Intrinsic Sex Differences in the Early Growth Hormone Responsiveness of Sex-Specific Genes in Mouse Liver

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Sex differences in liver gene expression are dictated by sex differences in circulating GH profiles. Presently, the pituitary hormone dependence of mouse liver gene expression was investigated on a global scale to discover sex-specific early GH response genes that could contribute to sex-specific regulation of downstream GH targets and to ascertain whether intrinsic sex differences characterize hepatic responses to plasma GH stimulation. Global RNA expression analysis identified two distinct classes of sex-specific mouse liver genes: genes subject to positive regulation (class I) and genes subject to negative regulation by pituitary hormones (class II). Genes activated or repressed in hypophysectomized (Hypox) mouse liver within 30–90 min of GH pulse treatment at a physiological dose were identified as putative direct targets of GH action (early response genes). Intrinsic sex differences in the GH responsiveness of a subset of these early response genes were observed. Notably, 45 male-specific genes, including five encoding transcriptional regulators that may mediate downstream sex-specific transcriptional responses, were induced by GH within 30 min in Hypox male but not Hypox female mouse liver. The early GH response genes were enriched in 29 male-specific targets of the transcription factor myocyte enhancer factor 2, whose activation in hepatic stellate cells is associated with liver fibrosis leading to hepatocellular carcinoma, a male-predominant disease. Thus, the rapid activation by GH pulses of certain sex-specific genes is modulated by intrinsic sex-specific factors, which may be associated with prior hormone exposure (epigenetic mechanisms) or genetic factors that are pituitary-independent, and could contribute to sex differences in predisposition to liver cancer or other hepatic pathophysiologies. **(*Molecular Endocrinology* 24: 667–678, 2010)**

Sex-specific gene expression in the liver is a characteristic of more than 1000 genes and affects a wide range of biological processes, including steroid, lipid, and foreign compound metabolism (1–3). This sexual dimorphism is determined by sex differences in circulating GH profiles (4–6). In many species, including rats, mice, and humans, the temporal pattern of pituitary GH secretion is sex-dependent (episodic in males and more frequent in females) and leads to sex differences in downstream signaling pathways in liver and perhaps other target tissues. In mice, GH is secreted in episodic bursts ranging up to 100 ng/ml in both sexes; however, female mice have more frequent peaks than males over a given time period (7). This differential frequency of plasma GH stimulation is an essential element for sex-specific liver gene expression (5).

GH signaling is initiated by the binding of GH to the extracellular domain of GH receptor, which activates multiple intracellular signaling pathways, including signaling by the transcription factor signal transducer and activator of transcription 5b (STAT5b) (8). Liver sexual dimorphism is ablated in male mice with STAT5b deficiency (1, 9). Liver STAT5b is directly responsive to the

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Abbreviations: GSEA, Gene set enrichment analysis; Hypox, hypophysectomized; Mef2, myocyte enhancer factor 2; STAT5b, signal transducer and activator of transcription 5b; TFS, total flagging sum.

male plasma GH profile, as demonstrated by the direct correlation between the occurrence of a plasma GH pulse and the presence of activated STAT5b in the nucleus in male rats, with active STAT5b being low or undetectable during the GH-free interpulse intervals (10). In contrast, in adult female rats, the near continuous presence of GH in circulation partially down-regulates GH signaling to STAT5b in hepatocytes, resulting in activated STAT5b levels that are generally low compared with peak male levels (11). Nuclear STAT5b activity is also reported to exhibit sexual dimorphism in mouse liver (12). The strong, repeated activation of liver STAT5 by each incoming plasma GH pulse that occurs in male but not female rats (10, 13) enables STAT5 to bind dynamically to chromatin in males at both low- and high-affinity binding sites, whereas in females, STAT5 binding to chromatin displays selectivity for high-affinity binding sites (14). Although STAT5b is required for sexual dimorphism of the liver, it is not sufficient, on its own, to establish and maintain sex-specific liver gene expression (10, 15). This finding suggests a requirement for additional factors, some of which may themselves be targets of STAT5b. This possibility, that GH and STAT5b activate indirect regulatory pathways and mechanisms, is consistent with the rather slow feminization of male-specific gene expression seen in intact male mice given a continuous GH infusion (female-like GH pattern) (16).

Global gene expression studies have provided important insights into the cellular and molecular mechanisms that determine liver sexual dimorphism. In one study, GH was shown to play a global regulatory role in sexually dimorphic gene expression in rat liver (17). Another study revealed a key role for STAT5b in sexual dimorphic gene expression in mouse liver, as demonstrated by the near-global loss of sex-specific gene expression in STAT5b-deficient male mice (1). A specific role for STAT5a, a minor liver STAT5 form, has been observed in female mouse liver (18). Genes directly activated (19) or repressed (20) by GH/STAT5b have been identified in rat liver; however, sex-specific early GH response genes were not investigated. In rats, hypophysectomy has a major impact on liver gene expression (21), including sex-specific gene expression (3). In the mouse, the impact of pituitary hormone ablation and the effects of GH restoration at a supraphysiological dose have been investigated for select genes using traditional assays of gene expression (16); however, the global impact of hypophysectomy on sex-specific genes and the ability of short-term physiological GH replacement to reverse the effects of pituitary hormone ablation have not been investigated. Finally, although several *in vitro* and *in vivo* studies have suggested sex differences in the intrinsic GH responsiveness of certain sex-specific genes and signaling pathways (22–26), such intrinsic sex differences have not been investigated on a genome-wide scale.

The present study used genome-wide expression microarrays: 1) to investigate the pituitary hormone dependence of liver sexual dimorphism and to identify sex-specific early GH response genes in a mouse model, and 2) to discover any intrinsic sex differences in the responsiveness of mouse liver to GH pulse stimulation. These studies used the hypophysectomized (Hypox) mouse model to eliminate potentially confounding effects of sex steroids or other pituitary-dependent factors. Importantly, GH was used at a physiological replacement dose to avoid artifacts that may be associated with the 10- to 100-fold supraphysiological doses of GH that have been widely used in earlier studies monitoring GH-induced changes in liver gene expression. Our findings reveal early GH responses in male liver that are not seen in female liver and that affect 45 male-specific genes, 29 of which are targets of the transcription factor myocyte enhancer factor 2 (Mef2).

Results

Experimental design

Expression microarrays were used to investigate the impact of hypopohysectomy on mouse liver gene expression, with a focus on sexually dimorphic gene expression, and to establish the short-term responsiveness of these genes to a physiological replacement dose of GH. RNA was isolated from livers of adult mice that were: intact males and intact females; Hypox males and Hypox females; Hypox male and Hypox female mice treated with a single GH pulse and killed either 30 or 90 min later; and Hypox male and Hypox female mice treated with two GH injections, spaced 4 h apart, and killed 30 min after the second GH injection (Hypox + two GH). Liver RNA samples representing each of these 10 treatment groups were analyzed in 11 sets of competitive hybridizations to 41,174-feature Agilent oligonucleotide microarrays. Normalized ratios and *P* values were calculated for all 11 datasets using Rosetta Resolver software; 7046 microarray probes met the threshold criteria for differential expression (average expression ratio >2-fold and a significance of $P < 0.005$) for at least one of the 11 datasets after elimination of redundant probes (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

Sex specificity in intact and Hypox mouse liver

Sex-specific expression in intact mouse liver was found for 1380 of the 7046 genes of interest (20%), with 864

TABLE 1. Sex specificity of liver-expressed genes in intact and Hypox mice

Sex-specificity in intact mouse liver	Sex specificity in Hypox mice	Gene count	%
Male-specific			
	Female	9	1
	Male	36	4
	Nonspecific	819	95
	Total	864	
Female-specific			
	Female	35	7
	Male	9	2
	Nonspecific	472	91
	Total	516	
Sex-independent			
	Female	99	2
	Male	135	2
	Nonspecific	5432	96
	Total	5666	

The 7046 liver-expressed genes meeting the criteria described in *Materials and Methods* are listed based on the sex specificity of their expression in intact mice and secondarily based on the sex specificity of their expression in Hypox mice.

genes showing male specificity (male/female >2.0) and 516 genes showing female specificity (male/female <0.5) (Table 1). Hypophysectomy abolished sex specificity for 95% of the male-specific genes and 91% of the female-specific genes (Table 1). Moreover, many of the 71 genes that retained sex specificity in Hypox mice showed a reduced sex specificity ratio. Overall, 94% of the genes showing sex specificity in mouse liver are pituitary hormone-dependent and perhaps GH-dependent (Table 1). This conclusion was also evident from the hierarchical clustering of sex-specific genes, presented as a heat map in Fig. 1A. The Hypox male *vs.* Hypox female array formed a branch on its own, reflecting the major effect of hypophysectomy on sex specificity. Sex-specific genes not affected by hypophysectomy (*i.e.* pituitary hormone-independent genes) include the Y chromosome-encoded male-specific genes *Ddx3y*, *Eif2s3y*, *Jarid1d/Kdm5d*, and *Uty* and the female-specific genes *Xist* and *Tsix*, which are involved in X-inactivation.

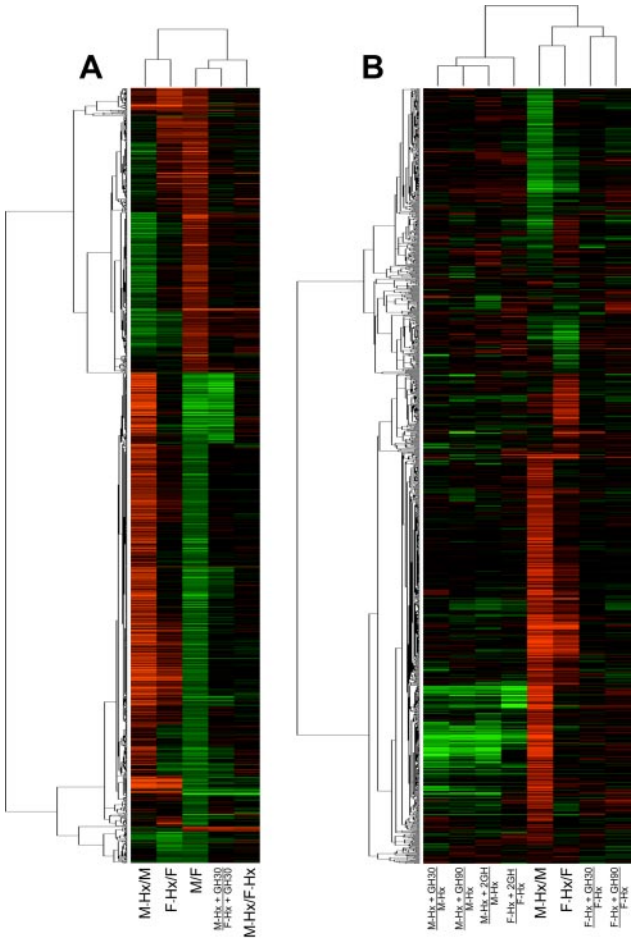

FIG. 1. Heat maps representing expression of 1380 sex-specific genes clustered by gene and by sample. Genes are depicted based on their average expression ratios across the 11 microarray experiments. *Colors* range from *bright green* (up-regulation) to *bright red* (down-regulation). Hierarchical clustering was performed based on Pearson's correlation of log ratios. A, Heat map showing expression of the 1380 sex-specific genes in the three male *vs.* female comparisons [Sham, Hypox, and Hypox + GH (30 min)] and the two Hypox *vs.* Sham comparisons (male and female). B, Heat map showing expression of the 1380 sex-specific genes in the two Hypox *vs.* Sham comparisons and the six GH-treated *vs.* Sham comparisons. The dendrogram at *top* identifies arrays that show the greatest similarity in their patters of expression.

Sex-specific response to hypophysectomy

Hypophysectomy had a significantly greater impact on sex-specific gene expression in male than in female mouse liver (Fig. 1A, lane 1 *vs.* lane 2). Substantial fractions of the male-specific genes (70%) and female-specific genes (54%) whose expression changed after hypophysectomy were affected in Hypox males only (Table 2). Moreover, of the sex-specific genes whose expression was changed in Hypox male liver only, 479 of the 480 male-specific genes were decreased in expression, whereas 183 of the 185 female-specific genes were increased in expression. In contrast, of the (many fewer) genes whose expression changed in Hypox female liver only, 50 of 58 male-specific genes were increased in expression, whereas 77 of 81 female-specific genes were decreased (Table 2). Thus, the male pituitary hormone profile maintains the expression of these male-specific genes and suppresses the expression of the female-specific genes in male liver, whereas the female pituitary hormone profile maintains female-specific gene expression and suppresses male-specific gene expression in female liver. Furthermore, many more sex-specific genes are dependent on male pituitary hormones as compared with female pituitary hormones for expression in mouse liver. In contrast, in the rat model, fewer sex-specific genes were altered in Hypox males only (41% of male-specific genes and 19% of female-specific genes),

TABLE 2. Impact of hypophysectomy on liver gene expression

Effect of Hypox on gene expression	Changed in M-Hypox only		Changed in F-Hypox only		Changed in both M-Hypox and F-Hypox	
	Gene count	%	Gene count	%	Gene count	%
Male-specific genes						
Increase	1	0	50	7	21	3
Decrease	479	70	8	1	112	16
Total	480		58		145[a]	
Female-specific genes						
Increase	183	53	4	1	39	11
Decrease	2	1	77	22	20	6
Total	185		81		80[b]	

The 7046 liver-expressed genes meeting the criteria described in *Materials and Methods* were initially sorted by sex specificity in intact, untreated mice and secondarily by response to hypophysectomy in males only, in females only and in both males and females. Percentages are calculated based on the 683 male-specific genes and 346 female-specific genes that respond to hypophysectomy. Many of the other sex-specific genes are affected by hypophysectomy but do not reach the threshold of more than 2.0-fold change in expression and $P < 0.005$; however, those genes do lose their sex specificity in hypophysectomized mice (<2.0-fold sex difference).

[a] Includes 12 male-specific genes down-regulated in M-Hypox and up-regulated in F-Hypox.

[b] Includes 21 female-specific genes down-regulated in F-Hypox and up-regulated in M-Hypox.

and many more sex-specific genes were changed in expression in Hypox females only (30% of male-specific genes and 48% of female-specific genes) (3). Given the major role of GH as a determinant of liver sex specificity (17), these findings indicate that male and female plasma GH profiles both make substantial contributions to liver sex specificity in rats, whereas the male plasma GH profile dominates in mice.

Classification of sex-specific genes

Two distinct classes of sex-specific genes were identified: genes whose expression decreases after hypophysectomy, indicating pituitary hormone is required for full expression (class I sex-specific genes), and genes whose expression increases after hypophysectomy, indicating repression by pituitary hormone (class II sex-specific genes) (Table 3). A large majority (88%) of the male-specific genes affected by hypophysectomy are class I genes (*i.e.* are induced by the male pituitary hormone profile), with only 10% being class II genes. In contrast, a majority (64%) of the pituitary-dependent female-specific genes are class II genes (*i.e.* are suppressed by male pituitary hormone profile).

The class I and II sex-specific genes were further subdivided based on their responses to hypophysectomy in males and females (Table 3). Class IA male genes are positively regulated by the male but not the female pituitary hormone profile (479 genes), whereas class IB male genes require pituitary hormone for full expression in both sexes (112 genes). Class IC male genes are positively regulated by the male pituitary hormone profile and are repressed by the female pituitary hormone profile (12 genes). Class IIA male genes do not require the male pi-

TABLE 3. Classification of pituitary hormone-dependent male- and female-specific genes

Sex specificity: class	Gene count	Subclass	Response in M-Hypox	Response in F-Hypox	Gene count	Examples (genes)
Male-specific class I	603 (88%)	IA	Down	—	479	*Ckmt2*, *Cml4*, *Gstp1*, *Myh1*
		IB	Down	Down	112	*Cyp7b1*, *Elovl3*, *Hsd3b4*, *Mup1/3/4/5*
		IC	Down	Up	12	*Acta1*, *Cyp4a12a*, *Myh4*
Male-specific class II	71 (10%)	IIA	—	Up	50	*Alas2*, *Mcm10*, *Nox4*
		IIB	Up	Up	21	*Gsta1/Gsta2*, *Grem2*, *Lpl*
					683 total[a]	
Female-specific class I	118 (34%)	IA	—	Down	77	*A1bg*, *Cyp3a41*, *Fmo3*, *Trim24*
		IB	Down	Down	20	*Ly6c2*, *Mfsd2*, *Ptgds*
		IC	Up	Down	21	*Acot3*, *Cyp2g1*, *Npal1*
Female-specific class II	222 (64%)	IIA	Up	—	183	*Cyp2b9*, *Cyp4a10*, *Hao3*, *Nnmt*, *Tox*
		IIB	Up	Up	39	*Cyp4a14*, *Serpinb1a*, *Serpinb1b*,
					346 total[a]	

The 1029 sex-specific genes affected by hypophysectomy in males only, in females only, or in both males and females (Table 2) were classified based on whether expression is suppressed (class I) or induced (class II) by hypophysectomy in males and females. The subclass indicates if the impact of hypophysectomy is seen in only one sex (A) or in both sexes (B). Subclass C includes genes that responded to hypophysectomy in both sexes but in the opposite direction. Percentages (*column 2*) are based on 683 male-specific and 346 female-specific genes. *Columns* 4 and 5 indicate whether the genes are down-regulated, up-regulated, or not changed significantly (*dash*) after hypophysectomy.

[a] Gene totals include nine other male-specific genes and six other female-specific genes.

tuitary hormone profile for expression but are repressed by the female pituitary hormone pattern (50 genes), whereas class IIB male genes are repressed by pituitary hormones in both sexes (21 genes). Class IA female genes are positively regulated by the female but not male pituitary hormone profile (77 genes), whereas class IB female genes require pituitary hormone for full expression in both sexes (20 genes). Class IC females genes are positively regulated by the female pituitary hormone profile but are repressed by the male pituitary profile (21 genes). Class IIA female genes are repressed by the male but not by the female pituitary hormone pattern (183 genes), whereas class IIB female genes are repressed by pituitary hormone in both males and females (39 genes).

Clustering by significance and differential expression

The 7046 genes that met the threshold criteria for at least one of the 11 datasets (Supplemental Table 1) were clustered using a binary flagging system (1), whereby each gene is assigned to a specific category, termed total flagging sum (TFS), based upon its expression ratio and *P* in each of the 11 microarrays (Supplemental Fig. 1). Genes were thus classified into groups and subgroups based on their sex specificity in intact mice, their response to hypophysectomy in both males and females, their sex specificity in Hypox mice, and their response to either one or two GH injections in Hypox male and Hypox female mice. These groupings are very useful in identifying major groups of genes (both sex specific and sex independent) based on their patterns of response under the 11 conditions investigated in this study, as summarized in Supplemental Tables 2 and 3. Select gene groups are discussed below.

Sex difference in response to short-term GH pulse treatment

We hypothesized that a subset of the sex-specific genes affected by hypophysectomy is a direct target of GH that can be detected as a rapid response to GH treatment. This was investigated in Hypox mice killed 30 or 90 min after a single GH injection, and in Hypox mice given two GH injections and killed 30 min after the second injection. These GH treatments were administered to both males and females to identify any sex differences in the early responses to GH. A majority of the class I sex-specific genes (74%) did not respond to short-term GH treatment; these genes may be regulated by GH indirectly (hence no rapid response to GH), or may require pituitary hormone(s) other than GH for expression. However, 147 class I male-specific genes and 17 class I female-specific genes were induced by short-term GH treatment in livers of Hypox male and/or Hypox female mice (Supplemental Table 4A; genes listed in Supplemental Table 4C). These responses to short-term GH treatment were sex-dependent: 70 of the 147 short-term GH-responsive class I male-specific genes were induced in Hypox males only, and 37 of the 147 genes were induced in Hypox females only. Moreover, GH induced 12 of the 17 short-term GH-responsive class I female genes in a sex-specific manner (Supplemental Table 4A).

Sex differences were also apparent from the time course of induction of the male-specific genes, which was more rapid in Hypox males than in Hypox females (Fig. 2; Supplemental Table 4B). Thus, 46 of the 70 class I male genes induced in Hypox males only were induced by either the 30- or 90-min time point, whereas none of the 37 class I male genes that responded to short-term GH treatment in Hypox females only were induced until 30 min after the second GH injection (*i.e.* 4.5 h after the first GH injection). Similarly, 32 of the 40 male-specific genes induced by GH in both Hypox males and Hypox females were up-regulated by either the 30- or 90-min time point in Hypox males, whereas 37 of the 40 genes required two GH injections (4.5 h) to respond in Hypox females. In contrast, the time course of response to short-term GH treatment was very similar in males and females for class I female-specific genes (Supplemental Table 4B). These sex differences in early GH responsiveness were evident from Fig. 1B, where the three GH-treated Hypox male arrays clustered together, and were most similar to Hypox females given two GH injections, indicating that

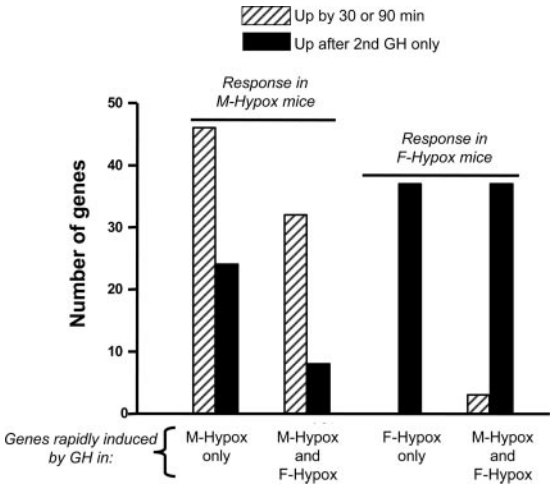

FIG. 2. Class I male-specific genes that respond to GH rapidly in M-Hypox and F-Hypox liver. Shown are the numbers of genes that are rapidly induced by a pulse of GH (within 30 or 90 min), or not until 30 min after a second pulse of GH given 4 h after the first pulse. The class I male-specific genes are divided into groups based on whether they are induced by short-term GH treatment in Hypox males only, Hypox females only, or in both sexes, as indicated on the x-axis. Early GH induction responses dominated in Hypox males, whereas the GH responses seen in Hypox females were almost exclusively late responses. See Supplemental Table 4B for further details.

genes that respond to GH after a single injection in Hypox males require two injections in Hypox females.

These findings indicate that the liver maintains intrinsic sex differences in GH responsiveness several weeks after hypophysectomy, *i.e.* despite the chronic absence of GH and other pituitary-dependent hormones (*e.g.* sex steroids). This sex difference in GH responsiveness was confirmed by a direct microarray comparison of the rapid (30 min) GH pulse-induced changes in liver gene expression seen in Hypox male *vs.* Hypox female mice (Supplemental Table 1, array 11). Notably, 45 of the class IA male-specific genes whose sex specificity was lost after hypophysectomy showed consistent early GH responses in males compared with females (genes listed in Supplemental Table 4D; quantitative PCR verification for select genes in Supplemental Figs. 2 and 3 and Supplemental Table 5). Five of these 45 genes have Gene Ontology terms indicating DNA binding or transcriptional regulation activity (see below).

Rapid repression of sex-specific genes by GH pulse treatment

Rapid down-regulation of gene expression is often difficult to detect by microarray analysis of mature RNAs due to the delay imposed by the intrinsic stability of preexisting RNAs. Nevertheless, 15 of the male-specific genes and 37 of the female-specific genes that were up-regulated in Hypox mouse liver (class II genes) were down-regulated by GH treatment in either one or both sexes (Supplemental Table 4E). Down-regulation was not apparent until 4.5 h after the first GH injection, as seen for 9 of the 15 male-specific genes and 30 of the 37 female-specific genes. Several of the short-term GH down-regulated class IIA female genes are also derepressed in male mouse liver upon ablation of STAT5b (1), suggesting that STAT5b mediates the short-term suppression by GH. These genes include *9030611O19Rik*, *Abcb1a*, *Abcd2*, *Akr1b7*, *Gadd45b*, *Odz3*, *Rab30* (1), and *Tox*, which encodes a class IIA female-specific transcription factor (27).

GH responsiveness of sex-independent genes

In contrast to the sex differences in GH responsiveness of the sex-specific genes, no major sex differences were apparent in the time course of GH responsiveness of the sex-independent genes, a majority of which were not induced or suppressed until 4.5 h after the first GH injection in both sexes (Supplemental Table 6). Genes showing consistent early GH responses are listed in Supplemental Table 7. Of note, six genes were up-regulated within 30 min and then remained elevated in both males and females; four of these genes encode well-established targets of the GH-activated transcription factor STAT5: *Igf1*, *Cish*, *Socs2*, and a *Socs2*-like sequence. The other two, *Phlda1* and an uncharacterized transcript (*AK053952*), may be novel targets of STAT5. *Phlda1* encodes a proline-histidine rich nuclear protein that may play an important role in the antiapoptotic effects of Igf1 (28).

DNA-binding proteins and transcriptional regulators

Forty-one male-specific genes and 28 female-specific genes were identified as DNA-binding proteins and transcriptional regulators by their Gene Ontology descriptors (Supplemental Table 8). Seven of these are male class IA genes that were induced by GH within 30–90 min in male but not female liver and could mediate the downstream transcriptional effects of GH on other sex-specific genes. These genes include: *Tbx15*, which encodes a transcriptional repressor required for skin and skeletal development (29); *Foxg1*, a corepressor of the androgen receptor that is overexpressed in hepatoblastoma (30, 31); *Vgll2*, a transcriptional cofactor required for skeletal muscle differentiation (32); and three members of the ankyrin repeat and suppressor of cytokine signaling box-containing protein family (*Asb11*, *Asb12*, and *Asb15*), of which *Asb15* plays a role in counteracting Igf1-induced myoblast differentiation (33). Two genes encoding sex-independent DNA-binding proteins and transcriptional regulators, *Onecut1* and *Mbd1*, were down-regulated in both Hypox male and Hypox female liver and were up-regulated in both sexes within 90 min of GH treatment (Supplemental Table 9). *Onecut1* encodes the liver-enriched transcription factor HNF6, and *Mbd1* encodes a methyl-CpG binding domain protein that can repress transcription from methylated gene promoters and contributes to epigenetic gene silencing (34).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was used to investigate whether any of the 11 microarray datasets shows enrichment for known transcription factor motifs, as determined using a set of 837 motif gene sets, comprised of genes for which a conserved motif is found within a 4-kb window centered on the transcription start site (35). Twelve motif gene sets associated with the transcription factor Mef2 (36) were enriched among genes up-regulated in three of the 11 microarray datasets: Hypox males + 30-min GH treatment, Hypox males + two GH (4.5-h GH time point), and Hypox female + two GH, all compared with the corresponding Hypox controls (Supplemental Table 10A). Moreover, GH induced a similar number of Mef2 target genes in Hypox males at the 90-min time point as at the other two time points (Supplemental Table 10B). Examination of the 41 Mef2 target genes induced at two or more of the six-GH time points

(30 min, 90 min, and 4.5 h, in both males and females) (Supplemental Table 11) revealed that 29 Mef2 target genes (71%) were male specific, all but one being class I male-specific genes. In contrast, only 25% of the non-Mef2 target genes showing the same pattern of up-regulation by GH were male specific (Supplemental Table 10C). The Mef2 target genes induced by GH are involved in diverse processes, most notably muscle cell development and differentiation (Supplemental Table 12).

Comparisons of sex specificity between mouse and rat

Genes common to the present mouse microarray platform and the rat microarray platform used in Ref. 3 were compared for liver sex specificity and response to hypophysectomy and GH treatment across species; 268 of the genes common to both species showed the same sex specificity at $P < 0.005$ (12 and 20% of the sex-specific genes in mouse and rat liver, respectively; Supplemental Fig. 6A). Many sex-specific genes are assigned species-specific gene names (*e.g.* members of the *Cyp* and *Mup* gene families), and this contributes to the low number of genes showing species-conserved sex specificity. To compare responses with hypophysectomy across species, the 268 sex-specific genes were classified as class I, class II, or nonresponsive to hypophysectomy. Approximately half of the 268 sex-specific genes belong to the same class in both species (Supplemental Fig. 6B), and showed class patterns similar to all sex-specific genes in the species (Supplemental Fig. 7 and Supplemental Table 13). Of the 37 genes that are class I male-specific genes in both species, 13 genes were induced by GH in Hypox male mice and 8 genes were induced by GH time in Hypox male rats. Genes common to both species and showing sex specificity in at least one species are listed in Supplemental Table 14.

Discussion

A large-scale gene expression study was conducted to investigate the pituitary hormone dependence of sex-specific gene expression in mouse liver and to identify sex-specific early GH response genes that might contribute to downstream signaling pathways. Sex-specific liver gene expression was shown to be highly pituitary hormone dependent, as indicated by the near global loss (94%) of sexual dimorphism in Hypox mice. Two distinct classes of sex-specific mouse liver genes were identified. Class I sex-specific genes, which are positively regulated by the pituitary gland, as shown by their down-regulation after hypophysectomy, comprise 88% of the pituitary-dependent genes that are male specific but only 34% of those that are female specific. In contrast, class II sex-specific genes, which are pituitary hormone repressed, comprise 64% of the female-specific genes but only 10% of the male-specific genes. Furthermore, the male pituitary hormone profile was shown to have a greater influence on mouse liver sexual dimorphism than the female hormone profile, as indicated by the greater impact of hypophysectomy on the expression of sex-specific genes in males. Finally, an intrinsic sex difference in the GH pulse responsiveness of the liver was identified, with a subset of sex-specific genes responding rapidly to a physiological pulse of GH in Hypox male but not Hypox female mouse liver. These studies were carried out using GH at a dose, 125 ng/g body weight, that approximates a physiological replacement dose, activates hepatic STAT5 rapidly, and gives plasma GH levels similar to peak levels in intact adult males (10, 37). Supraphysiological GH doses, routinely used in hormone replacement studies in Hypox rats and mice, can activate nonphysiological GH signaling pathways, such as tyrosine phosphorylation of STAT1 (37) and potentially other transcriptional regulators and signaling pathways and may contribute to GH responses seen in studies where high GH doses are used (*e.g.* Refs. 19, 20). Finally, it should be noted that the absence of other pituitary-dependent hormones (*e.g.* thyroid hormone and corticosteroids) might influence the GH response profiles reported in this study.

The global role of the pituitary gland in regulating mouse liver sexual dimorphism through both positive regulation (class I genes) and negative regulation (class II genes) shown here is consistent with the dominant role of plasma GH profiles in determining liver sex specificity, demonstrated in rat liver at both the RNA and protein level (17, 38). GH induced a subset of class I sex-specific genes within 30–90 min; these genes are putative direct targets of GH. Several of the early GH response genes are transcription factors (Supplemental Table 8), which could mediate the inductive effects of GH on downstream sex-specific genes. Class I genes induced after a second GH injection may be primary response genes whose induction is delayed, or could be secondary GH response genes. Some of the class I genes that did not respond to GH during the time frame of our experiments (4.5 h) might have a requirement for pituitary hormones other than GH. However, many of the class I sex-specific genes are likely to be regulated by GH indirectly, as indicated by the finding that several days of continuous GH stimulation are required to feminize liver gene expression in intact male mice (16). The 603 class I male-specific genes identified here include seven male-specific genes previously shown by quantitative PCR to be pituitary hormone-dependent in mouse liver (16), validating our microarray results. Individual examples of the

222 pituitary hormone-repressed class II female-specific genes were also identified previously (16). However, whereas examples of class II male-specific genes [*e.g. Cyp2a2* (39, 40)] and class I female-specific genes [*e.g. Cyp2c12* (23, 41)] were previously identified in the rat model, the 71 class II male-specific mouse genes and the 118 class I female-specific mouse genes (Table 3) represent novel classes of sex-specific mouse liver genes.

Genes that are up-regulated in Hypox mouse liver (class II genes) and repressed by short-term GH treatment are candidates for direct targets of the inhibitory action of GH. Although direct suppression by GH was observed for relatively few class II sex-specific genes, this may be the result of mRNA half-lives that are longer than the 4.5-h time frame of our GH-treatment experiments. Precedent is provided by a recent study in Hypox rats identifying sex-specific genes whose rapid suppression by GH was apparent at the hnRNA level but not at the mRNA level (3). Class II sex-specific genes that responded to GH slowly or not at all in the present study may be secondary GH response genes or perhaps may be repressed via the action of other pituitary hormones. Intrinsic sex differences in the inhibitory action of GH on sex-specific genes were also apparent.

The rapid induction by GH of liver gene expression (including sex-specific gene expression) may be mediated by STAT5b, which was previously shown to be essential for liver sexual dimorphism by microarray analysis (1) and in studies of the effect of GH pulse replacement in Hypox STAT5b-deficient mice (16, 42). Consistent with this hypothesis, several genes with characterized STAT5 response elements, including *Igf1* (43), *Cish* (44), *Spi2.1* (*Serpina3* family) (45), and *Onecut1/Hnf6* (14, 46), were rapidly induced by GH. The rapid induction by GH of certain female-specific genes (Supplemental Table 4C) could be mediated by STAT5a, a minor liver STAT5 form that is >90% identical to STAT5b but is apparently more important for sex-specific gene expression in female than male mouse liver (18). The short-term GH inhibitory effects on certain sex-specific genes (Supplemental Table 4E) may be dependent on STAT5b, which can effect rapid GH suppression in rat liver (20). Class II male-specific genes may be directly repressed by STAT5b during each plasma GH pulse and then derepressed during the plasma GH-free interpulse interval, as suggested by the rapid down-regulation of class II male-specific hnRNAs in Hypox rat liver after GH pulse treatment (3). In this manner, GH could both activate and repress sex-specific genes via the same transcription factor, namely STAT5b.

GSEA analysis was carried out to identify other transcription factors that might be involved in the short-term responses to GH. These analyses identified the transcription factor Mef2, which regulates processes such as neural survival and muscle differentiation (36). Mef2 target genes were induced at all three GH-treatment time points in Hypox males (30 min, 90 min, and 4.5 h/two GH injections), but induction was delayed until the 4.5-h time point in Hypox females, consistent with the sex difference in short-term GH-responsiveness discussed above. Strikingly, the GH-responsive Mef2 target genes were substantially enriched in class I male-specific genes (28 out of 41 GH-responsive Mef2 target genes) and account for 17% of all short-term GH-responsive class I male-specific genes (Supplemental Table 10D). The functional significance of the rapid activation of Mef2 target genes by GH in male liver is unknown. Two of the four vertebrate *Mef2* genes, *Mef2a* and *Mef2d*, are expressed at significant levels in mouse liver, and the other two forms are expressed at low levels, as judged by their microarray signal intensities. The *Mef2* genes themselves did not respond to GH treatment, suggesting that GH may regulate Mef2 activity posttranslationally, *e.g.* by phosphorylation. Other studies have reported that Mef2a, Mef2c, and Mef2d RNA and protein are present in hepatic stellate cells, which comprise 5–8% of resident liver cells and are activated in a Mef2-dependent manner to a myofibroblast-like phenotype under pathological conditions leading to liver fibrosis and cirrhosis (47, 48). The association of Mef2-regulated genes with myofibroblast/smooth muscle function is consistent with the preferential expression of Mef2 protein in this liver cell type and with the activation of these *Mef2* genes under conditions of hepatic stellate cell activation (47). Furthermore, hepatic stellate cells are strongly activated in hepatocellular carcinoma, where Mef2 protein is highly expressed (49), suggesting that Mef2 plays a key role in hepatocellular carcinoma pathogenesis. In this context our observation that male liver is more responsive to rapid GH induction of Mef2 target gene expression is intriguing, insofar as it suggests that GH activation of Mef2 signaling could be an important factor in the widespread greater susceptibility of males to liver cancer (50). Although STAT5 is a known mediator of GH-mediated gene regulation in the liver, its target genes were not enriched in our GSEA analysis, which was limited to transcription factors whose conserved binding sites are within 2 kb of the transcription start site, which is frequently not a characteristic of STAT5 binding sites (14, 51). Finally, the mechanism whereby GH induces Mef2 target genes in liver is unknown. The Mef2 target genes up-regulated by GH are involved in diverse processes, most notably muscle cell development and differentiation (Supplemental Table 12). Of note, during muscle cell differentiation, p38 signaling leading to the activation of

Mef2 converges with Igf1 activation of phosphatidylinositol kinase/AKT signaling at the level of chromatin to induce Mef2 target gene expression (52). Conceivably, there may be similar cooperation between Mef2 and Igf1 in the induction of GH-responsive genes in liver. Further study is required to determine whether Mef2 responses in liver are limited to hepatic stellate cells, or whether they might also contribute to GH responses associated with hepatocytes, the major liver cell type.

Hypophysectomy is presently shown to have a greater impact on sex-specific gene expression in male than in female mouse liver. Given the central role of pituitary GH secretory profiles in establishing and maintaining liver sexual dimorphism, discussed above, this implies that in the mouse, the male plasma GH profile is the dominant determinant of liver sexual dimorphism. This conclusion is consistent with our earlier finding that loss of GH-activated STAT5b has a much greater impact on sex-specific gene expression in male than in female mouse liver (1). By contrast, in the rat model, hypophysectomy of males had a greater effect on the expression of male-specific genes, whereas hypophysectomy of females had a greater impact on female-specific genes (3), indicating that both the male and the female GH profile make important contributions to liver sexual dimorphism in that species. This species difference likely results from the distinct plasma GH profiles of rats and mice, with GH circulating in a near continuous manner in female rats, whereas in female mice the plasma GH profile is highly pulsatile, albeit with noticeably shorter interpulse intervals than in male mice (7). It is unclear, however, how these species differences in plasma GH profiles might contribute to the species-dependent effects of hypophysectomy reported here.

A subset of the sex-specific genes responded to GH rapidly in Hypox male but not Hypox female mice. Given that this sex difference was seen in mice deficient in pituitary-dependent hormones, including gonadal hormones, for a period of several weeks, we conclude that intrinsic sex differences characterize the liver's response to GH. These intrinsic sex differences could result from an epigenetic memory set by the distinct male and female pituitary hormonal environments prior to hypophysectomy. Alternatively, they might be mediated by sex-specific genes encoding other modulatory factors that are expressed in the liver in a pituitary-independent manner, which would enable them to continue to impose their modulatory effects on pituitary/GH-responsive sex-specific genes after hypophysectomy. Indeed, several of the sex-specific genes whose expression is pituitary hormone-independent are chromatin modifying enzymes, *e.g.* the Y-chromosome genes *Jarid1d*, a histone H3 lysine 4 demethylase (53), and *Uty*, which exhibits 84% sequence similarity to the histone H3 lysine 27 demethylase *Utx* (54). These findings are reminiscent of intrinsic sex differences reported for the GH responsiveness of certain sex-specific cytochrome P450 genes (24–26), which may be linked to reduced activation of the JAK2/STAT5b signaling pathway in female as compared with male hepatocytes (22). An intrinsic, more extensive desensitization of JAK2/STAT5b signaling in female as compared with male hepatocytes could explain the reduced GH responsiveness and the delayed induction of certain sex-specific genes in Hypox female liver. Five of the male-specific genes rapidly induced by GH within 30 min in Hypox males but not Hypox females code for transcription factors (Foxg1, Asb11, Asb12, Tbx15, and Vgll2). Further studies will be required to determine whether any of these factors contribute to the GH-dependent regulation of downstream sex-specific genes, and to elucidate the cellular and molecular mechanisms that dictate the intrinsic sex differences in GH responsiveness of the liver.

Materials and Methods

Animal treatments and liver RNA isolation

Male and female crl:CD1 (ICR strain) mice were untreated or Hypox at 8 wk of age. Hypox male and female mice were given a single ip injection of rat GH (125 ng/g body weight) and killed 30 or 90 min later. Other Hypox male and Hypox female mice were given two ip injections of GH, spaced 4 h apart, and killed 30 min after the second GH injection. This dose of GH increases plasma GH to within the physiological range and stimulates robust activation of STAT5b tyrosine phosphorylation in Hypox rats (10, 37) and dwarf mice (55); it contrasts to the 12-fold higher GH replacement dose used in earlier studies investigating primary targets of STAT5b (19, 20). [Of note, activation/tyrosine phosphorylation of liver STAT1 is observed at supraphysiological GH doses (56) but is substantially weaker at physiological hormone replacement doses (37).] Total liver RNA was isolated from individual livers for the following 10 groups of mice (n = 6–8 mice/group): sham surgery-treated and vehicle-injected males and females (M-Sham and F-Sham, respectively), male and female Hypox mice (M-Hypox and F-Hypox, respectively), M-Hypox and F-Hypox mice given a single GH injection and killed 30 or 90 min later [M-Hypox + GH (30), F-Hypox + GH (30), M-Hypox + GH (90), and F-Hypox + GH (90), respectively], and M-Hypox and F-Hypox mice given two GH injections spaced 4 h apart and killed 30 min after the second injection (M-Hypox + two GH and F-Hypox + two GH, respectively). RNA integrity was validated using an Agilent Bioanalyzer 2100 (Agilent Technology, Palo Alto, CA).

Microarrays and data analysis

Liver RNA pools were prepared for two independent sets of biological replicates and used in 11 sets of competitive hybridization experiments in a loop design: 1) M-Sham *vs.* F-Sham, 2) M-Hypox *vs.* M-Sham, 3) M-Hypox + GH (30) *vs.* M-Hypox, 4) M-Hypox + GH (90) *vs.* M-Hypox, 5) M-Hypox + two GH

vs. M-Hypox, 6) F-Hypox *vs.* F-Sham, 7) F-Hypox + GH (30) *vs.* F-Hypox, 8) F-Hypox + GH (90) *vs.* F-Hypox, 9) F-Hypox + two GH *vs.* F-Hypox, 10) F-Hypox *vs.* F-Hypox, and 11) M-Hypox + GH (30) *vs.* F-Hypox + GH (30). Hybridization of fluorescent labeled RNA to Agilent Whole Mouse Genome oligonucleotide microarrays (catalog no. G4122F; Agilent Technology) was carried out (57), with dye swapping to eliminate dye bias, giving a total of 22 microarrays. Linear and LOWESS normalization and data analysis employed Rosetta Resolver software (58). The full set of normalized expression ratios and *P* values is available as Gene Expression Omnibus series GSE17644 (available at http://www.ncbi.nlm.nih.gov/geo). A filter ($P < 0.005$) was applied to the *P* values to determine the significance of each gene's differential expression for each of the 11 microarray experiments. A fold change filter of 2.0-fold was combined with the above *P* filter to reduce the false discovery rate to 0.78%. A system of binary and decimal flags (TFS) was applied for clustering the probes (genes) based on expression ratios obtained in all 11 microarray experiments, as described (Supplemental Fig. 1) (1). Microarray probe annotation is detailed in Supplemental Materials and Methods. Where two or more probes assigned the same gene name gave the same pattern of regulation, as indicated by assignment to the same TFS group (*i.e.* redundant probes), only the probe with the best *P* was retained. Probes associated with the same gene name but different TFS groups were retained. The number of regulated probes meeting the threshold criteria was thus reduced from 7775 to 7046. Hierarchical clustering and heat map generation for the 1380 genes identified as sex-specific was carried out using GenePattern (59). GSEA (60) was used to identify sets of regulated genes that have a common transcription factor binding set motif using the C3 motif gene sets (available at http://www.broadinstitute.org/gsea/index.jsp); cut-offs were set as false discovery rate less than 0.05 and normalized enrichment score more than or equal to 3. Mef2 target genes up-regulated at two or more GH-treatment time points were analyzed for enrichment of Gene Ontology, protein domain, pathway, and functional categories using DAVID (available at http://david.abcc.ncifcrf.gov).

Comparisons between mouse and rat

Sex differences in liver gene expression and response to hypophysectomy and GH treatment were compared between mouse liver (the present study) and rat liver (3). Comparisons were made for 11,295 genes represented by probes in both microarray platforms (defined as probes with the same gene name), and a threshold of $P < 0.005$ was used to identify regulated probes common to both species.

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References

1. **Clodfelter KH, Holloway MG, Hodor P, Park SH, Ray WJ, Waxman DJ** 2006 Sex-dependent liver gene expression is extensive and largely dependent upon STAT5b: STAT5b-dependent activation of male genes and repression of female genes revealed by microarray analysis. Mol Endocrinol 20:1333–1351
2. **Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, Drake TA, Lusis AJ** 2006 Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res 16:995–1004
3. **Wauthier V, Waxman DJ** 2008 Sex-specific early growth hormone response genes in rat liver. Mol Endocrinol 22:1962–1974
4. **Waxman DJ, Holloway MG** 2009 Centennial perspective: sex differences in the expression of hepatic drug metabolizing enzymes. Mol Pharmacol 76:215–228
5. **Waxman DJ, O'Connor C** 2006 Growth hormone regulation of sex-dependent liver gene expression. Mol Endocrinol 20:2613–2629
6. **Mode A, Gustafsson JA** 2006 Sex and the liver—a journey through five decades. Drug Metab Rev 38:197–207
7. **MacLeod JN, Pampori NA, Shapiro BH** 1991 Sex differences in the ultradian pattern of plasma growth hormone concentrations in mice. J Endocrinol 131:395–399
8. **O'Shea JJ, Gadina M, Schreiber RD** 2002 Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 109(Suppl):S121–S131
9. **Holloway MG, Cui Y, Laz EV, Hosui A, Hennighausen L, Waxman DJ** 2007 Loss of sexually dimorphic liver gene expression upon hepatocyte-specific deletion of Stat5a-Stat5b locus. Endocrinology 148:1977–1986
10. **Choi HK, Waxman DJ** 2000 Plasma growth hormone pulse activation of hepatic JAK-STAT5 signaling: developmental regulation and role in male-specific liver gene expression. Endocrinology 141:3245–3255
11. **Choi HK, Waxman DJ** 1999 Growth hormone, but not prolactin, maintains, low-level activation of STAT5a and STAT5b in female rat liver. Endocrinology 140:5126–5135
12. **Sueyoshi T, Yokomori N, Korach KS, Negishi M** 1999 Developmental action of estrogen receptor-α feminizes the growth hormone-Stat5b pathway and expression of Cyp2a4 and Cyp2d9 genes in mouse liver. Mol Pharmacol 56:473–477
13. **Waxman DJ, Ram PA, Park SH, Choi HK** 1995 Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, Stat 5-related DNA binding protein. Proposed role as an intracellular regulator of male-specific liver gene transcription. J Biol Chem 270:13262–13270
14. **Laz EV, Sugathan A, Waxman DJ** 2009 Dynamic *in vivo* binding of STAT5 to growth hormone-regulated genes in intact rat liver. Sex-specific binding at low- but not high affinity STAT5 sites. Mol Endocrinol 23:1242–1254
15. **Verma AS, Dhir RN, Shapiro BH** 2005 Inadequacy of the Janus kinase 2/signal transducer and activator of transcription signal transduction pathway to mediate episodic growth hormone-dependent regulation of hepatic CYP2C11. Mol Pharmacol 67:891–901
16. **Holloway MG, Laz EV, Waxman DJ** 2006 Co-dependence of growth hormone-responsive, sexually dimorphic hepatic gene expression on signal transducer and activator of transcription 5b and hepatic nuclear factor 4-α. Mol Endocrinol 20:647–660
17. **Ahluwalia A, Clodfelter KH, Waxman DJ** 2004 Sexual dimorphism of rat liver gene expression: regulatory role of growth hormone

revealed by deoxyribonucleic acid microarray analysis. Mol Endocrinol 18:747–760
18. **Clodfelter KH, Miles GD, Wauthier V, Holloway MG, Zhang X, Hodor P, Ray WJ, Waxman DJ** 2007 Role of STAT5a in regulation of sex-specific gene expression in female, but not male, mouse liver revealed by microarray analysis. Physiol Genomics 31:63–74
19. **Vidal OM, Merino R, Rico-Bautista E, Fernandez-Perez L, Chia DJ, Woelfle J, Ono M, Lenhard B, Norstedt G, Rotwein P, Flores-Morales A** 2007 *In vivo* transcript profiling and phylogenetic analysis identifies suppressor of cytokine signaling 2 as a direct signal transducer and activator of transcription 5b target in liver. Mol Endocrinol 21:293–311
20. **Ono M, Chia DJ, Merino-Martinez R, Flores-Morales A, Unterman TG, Rotwein P** 2007 Signal transducer and activator of transcription (Stat) 5b-mediated inhibition of insulin-like growth factor binding protein-1 gene transcription: a mechanism for repression of gene expression by growth hormone. Mol Endocrinol 21:1443–1457
21. **Flores-Morales A, Ståhlberg N, Tollet-Egnell P, Lundeberg J, Malek RL, Quackenbush J, Lee NH, Norstedt G** 2001 Microarray analysis of the *in vivo* effects of hypophysectomy and growth hormone treatment on gene expression in the rat. Endocrinology 142:3163–3176
22. **Dhir RN, Thangavel C, Shapiro BH** 2007 Attenuated expression of episodic growth hormone-induced Cyp2c11 in female rats associated with suboptimal activation of the Jak2/Stat5b and other modulating signaling pathways. Drug Metab Dispos 35:2102–2110
23. **Pampori NA, Shapiro BH** 1999 Gender differences in the responsiveness of the sex-dependent isoforms of hepatic P450 to the feminine plasma growth hormone profile. Endocrinology 140:1245–1254
24. **Thangavel C, Dworakowski W, Shapiro BH** 2006 Inducibility of male-specific isoforms of cytochrome p450 by sex-dependent growth hormone profiles in hepatocyte cultures from male but not female rats. Drug Metab Dispos 34:410–419
25. **Thangavel C, Garcia MC, Shapiro BH** 2004 Intrinsic sex differences determine expression of growth hormone-regulated female cytochrome P450s. Mol Cell Endocrinol 220:31–39
26. **Waxman DJ, Ram PA, Pampori NA, Shapiro BH** 1995 Growth hormone regulation of male-specific rat liver P450s 2A2 and 3A2: induction by intermittent growth hormone pulses in male but not female rats rendered growth hormone deficient by neonatal monosodium glutamate. Mol Pharmacol 48:790–797
27. **Laz EV, Holloway MG, Chen CS, Waxman DJ** 2007 Characterization of three growth hormone-responsive transcription factors preferentially expressed in adult female liver. Endocrinology 148:3327–3337
28. **Toyoshima Y, Karas M, Yakar S, Dupont J, Lee Helman, LeRoith D** 2004 TDAG51 mediates the effects of insulin-like growth factor I (IGF-I) on cell survival. J Biol Chem 279:25898–25904
29. **Farin HF, Bussen M, Schmidt MK, Singh MK, Schuster-Gossler K, Kispert A** 2007 Transcriptional repression by the T-box proteins Tbx18 and Tbx15 depends on Groucho corepressors. J Biol Chem 282:25748–25759
30. **Obendorf M, Meyer R, Henning K, Mitev YA, Schröder J, Patchev VK, Wolf SS** 2007 FoxG1, a member of the forkhead family, is a corepressor of the androgen receptor. J Steroid Biochem Mol Biol 104:195–207
31. **Adesina AM, Nguyen Y, Guanaratne P, Pulliam J, Lopez-Terrada D, Margolin J, Finegold M** 2007 FOXG1 is overexpressed in hepatoblastoma. Hum Pathol 38:400–409
32. **Chen HH, Maeda T, Mullett SJ, Stewart AF** 2004 Transcription cofactor Vgl-2 is required for skeletal muscle differentiation. Genesis 39:273–279
33. **McDaneld TG, Spurlock DM** 2008 Ankyrin repeat and suppressor of cytokine signaling (SOCS) box-containing protein (ASB) 15 alters differentiation of mouse C2C12 myoblasts and phosphorylation of mitogen-activated protein kinase and Akt. J Anim Sci 86:2897–2902
34. **Sakamoto Y, Watanabe S, Ichimura T, Kawasuji M, Koseki H, Baba H, Nakao M** 2007 Overlapping roles of the methylated DNA-binding protein MBD1 and polycomb group proteins in transcriptional repression of HOXA genes and heterochromatin foci formation. J Biol Chem 282:16391–16400
35. **Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M** 2005 Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. Nature 434:338–345
36. **Potthoff MJ, Olson EN** 2007 MEF2: a central regulator of diverse developmental programs. Development 134:4131–4140
37. **Ram PA, Park SH, Choi HK, Waxman DJ** 1996 Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. Differential kinetics of hormone desensitization and growth hormone stimulation of both tyrosine phosphorylation and serine/threonine phosphorylation. J Biol Chem 271:5929–5940
38. **Laz EV, Wiwi CA, Waxman DJ** 2004 Sexual dimorphism of rat liver nuclear proteins: regulatory role of growth hormone. Mol Cell Proteomics 3:1170–1180
39. **Agrawal AK, Shapiro BH** 2001 Intrinsic signals in the sexually dimorphic circulating growth hormone profiles of the rat. Mol Cell Endocrinol 173:167–181
40. **Waxman DJ, LeBlanc GA, Morrissey JJ, Staunton J, Lapenson DP** 1988 Adult male-specific and neonatally programmed rat hepatic P-450 forms RLM2 and 2a are not dependent on pulsatile plasma growth hormone for expression. J Biol Chem 263:11396–11406
41. **MacGeoch C, Morgan ET, Gustafsson JA** 1985 Hypothalamo-pituitary regulation of cytochrome P-450(15) β apoprotein levels in rat liver. Endocrinology 117:2085–2092
42. **Davey HW, Park SH, Grattan DR, McLachlan MJ, Waxman DJ** 1999 STAT5b-deficient mice are growth hormone pulse-resistant. Role of STAT5b in sex-specific liver p450 expression. J Biol Chem 274:35331–35336
43. **Eleswarapu S, Gu Z, Jiang H** 2008 Growth hormone regulation of insulin-like growth factor-I gene expression may be mediated by multiple distal signal transducer and activator of transcription 5 binding sites. Endocrinology 149:2230–2240
44. **Verdier F, Rabionet R, Gouilleux F, Beisenherz-Huss C, Varlet P, Muller O, Mayeux P, Lacombe C, Gisselbrecht S, Chretien S** 1998 A sequence of the CIS gene promoter interacts preferentially with two associated STAT5A dimers: a distinct biochemical difference between STAT5A and STAT5B. Mol Cell Biol 18:5852–5860
45. **Wood TJ, Sliva D, Lobie PE, Goullieux F, Mui AL, Groner B, Norstedt G, Haldosén LA** 1997 Specificity of transcription enhancement via the STAT responsive element in the serine protease inhibitor 2.1 promoter. Mol Cell Endocrinol 130:69–81
46. **Lahuna O, Rastegar M, Maiter D, Thissen JP, Lemaigre FP, Rousseau GG** 2000 Involvement of STAT5 (signal transducer and activator of transcription 5) and HNF-4 (hepatocyte nuclear factor 4) in the transcriptional control of the hnf6 gene by growth hormone. Mol Endocrinol 14:285–294
47. **Wang X, Tang X, Gong X, Albanis E, Friedman SL, Mao Z** 2004 Regulation of hepatic stellate cell activation and growth by transcription factor myocyte enhancer factor 2. Gastroenterology 127:1174–1188
48. **Sato M, Suzuki S, Senoo H** 2003 Hepatic stellate cells: unique characteristics in cell biology and phenotype. Cell Struct Funct 28:105–112
49. **Bai X, Wu L, Liang T, Liu Z, Li J, Li D, Xie H, Yin S, Yu J, Lin Q, Zheng S** 2008 Overexpression of myocyte enhancer factor 2 and histone hyperacetylation in hepatocellular carcinoma. J Cancer Res Clin Oncol 134:83–91
50. **De Maria N, Manno M, Villa E** 2002 Sex hormones and liver cancer. Mol Cell Endocrinol 193:59–63
51. **Nelson EA, Walker SR, Alvarez JV, Frank DA** 2004 Isolation of unique STAT5 targets by chromatin immunoprecipitation-based gene identification. J Biol Chem 279:54724–54730
52. **Serra C, Palacios D, Mozzetta C, Forcales SV, Morantte I, Ripani M, Jones DR, Du K, Jhala US, Simone C, Puri PL** 2007 Functional interdependence at the chromatin level between the MKK6/p38 and IGF1/PI3K/AKT pathways during muscle differentiation. Mol Cell 28:200–213

53. **Lee MG, Norman J, Shilatifard A, Shiekhattar R** 2007 Physical and functional association of a trimethyl H3K4 demethylase and Ring6a/MBLR, a polycomb-like protein. Cell 128:877–887
54. **Hong S, Cho YW, Yu LR, Yu H, Veenstra TD, Ge K** 2007 Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci USA 104:18439–18444
55. **Miquet JG, Sotelo AI, Dominici FP, Bonkowski MS, Bartke A, Turyn D** 2005 Increased sensitivity to GH in liver of Ames dwarf (Prop1df/Prop1df) mice related to diminished CIS abundance. J Endocrinol 187:387–397
56. **Gronowski AM, Rotwein P** 1994 Rapid changes in nuclear protein tyrosine phosphorylation after growth hormone treatment in vivo. Identification of phosphorylated mitogen-activated protein kinase and STAT91. J Biol Chem 269:7874–7878
57. **Holloway MG, Miles GD, Dombkowski AA, Waxman DJ** 2008 Liver-specific HNF4-α deficiency: greater impact on gene expression in male than in female mouse liver. Mol Endocrinol 22:1274–1286
58. **Weng L, Dai H, Zhan Y, He Y, Stepaniants SB, Bassett DE** 2006 Rosetta error model for gene expression analysis. Bioinformatics 22:1111–1121
59. **Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP** 2006 GenePattern 2.0. Nat Genet 38:500–501
60. **Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP** 2005 Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA 102:15545–15550