

Insulin-like growth factor-binding protein 5 (*Igfbp5*) compromises survival, growth, muscle development, and fertility in mice

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The insulin-like growth factors (IGFs) are essential for development; bioavailable IGF is tightly regulated by six related IGF-binding proteins (IGFBPs). *Igfbp5* is the most conserved and is developmentally up-regulated in key lineages and pathologies; *in vitro* studies suggest that IGFBP-5 functions independently of IGF interaction. Genetic ablation of individual *Igfbps* has yielded limited phenotypes because of substantial compensation by remaining family members. Therefore, to reveal *Igfbp5* actions *in vivo*, we generated lines of transgenic mice that ubiquitously overexpressed *Igfbp5* from early development. Significantly increased neonatal mortality, reduced female fertility, whole-body growth inhibition, and retarded muscle development were observed in *Igfbp5*-overexpressing mice. The magnitude of the response in individual transgenic lines was positively correlated with *Igfbp5* expression. Circulating IGFBP-5 concentrations increased a maximum of only 4-fold, total and free IGF-I concentrations increased up to 2-fold, and IGFBP-5 was detected in high M_r complexes; however, no detectable decrease in the proportion of free IGF-I was observed. Thus, despite only modest changes in IGF and IGFBP concentrations, the *Igfbp5*-overexpressing mice displayed a phenotype more extreme than that observed for other *Igfbp* genetic models. Although growth retardation was obvious prenatally, maximal inhibition occurred postnatally before the onset of growth hormone-dependent growth, regardless of *Igfbp5* expression level, revealing a period of sensitivity to IGFBP-5 during this important stage of tissue programming.

The insulin-like growth factors (IGF-I and -II) are essential for growth and development (1). Six high-affinity IGF-binding proteins (IGFBP-1 to IGFBP-6; refs. 2 and 3) strictly orchestrate IGF action. Despite their considerable sequence homology, each exhibits a discrete expression pattern and possesses an individual subset of motifs, signifying that although IGFBPs have common actions, they may also have unique properties.

IGFBP-5 is the most conserved of the IGFBPs (4) and has been highlighted as a focal regulatory factor during the development of several key cell lineages, e.g., myoblasts (5) and neural cells (6, 7). In mice, *Igfbp5* is expressed in the embryo from early development, principally in the myotomal component of the somites and developing central nervous system (8). Postnatally, serum IGFBP-5, in common with IGFBP-3, forms a ternary complex with IGF-I or IGF-II and the acid-labile subunit (9). *Igfbp5* is up-regulated in the aggressive pediatric cancer, rhabdomyosarcoma (10), in the progression of prostate cancers to androgen independence (11), and in smooth muscle-derived uterine leiomyoma (12), indicating a function in neoplasia.

IGFBP-5 initially binds IGFs with high affinity, principally by an N-terminal motif (13), and inhibits IGF activity by preventing IGF interaction with the type 1 receptor. It is further subject to regulated posttranslational modifications (3) to induce conformational changes that decrease its affinity for IGFs and allow their effective delivery to the type 1 receptor. However, IGFBPs are also multifunctional proteins that can regulate cell function independent of IGF signaling (14–16); key motifs that may mediate these functions

include a consensus nuclear localization signal (17) and serine/threonine phosphorylation sites. These primary motifs are conserved, signifying that putative IGF-dependent and -independent functions have been maintained.

We have therefore pursued the hypothesis that IGFBP-5 has a significant role in growth and development. IGFBP function has been investigated *in vivo* by using gene ablation by homologous recombination (e.g., ref. 18); however, to date *Igfbp*-null mice have exhibited a limited phenotype because of compensation and redundancy from remaining IGFBPs. We therefore decided to take an alternative approach and generate transgenic mice overexpressing *Igfbp5*. These mice are significantly growth-retarded and exhibit the most severe phenotype of any *Igfbp* genetic manipulation *in vivo* to date, providing convincing evidence that IGFBP-5, by either IGF-dependent or -independent mechanisms, is important in determining cell fate.

Materials and Methods

Transgene Construction. The murine *Igfbp5*-coding region [in pEMSVscribe $\alpha 2$ (5), P. Rotwein, Oregon Health Sciences University, Portland] was amplified by PCR (primer sequences are given in *Supporting Text*, which is published as supporting information on the PNAS web site) with use of *PfuTurbo* (Stratagene) to include the native translation initiation sequence of *Igfbp5* and to introduce a C-terminal *myc* epitope. The *EcoRI*-restricted, 893-bp *Igfbp5*-*myc* amplicon was inserted downstream of the cytomegalovirus enhancer, chicken β -actin promoter, and rabbit β -globin splice acceptor, and upstream of the simian virus 40 polyadenylation signal of a pCAGGS expression vector (J. Miyazaki, Osaka University Medical School, Osaka; ref. 19 and Fig. 1A). The *CMV- β A/Igfbp5*-*myc* transgene was isolated from the plasmid by *PstI/SalI* double digestion, QIAquick gel purification (Qiagen, Valencia, CA) and serial ethanol precipitations.

Generation of *Igfbp5*-Overexpressing Mice. Transgenic mice were generated by pronuclear injection of the linear transgene into fertilized zygotes from C57BL/6J \times CBA/CA superovulated dams mated to males of the same genotype. Founders were identified by Southern blot analysis and PCR.

Growth and Development of *Igfbp5* Mice. Mice were housed in a controlled-barrier unit (12 h light/12 h dark at $21 \pm 2^\circ\text{C}$) and fed standard laboratory diet ad libitum. WT ($-/-$), hemizygous ($-/+$), and homozygous ($+/+$) animals were weighed weekly from birth to 8 wk and killed by decapitation (postneonatal mice were anesthetized first). Selected tissues were dissected; muscle was represented by the gastrocnemius, plantaris, and soleus muscle bundle.

Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; Tg, transgenic; en, embryonic day *n*.

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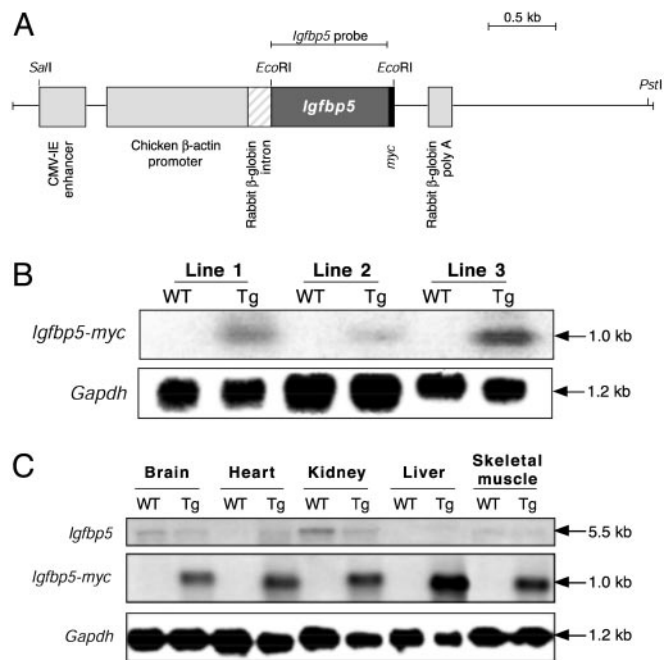


Fig. 1. *Igfbp5-myc* expression is widespread but at different levels in the three lines. (A) Schematic of the *Igfbp5-myc* construct. CMV-IE, immediate early cytomegalovirus. (B) Hybridization of *Igfbp5* cDNA probe to mRNA isolated from hemizygous skeletal muscle at 8 wk (mice from WT females × Tg males). (C) Endogenous and Tg mRNA levels from key tissues of line 3 mice at 8 wk.

Southern and Northern Blot Analyses. Tail genomic DNA was digested with *EcoRI* for Southern blot analysis. Slot-blot analysis of genomic DNA, in parallel with WT genomic DNA that had been spiked using a serial dilution of transgene, confirmed copy number, and distinguished homo- and hemizygotes. Blots were hybridized with a random-primed, labeled (High Prime protocol, Roche Diagnostics), full-length mouse *Igfbp5* cDNA template (S. L. Drop, Erasmus University, Rotterdam, The Netherlands) and *H19* probe as a control.

Total RNA was purified from key tissues by using the RNeasy Midi protocol (Qiagen) and analyzed by Northern blotting with random-primed, labeled cDNA probes; *Gapdh* was used as a loading control. Southern and Northern blot signals were quantified by phosphorimaging.

Circulating IGFBP, Total, and Free IGF-I Concentrations. Serum IGFBP levels were analyzed by ligand blotting (20) with biotinylated human IGF-I (GroPep, Adelaide, Australia). Immunoblotting was performed with anti-human IGFBP-5 polyclonal rabbit Ab (1:250; Upstate Biotechnology, Lake Placid, NY) or anti-myc polyclonal rabbit Ab (1:125; Upstate Biotechnology). Serum total IGF-I concentrations were measured by using a rat IGF-I enzyme immunoassay from Diagnostics Systems Laboratories (DSL; Webster, TX). To measure total serum IGF-I levels, IGF-I was dissociated from IGFBPs before the enzyme immunoassay by using three different methods (21): (i) acid/ethanol extraction, according to the DSL protocol, (ii) acidification and ultrafiltration through a Microcon YM-30 device (Millipore), and (iii) acidification and gel filtration by using a Superdex 75 Precision Column (3.2 × 300 mm; Amersham Pharmacia). Free IGF-I levels were measured directly in ultrafiltrates by using the DSL enzyme immunoassay after centrifugation of 50 μl of serum with Microcon YM-30 devices.

IGFBP-5-myc in serum binary or ternary complexes was examined by crosslinking proteins in 3 μl of serum for 30 min at room temperature with 0.25 mM disuccinimidyl suberate and 10%

DMSO, followed by nonreducing 7.5% SDS/PAGE (22) and immunoblotting with anti-myc Ab.

Real-Time PCR. Total RNA (2 μg) was reverse-transcribed with SuperScript II RT (Invitrogen), and resultant cDNA was subjected to real-time PCR (Applied Biosystems). The mRNA levels of *Igf1*, *Igfbp3*, *Igfbp5*, and *β-actin* were assessed (primer sequences given in Supporting Text). The fluorescence of SYBR Green bound to double-stranded DNA was measured after each PCR cycle as the reaction proceeded to completion. A *β-actin* dilution series during the linear phase was used to normalize RNA levels between samples.

Histology. Embryos were embedded in optimum cutting temperature compound and frozen in dry-ice-cooled isopentane. Sagittal sections (10 μm) were stained with Gills III hematoxylin/eosin (BDH).

Statistics. Mice were grouped according to line, sex, and genotype. Differences between genotypes and lines at selected times were determined with Student's *t* test (confirmed by using the Mann-Whitney test) or by two-way ANOVA. Values are expressed as the mean ± SEM in all figures.

Results

Derivation of *Igfbp5*-Overexpressing Lines: Low Copy Number. Zygotes were microinjected with *Igfbp5-myc* transgene to generate three *Igfbp5*-overexpressing lines. Southern and slot blot analyses (Fig. 7, which is published as supporting information on the PNAS web site) revealed differential sites of integration and a single copy of *Igfbp5-myc* per haploid genome for all founders. Other transgenes driven by this promoter have produced lines containing up to 20 copies of transgene (23), and mouse models overexpressing other *Igfbps* have invariably displayed multiple copy numbers (e.g., ref. 24).

Ubiquitous Expression of *Igfbp5-myc*. Northern blot analyses revealed relative transgene expression levels between lines (transcript at 1.0 kb) and demonstrated that line 3 mice exhibited the highest *Igfbp5-myc* mRNA abundance, with line 1 and 2 mice having intermediate and low expression levels, respectively (Fig. 1B). These between-line differences were consistently observed for all tissues and ages examined between birth and 8 wk in hemizygous animals and were corroborated by real-time RT-PCR (data not shown). Endogenous *Igfbp5* mRNA (5.5 kb) was confirmed in all tissues examined, except liver (Fig. 1C); the greatest levels were in kidney, brain, and skeletal muscle. Real-time PCR analysis determined that *Igfbp5* mRNA levels were increased by 10- to 20-fold in skeletal muscle of line 3 adults (data not shown). Transgene *Igfbp5-myc* tended to down-regulate endogenous *Igfbp5* expression.

Reduced Litter Size and Increased Morbidity Correlated with High *Igfbp5* Expression. Litter size at birth was decreased in direct correlation with maternal *Igfbp5* expression levels when transgenic (Tg) hemizygous females were used for matings with either hemizygous (Fig. 2) or WT males (although only a few successful matings occurred for line 3 mice because of the size difference in males and females for this cross; data not shown). Reduced litter size was apparent from as early as embryonic day 10.5 (e10.5). Hemizygous males generated WT litter sizes when crossed with WT females.

Litters of *Igfbp5-myc*-overexpressing mice derived from hemizygous parents also had higher rates of mortality than from WT parents (Fig. 8, which is published as supporting information on the PNAS web site); line 3 mice exhibited an 8-fold increase in morbidity ($P < 0.01$), most occurring by 24 h postnatally. Mendelian inheritance was observed in all lines at birth, but no line 3 homozygotes survived (proportions in 8-wk-old line 3 mice: -/-, 10; -/+, 25; +/+, 0; total = 35; $P < 0.001$). Northern blot analysis

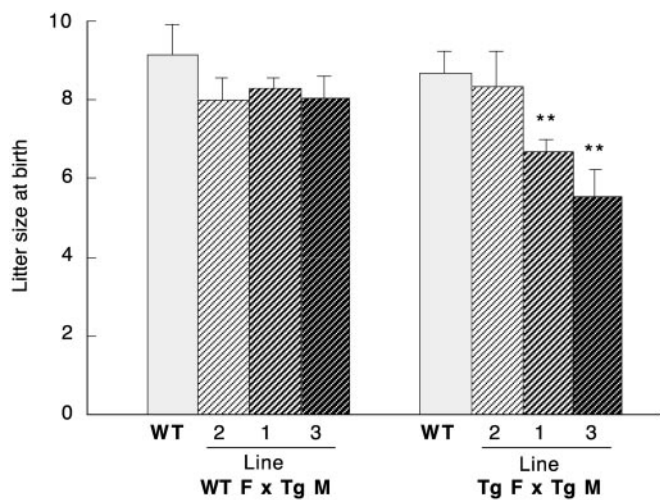


Fig. 2. Reduced litter size in *Igfbp5-myc* hemizygous females. Gray columns, mice generated from WT siblings; left shaded columns, mice generated from WT mothers and hemizygous fathers; right shaded columns, mice derived from hemizygous mothers and fathers. **, $P < 0.01$ Tg versus WT litters; mean \pm SEM of four to ten litters per line.

revealed higher *Igfbp5-myc* mRNA levels in homozygous than hemizygous mice for all lines (data not shown). We conclude that reduced litter size was due to maternal compromise but that high expression of *Igfbp5* was lethal in the early postnatal period.

Growth Retardation in *Igfbp5*-Overexpressing Mice. Birth weights were reduced for hemizygotes expressing high levels of *Igfbp5* (WT, 1.445 ± 0.024 g; line 1, $99.4 \pm 2.7\%$; line 2, $91.7 \pm 5.0\%$, $P = 0.18$; line 3, $81.9 \pm 4.1\%$, $P < 0.01$; % WT weight; generated from WT females \times hemizygous males). Birth weight was reduced even further when hemizygous parents were crossed (Fig. 3A). Line 3 homozygous pups were only 64%, and nontransgenic littermates were 92% WT weight, suggesting that IGFBP-5 influenced prenatal growth by modest indirect effects by way of the mother but by major direct effects on the fetus.

Postnatally, all hemizygous pups were significantly growth-retarded by 3 wk to $83.1 \pm 5.3\%$ ($P = 0.011$, line 2), $70.3 \pm 4.8\%$ ($P < 0.001$, line 1), and $67.0 \pm 2.5\%$ ($P < 0.001$, line 3) of WT weight (Fig. 3B); all WT siblings exhibited control weights. By 8 wk of age, line 1 and 3 females remained growth-retarded (line 1, $77.7 \pm 2.2\%$, $P < 0.001$; line 3, $65.1 \pm 1.5\%$, $P < 0.001$), whereas line 2 females displayed some catch-up growth (line 2, $97.7 \pm 1.8\%$, NS). For males, growth retardation induced by *Igfbp5* overexpression was similar to that of females (8-wk weight as % WT: line 2, 96.3 ± 1.7 , NS; line 1, 78.0 ± 1.4 , $P < 0.001$; line 3, 63.4 ± 1.11 , $P < 0.001$).

The dependence of growth on *Igfbp5* gene dose was further confirmed by crossing hemizygous parents (Fig. 9, which is published as supporting information on the PNAS web site). At 8 wk this was similar for line 1 homozygous and line 3 homozygous mice (60–65%), indicating a threshold before *Igfbp5* levels become lethal. The mild prenatal growth retardation in WT siblings derived from line 3 hemizygous mothers was overcome by 8 wk, suggesting that any maternal compromise was reversible.

Tissue-Specific Effects of *Igfbp5* Overexpression. Absolute tissue weights, except brain tissue, were significantly reduced in transgenic mice that exhibited decreased whole-body weights at 8 wk. Fractional tissue weights (tissue weight/whole bodyweight) were calculated for mice derived from hemizygote parents (Fig. 4) and from WT mothers crossed with hemizygous fathers (Fig. 10, which is published as supporting information on the PNAS web site). These

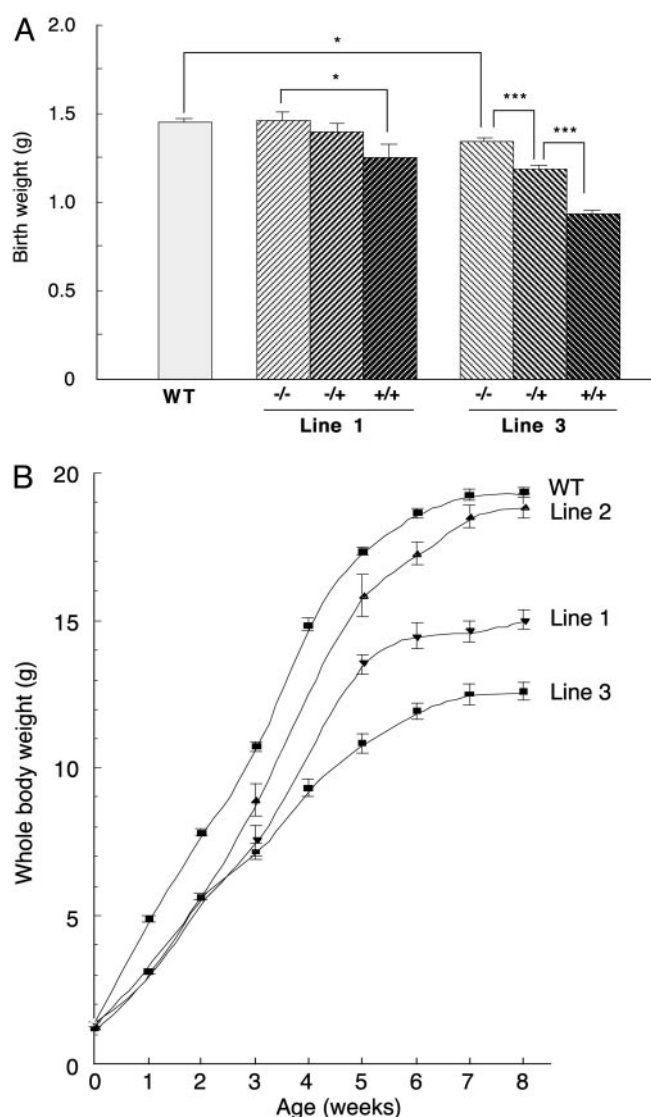


Fig. 3. Growth inhibition in *Igfbp5-myc*-overexpressing mice. (A) Birth weights of nongenetically manipulated WT, WT siblings ($-/-$), hemizygous ($-/+$), and homozygous ($+/+$) *Igfbp5-myc*-expressing mice; mean \pm SEM of three to eight mice per group. (B) Whole-body weight gain of female WT and hemizygous siblings derived from a WT mother and *Igfbp5* hemizygous father. *, $P < 0.05$; ***, $P < 0.001$; mean \pm SEM of 4–16 mice per group.

weights increased significantly for brain and liver, and decreased for muscle and spleen; these individual tissue responses correlated with transgene expression across lines. Changes in relative tissue weight appeared to be initiated by birth, although these changes did not always reach statistical significance (data not shown). The most significant deviation in relative tissue mass was observed for skeletal muscle, which decreased by 31%. Relative tissue weights for male and female mice responded similarly.

Histological studies corroborated the changes in embryo and tissue weights, with transgenic mice consistently appearing to have a large head relative to the body (Fig. 5A), an observation supported by the increase in fractional brain weight. Diaphragm muscles of the *Igfbp5* animals were thinner (width: WT, 143.6 ± 8.2 ; line 3 $+/+$, 101.8 ± 6.4 μm ; $n = 3$, $P < 0.05$; Fig. 5B). To assess the mechanism responsible, three adjacent intercostal muscles from each of three WT and line 3 mice were analyzed by two-way ANOVA, because these muscles are discrete bundles. Total bundle area decreased (WT, 0.160; line 3 $+/+$, 0.099; pooled SEM \pm 0.011 mm^2 ; $P < 0.01$),

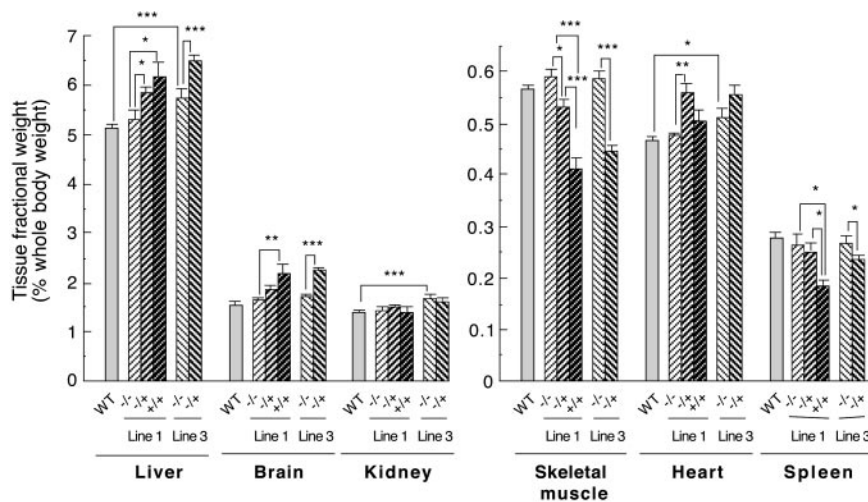


Fig. 4. *Igfbp5-myc* overexpression exerts differential effects on fractional tissue weight. Fractional tissue weights for female nonmanipulated WT, WT sibling ($-/-$), hemizygous ($-/+$), and homozygous ($+/+$) mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; mean \pm SEM of five to ten mice per group.

whereas the proportion occupied by muscle fibers increased in *Igfbp5*-overexpressing mice (WT, 67.6; line 3 $+/+$, 78.0; pooled SEM \pm 3.1%; $P < 0.05$), and the interfiber tissue area correspondingly decreased. Individual fiber area decreased by $>25\%$, although only at the 6% significance level (WT, 160; line 3 $+/+$, 116; pooled SEM \pm 15 μm^2 ; $P = 0.06$), whereas total fiber number per bundle remained constant (WT, 697; line 3 $+/+$, 666; pooled SEM \pm 31; NS). Taken together, these data suggest that *Igfbp5* overexpression decreased muscle fiber hypertrophy and interfiber tissue formation but did not affect fiber number, and, therefore, each muscle contained a similar number of smaller, more densely packed fibers.

Further, the lungs of homozygotes were more cellular with decreased alveolar separation than in WT animals (Fig. 5C).

Decreased Serum IGFBP-3 Levels and Increased Total and Free IGF-1 Concentrations in *Igfbp5*-Overexpressing Mice. At 8 wk of age IGFBP-3 (40–45 kDa) was the most abundant serum IGFBP in WT mice, with lower levels of IGFBP-1 and endogenous IGFBP-5 (both 28–30 kDa; Fig. 6A). In *Igfbp5-myc* mice, an extra band was observed at 31 kDa, representing myc-tagged IGFBP-5 (confirmed by immunoblotting with anti-myc and -IGFBP-5 Abs). The intensity of the 31-kDa band was proportional to tissue *Igfbp5-myc* mRNA levels [RIA studies determined that circulating IGFBP-5 concentrations were increased by up to 4-fold in 8-wk-old line 3 hemizygous mice [WT, 225 ± 25 ($n = 15$); line 3, 863 ± 55 ($n = 13$) ng/ml; $P < 0.001$; S. Mohan, personal communication]}. The intensity of the IGFBP-3 doublet was significantly decreased in transgenic mice and correlated with the intensity of the IGFBP-5-myc band. In WT animals, IGFBP-3 levels were low at birth and increased gradually up to 8 wk (Fig. 6B), but the consistent presence of IGFBP-5-myc suppressed this developmental increase.

Total serum IGF-I concentrations were 640 ng/ml in control mice (Fig. 6C) and paradoxically increased by 33% ($P < 0.05$) and 89% ($P < 0.001$) in line 1 and line 3 mice, respectively (corroborated by all three methods of IGFBP extraction). Moreover, mRNA levels of *Igf1* or *Igfbp3* in key tissues were similar in WT and transgenic mice, assessed by real-time RT-PCR (Fig. 11, which is published as supporting information on the PNAS web site), suggesting that alterations in IGF-I and IGFBP-3 levels observed in *Igfbp5* transgenic mice were not due to adaptations in expression.

The ratio of free to total serum IGF-I may represent bioactive IGF (25). Free to total serum IGF-I levels tended to increase ($P = 0.066$) in *Igfbp5* line 3 hemizygote adults ($3.60 \pm 0.67\%$; $n = 9$) versus WT siblings ($1.03 \pm 0.94\%$; $n = 5$), thus total free IGF-I concentrations increased significantly in the transgenic mice (Tg, 4.79 ± 0.63 ; WT, 2.05 ± 0.90 ng/ml; $P < 0.05$). IGFBP-5-myc was observed at molecular weights equivalent to those observed for ternary and binary complexes (9) and for free protein, indicating possible interaction with IGFs and acid-labile subunit (Fig. 12, which is published as supporting information on the PNAS web site).

Discussion

Our findings reveal that IGFBP-5 has an important role in growth and development. Three lines of *Igfbp5* transgenic mice were generated that expressed IGFBP-5 at low, intermediate, and high levels; resultant phenotypes spanned a wide physiological range of IGFBP-5 action, from mild growth retardation with catch-up

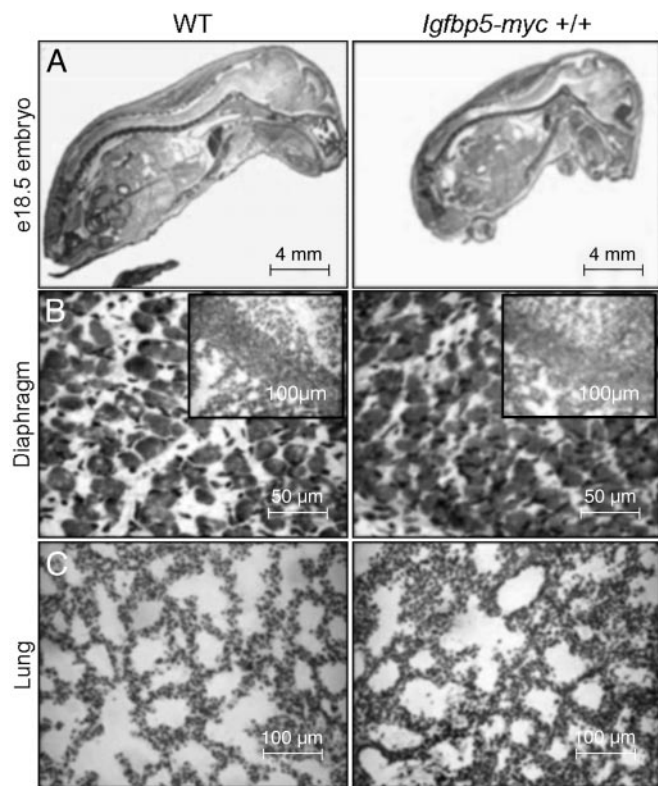


Fig. 5. Histology of *Igfbp5* transgenic embryos at e18.5. (A) Hematoxylin/eosin-stained sagittal sections of a line 3 homozygote ($+/+$) and WT sibling derived from hemizygous parents. (B) Diaphragm muscle. Insets demonstrate diaphragm width. (C) Lung.

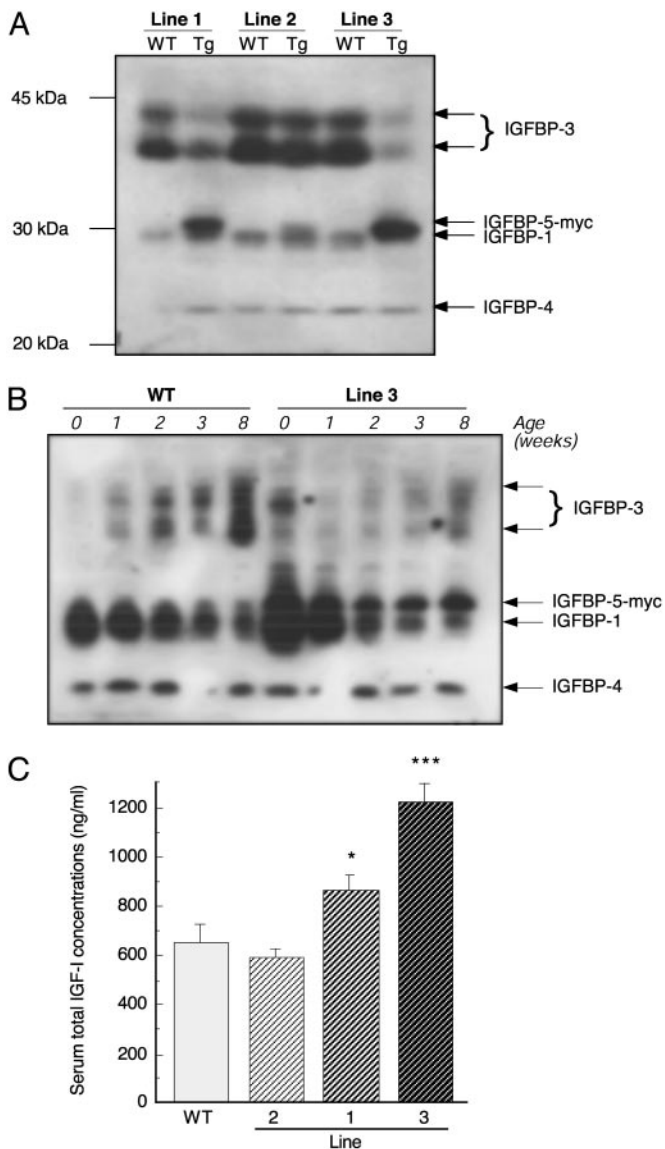


Fig. 6. *Igfbp5-myc* overexpression changes serum IGFBP and IGF-I concentrations postnatally. (A) Ligand blot to display IGFBP levels in serum derived from 8-wk-old hemizygous *Igfbp5*-overexpressing mice (Tg) and WT littermates. (B) Ligand blot to demonstrate changes in IGFBP levels between birth and 8 wk in serum derived from line 3 hemizygous and WT littermates. (C) Serum total IGF-I concentrations in 8-wk-old hemizygotes and WT siblings. *, $P < 0.05$; ***, $P < 0.001$ compared with WT; mean \pm SEM of six to seven mice per group. Serum was collected from offspring of WT mothers and hemizygous fathers.

growth, to severe growth retardation and lethality. In contrast to other genetic studies, this report demonstrates a clear effect for an IGFBP *in vivo*. The significance of these findings is that changes in *Igfbp5* expression could modulate developmental progression, in particular, during the critical immediate postnatal period when tissue programming for adaptation to adult metabolism occurs.

The complexity of IGFBP-5 action (3) dictates that its role in development could be due to (i) IGFBP-5 inhibition of IGF-I and/or IGF-II activity, (ii) IGF-potentiating effects of IGFBP-5, or (iii) IGF-independent actions of IGFBP-5. The first could be considered “IGFBP-generic” and, therefore, *Igfbp5*-overexpressing mice would exhibit characteristics common to other *Igfbp*-overexpression models, but the latter two options would be IGFBP-specific. At one extreme, IGFBP-5 action would inhibit IGF-I and -II activity, and thus the phenotype of the mice would resemble *Igf1*

and *Igf2* double-null mice. Alternately, IGF-independent actions of IGFBP-5 may be revealed as phenotypic properties divergent from those displayed by *Igf1*- or *Igf2*-null mice.

Inhibition of IGF Activity. Overall, the *Igfbp5*-overexpressing mice demonstrated a consistent and dose-dependent phenotype between lines that, in part, conformed to that for *Igf*-null animals. The highest expressing *Igfbp5* transgenic mice had birth weights that were almost as growth-retarded as either *Igf1*- or *Igf2*-null mice (1, 26, 27) and therefore not as extreme as *Igf* double-null mice (28), implying that *Igfbp5* only partially ablated IGF-I and/or IGF-II activity. Surprisingly, when growth rate was considered, *Igfbp5*-overexpressing mice did not conform to the growth pattern of either *Igf1*- or *Igf2*-null mice. All surviving *Igfbp5* transgenic mice became maximally growth-retarded between birth and 3 wk; the lowest expressing line 2 mice displayed catch-up growth, possibly due to increased postpubertal IGF-I overcoming the modest *Igfbp5-myc* expression. In contrast, *Igf1*-null mice become progressively more growth-retarded than WT mice after 3 wk postnatally (29) at the onset of growth hormone-dependent growth (30), whereas *Igf2*-null mice remain at a constant proportion of WT weight from birth (27).

Our data therefore reveal a critical period of sensitivity to *Igfbp5* expression levels between birth and before the onset of growth hormone-stimulated IGF-I synthesis (2–3 wk). This sensitivity is possibly because *Igf2* expression declines during the first 3 wk in mice before increased *Igf1* expression. In support of this hypothesis, serum concentrations of the main circulating IGFBP (IGFBP-3) remain suppressed until after 2–3 wk postnatally (Fig. 6 and ref. 3). In terms of overall growth rate, the phenotype of *Igfbp5*-overexpressing mice more closely resembled *Igf2*- than *Igf1*-null mice, reflecting the higher affinity of IGFBP-5 for IGF-II (13). Further support for inhibition of IGF-II action is derived from the strongly compromised muscle development observed in the *Igfbp5* transgenics; *Igf2* and *Igfbp5* are highly up-regulated in differentiating myoblasts (4), and, therefore, our observations are consistent with the suggestion that they have critical roles in myogenesis (31).

Potentiation of IGF Activity. A striking phenotype observed for *Igfbp5*-overexpressing mice was the increased brain fractional weight. In marked contrast, overexpression of *Igfbp1* or *Igfbp3* induced decreased fractional brain weight (24, 32) with *Igfbp2* overexpression having little effect (33). IGFBP-5 has been proposed to potentiate IGF-I activity in neural tissue (7), and *Igfbp5* and *Igf1* are coexpressed in brain (34); thus, it is possible that the relative increase in brain weight was due to the targeting of IGF activity to type 1 receptors by IGFBP-5. Even though hepatic tissue does not express *Igfbp5* in WT mice, liver fractional weight was also increased in *Igfbp5*-overexpressing mice. Because IGF type 1 receptors are only expressed in fetal not adult liver, *Igfbp5* “abnormally” expressed in the livers of transgenic mice could have performed a similar potentiating function prenatally, with postnatal effects due to IGF-independent actions of IGFBP-5.

IGF-Independent Actions of IGFBP-5. A proportion of *Igf1*-null mice die at birth, depending on genetic background (1). Because we have evidence for only partial ablation of IGF-I activity, the absolute morbidity of the highest expressing *Igfbp5* homozygous mice was unexpected. “Midi” mice have very reduced *Igf1* expression levels and are about the same size as the high-expressing hemizygous *Igfbp5* transgenic mice, yet they are completely viable (35). Further, *Igf2*-null mice are smaller and are viable (27). Therefore, the lethality observed in *Igfbp5* transgenics is unlikely because of their reduced birth weight. However, until the cause of death in *Igfbp5* transgenics is determined, it is difficult to conclude whether mainly IGF-dependent or -independent actions of IGFBP-5 were responsible. In addition, a dose-dependent sexually dimorphic decrease in fertility was observed in *Igfbp5*-overexpressing mice, with female but not male transgenic parents generating litters of reduced size.

Igf2 nulls have no defects in fertility, but surviving *Igf1* nulls of both genders are infertile (30). Recently, it has been suggested that IGFBP-5 may inhibit cell growth and be proapoptotic in an IGF-independent manner (36), which might provide a mechanism for the surprising morbidity and reduced female fertility.

Comparison of the *Igfbp5* transgenic mice generated here with other models of constitutive *Igfbp* overexpression provides further insight into generic versus unique actions of the IGFBP family. *Igfbp5*-overexpressing mice were much more growth-retarded than other *Igfbp* transgenics despite expression of equivalent amounts of IGFBP protein. Indeed, the lack of phenotype in other *Igfbp* overexpression models has always been surprising. For example, the cytomegalovirus and phosphoglycerate kinase promoters used to overexpress *Igfbp1* (37), *Igfbp2* (33), and *Igfbp3* (24) displayed negligible or only modest changes in growth rate. These phenotypes could often only be observed in homozygous animals despite substantial increases in circulating IGFBP concentrations that exceeded the 4-fold increase we observed in the highest *Igfbp5*-expressing line. Further, any decreased growth usually occurred by birth or after the onset of growth hormone-dependent growth, consistent with inhibition of IGF-I activity, in contrast to the critical period of growth inhibition observed between birth and 3 wk for the *Igfbp5*-overexpressing mice. Collectively, these data suggest that IGFBP-5 may have important functions that are not related to modulation of IGF activity and support the increasing number of observations that IGFBP-5 has additional IGF-independent actions (15, 16, 36).

Clinical Significance of Increased *Igfbp5* Expression. The importance of circulating IGF and IGFBP concentrations has been unequivocally demonstrated by using mice doubly null for liver *Igf1* and *Als* (25). Further, the molecular form of serum IGF is crucial, i.e., whether it is free or bound to individual IGFBPs, each of which may be modified in various ways to modify their interaction with the IGFs. In this study, despite increased total serum IGF-I levels, *Igfbp5* transgenics were growth-retarded, consistent with an inhibition of IGF access to tissue receptors. It was surprising, therefore,

that the proportion of free IGF-I in serum tended to increase in *Igfbp5*-overexpressing mice, although this also occurred in the growth-retarded liver *Igf1* and *Als* double-null mice (25). The proportion of different IGFBPs in serum is also important, and, in this regard, IGFBP-5 can effectively compete with IGFBP-3 for serum ternary complex formation with the acid-labile subunit *in vivo* (38). The demonstration of IGFBP-5-myc protein at an appropriate M_r for ternary complex formation suggests that IGFBP-5 could have displaced IGFBP-3 from this complex, retaining circulating IGF in a relatively tightly bound ternary complex (39). Because noncomplexed IGFBP-3 is susceptible to proteolysis (40), the decrease in circulating IGFBP-3 concentrations was consistent with degradation of displaced IGFBP-3.

Elevations in circulating IGF-I concentrations have been associated with increased risk of colorectal (41), breast (42), and prostate (43) cancers, clinically and experimentally (44). This relationship is strengthened when circulating IGFBP-3 concentrations are also considered (41, 43), which are negatively correlated with cancer risk and are proposed to inhibit or counteract IGF-I activity. Because the phenotype of the *Igfbp5*-overexpressing mice in this study displays some properties consistent with inhibition of IGF-I activity, the propensity for neoplasia may be reduced in the *Igfbp5*-overexpressing mice. Indeed, a recent study suggests that IGFBP-5 inhibits survival of breast cancer cells (36).

In summary, we demonstrate a significant role for IGFBP-5 in growth and development. Our findings suggest key differences between *Igfbp5*-overexpressing and *Igf*-null or other *Igfbp*-overexpressing mice. We therefore reveal a dual function for IGFBP-5, as a modulator of IGF activity and, significantly, as an IGF-independent bioactive peptide.

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