for these studies was approved by the UCSF Institutional Animal Care and Use Committee. Mice were maintained on a 12-h light, 12-h dark cycle and were fed PicoLab Diet Mouse Diet 20 (*5058) *ad libitum*. Mice with *loxP* sites flanking the first exon of *Jak2* were generated and described previously (23). Hepatocyte-specific JAK2-deficient mice (JAK2L) were generated by mating floxed JAK2 mice ($JAK2^{F/F}$) in a mixed (C57Bl/6:129Sv) background to mice carrying an *Alb* promoter-regulated Cre transgene ($cre^{+/-}$) on a 100% C57Bl/6 background purchased from The Jackson Laboratory (Bar Harbor, ME) (24). Male and female wild-type C57Bl/6 mice and little mice carrying the $Ghrhr^{lit}$ mutation (referred to as lit^{m}) were also purchased from The Jackson Laboratory. The little mice are on a 100% C57Bl/6 background and were maintained as female $lit^{m/m}$ mated to male $lit^{m/+}$ intercrosses. JAK2L and *little* mice were intercrossed by mating female $lit^{m/m}$ with male JAK2L mice and subsequently intercrossing mice heterozygous for the little mutation ($lit^{m/+}$) with mice carrying both a single copy of the albumin Cre and a floxed JAK2 allele ($JAK2^{F/F}cre^{+/-}$). The cross was designed to generate and compare animals of two genotypes including: $lit^{m/m}/JAK2^{F/F}cre^{-/-}$ (Lit-Con) and $lit^{m/m}/JAK2^{F/F}cre^{+/-}$ (Lit-JAK2L). An equal number of males and females were assessed in each group, but no statistical sex differences were found in any measures, justifying the combining of male and female data for our final report. For each group, n =15–18 unless otherwise indicated.

Implantation

Mouse recombinant GH was purchased from A.F. Parlow at the National Hormone and Pituitary Program (UCLA Medical Center, Torrance, CA). GH was resuspended in 0.03 M $NaCO_3$, 0.15 M NaCl, pH 9.5. Dilutions were calculated for individual animals to deliver 3 μg of GH per gram of mouse weight for 28 d. Alzet Model 1004 28-d mini-pumps (DURECT Corp., Cupertino, CA) were filled with GH, according to the manufacturer's instructions. Pumps were implanted sc on the back of isofluorane-anesthetized animals through a small incision, which was subsequently closed with wound clips.

Gene expression

Real-time quantitative PCR was performed using Taqman primer/probe sets (5′FAM/3′BHQ; Biosearch Technologies, Novato, CA) designed using Primer Express software (Applied Biosystems, Foster City, CA; see Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org for a complete list and primer/probe sequences). Total RNA was isolated from mouse livers and hearts with TRIZOL (Invitrogen, Carlsbad CA) extraction, followed by purification with RNeasy Mini Column (QIAGEN, Valencia, CA). First-strand cDNA synthesis was performed using the Superscript First-Strand Synthesis System (Invitrogen) and oligo-dT primers. Quantitative real-time PCR was performed in a 384-well format using Platinum qPCR mix (Invitrogen) and total reaction volumes of 10 μl on an ABI 7900HT (Applied Biosystems). Relative gene expression compared with untreated Lit-Con mice was quantified using the $2^{-\Delta\Delta CT}$ method and the control gene, *Gapdh*, which was expressed at similar levels in all four groups based on raw CT values (11).

Serum measurements

Blood was drawn via retroorbital puncture with a 1.5-cm segment of uncoated glass microcapillary tube into empty tubes, incubated overnight at 4 C, and serum was separated by double spinning at 13,000 × G for 15 min. Mouse IGF-I (R&D Systems, Minneapolis, MN) and mouse GH (Millipore Corp., Billerica, MA) were measured using standard ELISA assays according to the manufacturer's instructions.

Body composition

Lean mass, fat mass, and BMD were determined by dual-energy x-ray absorptiometry. Live animals were anesthetized with isoflurane and scanned on the Lunar PIXImus densitometer (GE Medical Systems, Madison, WI). Lean and fat mass were calculated by the PIXImus software and percent lean and fat were determined by manually dividing mass by actual weight. To determine femur parameters, a region of interest was drawn around one femur manually and blind to genotype. The length of the region of interest was recorded as femoral length, and the density within this region was recorded as femoral BMD.

Liver TG content

A portion of liver from each mouse was homogenized in Buffer A (250 mM sucrose, 50 mM Tris, pH 7.4) at a concentration of 50 mg tissue per 1 ml of buffer. Lysate was added to Infinity Triglyceride Reagent (Thermo Scientific, Waltham, MA) to determine TG content, according to manufacturer's instructions.

Statistics

A Student's *t* test was used to determine significance in cases where two groups are compared. For comparison of three or more groups, a one-way ANOVA was employed followed by Bonferroni's Multiple Comparison Test for four selected comparisons: 1) untreated Lit-Con *vs.* untreated Lit-JAK2L; 2) untreated Lit-Con *vs.* GH-treated Lit-Con; 3) untreated Lit-JAK2L *vs.* GH-treated Lit-JAK2L; and 4) GH-treated Lit-Con *vs.* GH-treated Lit-Con. Analysis of three or more groups over time was carried out using a two-way ANOVA followed by Bonferroni's Multiple Comparison Test. An α value of 0.05 was set for all statistical tests. Data are presented as mean ± SEM, and the 95% Confidence Intervals (95% CI) for the difference of the means is reported for select, nonsignificant data. All statistical analyses were performed in GraphPad Prism Statistical Software version 5 (GraphPad Software, La Jolla, CA).

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