Genes Nutr (2015) 10:443
DOI 10.1007/s12263-014-0443-1

RESEARCH PAPER

Effects of strain and age on hepatic gene expression profiles in murine models of HFE-associated hereditary hemochromatosis

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Received: 13 August 2014 / Accepted: 15 November 2014 / Published online: 27 November 2014
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Abstract Hereditary hemochromatosis is an iron overload disorder most commonly caused by a defect in the *HFE* gene. While the genetic defect is highly prevalent, the majority of individuals do not develop clinically significant iron overload, suggesting the importance of genetic modifiers. Murine hfe knockout models have demonstrated that strain background has a strong effect on the severity of iron loading. We noted that hepatic iron loading in *hfe−/−* mice occurs primarily over the first postnatal weeks (loading phase) followed by a timeframe of relatively static iron concentrations (plateau phase). We thus evaluated the effects of background strain and of age on hepatic gene expression in Hfe knockout mice (*hfe−/−*). Hepatic gene expression profiles were examined using cDNA microarrays in 4- and 8-week-old *hfe−/−* and wild-type mice on two different genetic backgrounds, C57BL/6J (C57) and AKR/J (AKR). Genes differentially regulated in all *hfe−/−* mice groups, compared with wild-type mice, including those involved in cell survival, stress and damage responses and lipid metabolism. AKR strain-specific changes in lipid metabolism genes and C57 strain-specific changes in cell adhesion and extracellular matrix protein genes were detected in *hfe−/−* mice. Mouse strain and age are each significantly associated with hepatic gene expression profiles in *hfe−/− mice*. These affects may underlie or reflect differences in iron loading in these mice.

Keywords Hereditary hemochromatosis · AKR/J · C57BL/6J · Mouse strains · Iron loading status

Introduction

Hereditary hemochromatosis (HH), caused by defective HFE function, is an iron overload disorder that affects one in 400 people with a carrier frequency of one in 10 individuals of Northern European descent (Feder et al. 1996; Fleming and Sly 2002). Wild-type HFE forms a complex with β_2-microglobulin in the endothelium reticulum (ER) and translocates to the cell surface (Feder et al. 1998; Gross et al. 1998; Roy et al. 1999). A current model suggests that the HFE protein acts by forming a complex with transferrin receptors 1 or 2 and influencing hepatocellular signaling in the regulation of the hepcidin gene (Feder et al. 1998; Gross et al. 1998; Roy et al. 1999). A point mutation in *HFE* (845G → A), which accounts for the vast majority cases of HH, results in the amino acid substitution of C282Y (Feder et al. 1996). The C282Y mutation disrupts a disulfide bond required for the interaction with β_2-microglobulin, and the heterodimer fails to move to the plasma membrane (Feder et al. 1997; Waheed et al. 1997). Heterozygotes for HFE gene are demonstrated to have elevated serum levels of body iron store indicators such as serum

Electronic supplementary material The online version of this article (doi:10.1007/s12263-014-0443-1) contains supplementary material, which is available to authorized users.

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ferritin and transferrin saturation (Bulaj et al. 1996). More than 90 % of patients with HH ascertained by clinical manifestations of liver disease are homozygous for the C282Y mutation in the HFE gene (Bacon et al. 1999). However, population-wide surveys demonstrate that fewer than 10 % of individuals with homozygous C282Y have clinical manifestations (Mura et al. 2001; Beutler et al. 2002; Sanchez et al. 2003). The low penetrance in *HFE*-associated hemochromatosis suggests that other factors such as genetic variation in other genes likely underlies differences in iron loading in people with this mutation (Rochette et al. 2010).

Mouse models for HH that were generated by knocking out the Hfe gene in C57 and AKR backgrounds showed strain effects on the levels of hepatic iron loading in *hfe*−/− mice (Fleming et al. 2001). Ten-week-old *hfe*−/− mice from the AKR background showed two to three times higher hepatic iron accumulation as compared to age-matched *hfe*−/− mice from a C57 background (Fleming et al. 2001). Differences in hepatic iron levels between *hfe*−/− mice and wild-type mice start to increase after 2 weeks of age in both strains (Fleming et al. 2001). Moreover, to reach complete saturation of serum transferrin, C57 mice needed a 10 times higher oral dose of carbonyl iron as compared to AKR mice (Leboeuf et al. 1995).

We aimed to characterize the effects of strain and/or age-associated iron loading stages on gene expression profiles in *hfe*−/− mice. We carried out cDNA microarray studies using *hfe*−/− 8-week-old C57 mice, 4-week-old AKR mice, 8-week-old AKR and wild-type mice. We first investigated whether there were differences in several selected genes known to be involved in iron metabolism by real-time PCR. Subsequently, we screened a set of ~11,000 genes by expression profiling using microarrays to identify differentially expressed genes that may play a role in strain-specific variation in iron loading in *hfe*−/− mice.

Materials and methods

Mice

hfe−/− male mice and wild-type AKR and C57 male mice were fed a standard chow (270 mg/kg of iron) ad libitum. Four-week-old and 8-week-old AKR mice and 8-week-old C57 mice were killed (8-week-old C57 group). Prior to killing, mice were fasted overnight and euthanized before liver tissue samples were collected. The studied mouse population consisted of 6–8 of *hfe*−/− mice. The animal protocols were approved by the Animal Care and Use Committees of Saint Louis University.

Reverse transcription (RT) for microarray

We used 12.5 μg of pooled total RNA samples isolated from the liver of 6 to 8 *hfe*−/− or wild-type mice in each group to synthesize cDNA. Only RNA samples with the ratio of A260 to A280 higher than 2.0 and the presence of two clear bands indicating intact 18S rRNA and 28S rRNA when run on a RNA gel electrophoresis were used for the study. Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) primed by Oligo dT in the presence of aminoallyl dUTP. The resulting amino-modified cDNA was labeled using Cy3 or Cy5 fluorescent dyes (Amersham Biosciences, Piscataway, NJ).

BMAP microarray slide

The cDNA microarrays were produced from Brain Mouse Anatomy Project (BMAP) mouse brain UniGene set (Open Biosystems, Huntsville, AL). cDNAs were amplified using primers adjacent to cloning sites by polymerase chain reaction (PCR). Purified PCR products were spotted onto poly-lysine-coated slides using a custom built arrayer at the College of Natural Resources Genomics Facility at the University of California, Berkeley.

Microarray hybridization and image acquisition

We performed eight hybridizations for each experimental group (4- and 8-week-old AKR groups and 8-week-old C57 group) to compare hepatic expression profiles from *hfe*−/− mice to those of wild-type mice. The Cy3- and Cy5-labeled cDNAs were mixed prior to hybridization to cDNA microarrays. Hybridization was performed at 65 °C for 16–20 h. Microarray images were obtained by scanning slides using an ArrayWoRx Biochip Reader (Applied Precision, Issaquah, WA). Images were quantified using GenePix 3.01 (Molecular Devices, Sunnyvale, CA) to obtain an average hybridization signal intensity for each spot and the background of the surrounding area.

Data analysis

Spot intensity values for each array were analyzed using our exploratory differential gene expression analysis algorithm (Loguinov et al. 2004). Spot intensities were normalized by nonparametric regression smoothing (super smoother, *supsmu,* also known as *lowess*). Using this method, data were grouped into subsets of equal size (print tip groups) and then regression smoothers were applied to the median absolute residual against the median of log2-transformed Cy3 for each box plot group. Log2-transformed normalized ratios were used for subsequent data

analysis to identify candidate genes for differential expression. A general α-outlier model for residuals was adapted, and smoothed simultaneous tolerance intervals (STIs) were applied to account for heteroscedasticity of residual variance. We selected candidate genes with a p value lower than 0.001 that were either up- or down-regulated in at least four out of eight hybridizations. Candidate genes were analyzed for their association with biological functions and/or diseases by Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA).

Reverse transcription (RT) and real-time quantitative polymerase chain reaction (RT-qPCR)

2 μg of DNaseI-treated total RNA that was isolated using Trizol reagent (Invitrogen) was reverse transcribed at 42 °C for 1 h using random primer and SuperscriptII reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was diluted ten times in autoclaved DEPC water and subjected to qPCR. qPCR was performed with the ABI Prism 7700 and SYBR Green Reagents (Applied Biosystems, Foster City, CA). Two-step qPCR protocol was used with 40 cycles of 95 °C for 15 s and 60 °C for 60 s. PCR efficiency was confirmed by dilution curves from serially diluted cDNA samples. The relative abundances of mRNAs were obtained based on the $2^{-\Delta\Delta Ct}$ method calculation (Beutler et al. 2002) and normalized to the amounts of 18S ribosomal RNA. The used primers are following: 18S ribosomal RNA F5′-GTAACC CGTTGAACCCCATT-3′, R 5′-CCATCCAATCGGTAG-TAGCG-3′; Transferrin receptor 1 (*Tfr1*) F5′-TCATGAG GGAAATCAATGATCGTA-3′, R 5′-GCCCCAGAAGA TATGTCGGAA-3′; Transferrin receptor 2 (*Tfr2*) F5′-CCCATGGACAGCAGTGCATAT-3′, R 5′-CGTGTGCA GGAATGGGTACA-3′; Ferritin heavy chain (*Fth*) F5′-TGCACAAACTGGCTACTGACAA-3′, R 5′-GGTCACC CAGTTCTTTAATGGATT-3′; Ferroportin (*Fpn1*or *Slc40a1*) F 5′-GGGAGCCATCATTGGTGACT-3′, R 5′-GATGATTCCGCAGAGGATGAC-3′; Hepcidin 1 (*Hamp1*) F 5′-GGCAGACATTGCGATACCAA-3′, R 5′-TGGCTCTAGGCTATGTTTTGCA-3′; Hepcidin 2 (*Hamp2*) F 5′-GCAGACATTGCGATCCCAA-3′, R 5′-CTCTCTATTCTTCACAACAGATACCACA-3′.

Results

Regulation of iron gene expression is affected by strains and iron loading status in *hfe*−/− mice

We observed that AKR mice accumulate iron during the first 8 weeks of life (loading phase), followed by relatively constant iron content (plateau phase) afterward (Fig. 1).

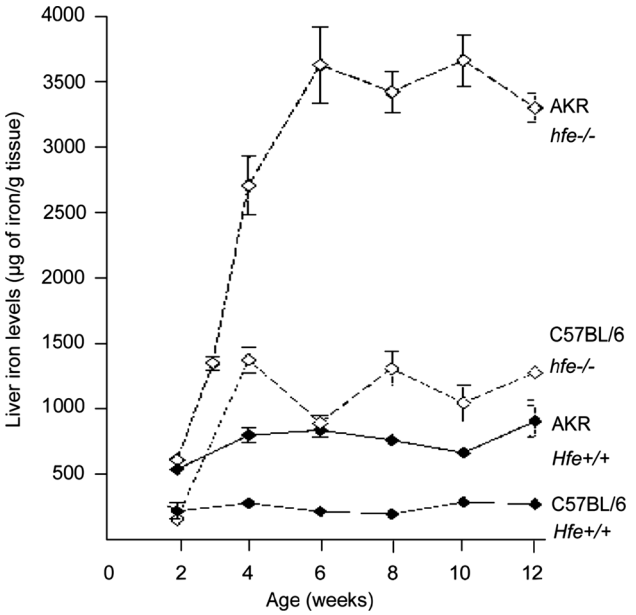

Fig. 1 Comparison of hepatic iron loading between *hfe*−/− AKR and C57 mice. The results are expressed as mean ± SD

The AKR wild-type mice demonstrate more hepatocellular iron than the C57 wild-type mice at all ages (Fig. 1). We thus compared hepatic gene expression profiles in AKR *hfe* −/− mice during "loading" (4 weeks old) and "plateau" (8 weeks old) phases with age-matched wild-type AKR mice. We also compared AKR hfe−/− mice with C57 hfe−/− mice at 8 weeks of age. We initially analyzed mRNA levels of selected genes: transferrin receptor (Tfr1 and Tfr2), hepcidin (Hamp1 and Hamp2), and Ferroportin 1 (Slc40a1) using pooled RNA samples by RT-qPCR (Fig. 2). Iron loading phase (4 week age) AKR *hfe*−/− mice showed a significant reduction in Tfr1, Hamp1, Hamp2, and Slc40a1 mRNA levels, but no changes in Tfr2 mRNAs, compared to age-matched WT mice (Fig. 2). Iron plateau phase (8 week age) AKR *hfe*−/− mice displayed a significant decreases in Tfr2, Hamp1, and Hamp2 mRNAs, an increase in Slc40a1 mRNA, and no changes in Tfr1 (Fig. 2) compared with wild-type mice of same age. Iron plateau phase (8 week age) C57 *hfe*−/− mice had elevated mRNA levels of Tfr1, Hamp1, and Hamp2, but decreased levels of Slc40a1, and no changes in Tfr2 as compared to wild-type C57 mice.

Differences in strains and iron loading status cause few hepatic genes to be commonly influenced by *hfe*−/−

We expanded the repertoire of analyzed genes to include all those represented in an 11K BMAP microarray. We identified 26, 51, and 79 genes to be differentially expressed in the 4-week AKR, 8-week AKR, and 8-week

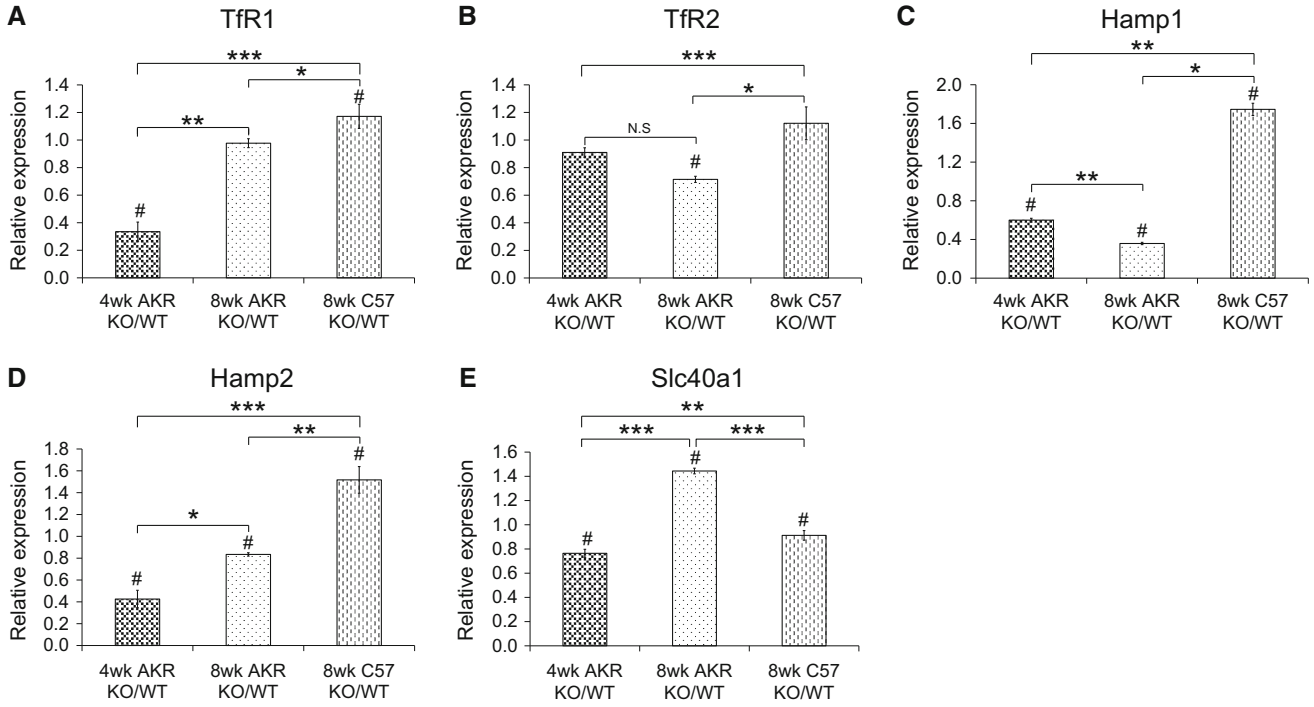

Fig. 2 Relative mRNA levels of iron-related genes in AKR and C57 *hfe*−/− mice compared to wild-type mice. RNA pooled from five to eight individuals in each mice group was used for quantitative real-time PCR using SYBR green. Expression values were normalized to the 18SrRNA. Transcript levels of Tfr1 (**a**), Tfr2 (**b**), Hamp1 (**c**), Hamp2 (**d**), and Slc40a1 (**e**) from *hfe*−/− mice were compared to those from wild-type mice. The results are expressed as mean ± SD. # Comparisons of the expression levels of genes between KO and WT mice were performed by Student's *t* test, $p < 0.05$; comparisons of the expression ratio of KO to WT between two groups were performed by Student's *t* test, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$

C57BL6/J *hfe*−/− mice, respectively, as compared to WT controls (Supplementary Table 1–3). Interestingly, no gene was commonly dysregulated in all *hfe*−/− groups (Fig. 3). Comparisons between two groups either by strain or by iron plateau phase revealed that most differentially regulated genes were specific to each group, and few genes were commonly regulated (Fig. 3). The iron loading phase (4 weeks) and the iron plateau phase (8 week age) of AKR *hfe*−/− mice had only two genes commonly up-regulated, which were Zdhhc14 (zinc finger, DHHC domain containing 14) and Gabra6 (GABA-A receptor, subunit alpha6) (Table 1). AKR and C57 *hfe*−/− strains in the iron plateau phase shared one unidentified EST gene (Mm.396822) that was regulated in a similar fashion (Table 1). Eight-week-old AKR *hfe*−/− mice have higher hepatic iron concentrations than do the other *hfe*−/− mouse groups (8-week-old C57 *hfe*−/− and 4-week-old AKR *hfe*−/− mouse groups) but demonstrated differential regulation of only Nfatc1 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1) (Table 1). The lack of a common differential gene expression profile in the *hfe*−/− mouse groups demonstrates the profound influences of strain and age on the consequences of loss of Hfe.

Biological functional groups of cell survival, stress response, and lipid metabolism were disturbed in all *hfe*−/− mouse groups

We categorized the differentially expressed genes in each *hfe*−/− mouse group based on the known or predicted biological function of the protein product using Ingenuity Pathways Analysis. Functional groups of cell survival/growth, stress/damage response, and lipid metabolism were affected in all three *hfe*−/− mouse groups, representing 31, 38, and 25 % of the total number of genes identified in 4-week-old AKR, 8-week-old AKR, and 8-week-old C57 groups, respectively (Fig. 4). Although genes involved in biological processes were different among the *hfe*−/− mouse groups, cell survival/growth and stress/damage response pathways were up-regulated in *hfe*−/− mouse groups (Table 1). AKR *hfe*−/− mouse groups had common disturbances in a heme-related genes, representing 8 % of total genes in the iron loading phase (4 week age) group and 4 % of the total genes in the iron plateau phase (8 week age) (Fig. 5). Genes participating in cellular signaling were dysregulated in both iron plateau phases of AKR (4 % of the total) and C57 (3 % of the total) *hfe*−/− mouse groups (Fig. 5).

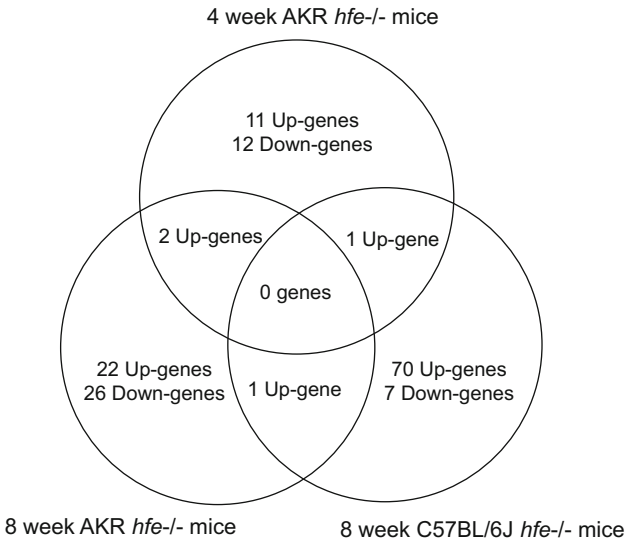

Fig. 3 Number of uniquely and commonly regulated genes in each group

Strains and iron loading phases affect different biological functional groups in *hfe*−/− mouse groups

Analysis of differences in iron loading status of AKR *hfe*−/− mouse groups revealed that groups of genes related to drug metabolism, immune response, and extracellular matrix are dysregulated specifically in the iron loading phase of the AKR *hfe*−/− mouse group. Dysregulation of genes in transport, fibrosis, and signaling is specific to the iron plateau phase of the AKR *hfe*−/− mouse group (Fig. 4). When the iron plateau phases of AKR and C57 *hfe*−/− groups were compared, the AKR strain-specific dysregulation was seen in genes in transport, fibrosis, and heme-related functions. Immune response, extracellular matrix, cell adhesion, vesicle trafficking, RNA splicing, metabolism, and calcium regulation were only dysregulated in the C57 strain (Fig. 4).

Discussion

There are considerable differences in clinical findings in patients with HH (Beutler et al. 2002; Olynyk et al. 1999); for example, body iron indices range widely from 253 to 9,530 μg/L of serum ferritin and 1,926 to 50,887 μg/g of liver iron (Adams et al. 2006a). Some HH patients develop liver fibrosis (Olynyk et al. 2005; Adams et al. 2006b), cirrhosis (Asberg et al. 2001), hepatic steatosis (Adams et al. 2006a), steatohepatitis (Adams et al. 2006a), and coronary heart disease (Rasmussen et al. 2001), while most do not. In addition, biochemical parameters for iron loading including serum ferritin and/or transferrin saturation are not always elevated in C282Y homozygotes (Asberg et al. 2001; Beutler et al. 2002; Olynyk et al. 1999). Different family members who were homozygous for C282Y mutations demonstrated different clinical outcomes, suggesting that the existence of modifier genes to influence manifestation of the disease phenotype of HFE (Bulaj et al. 2000). Other genetic factors could include another mutation in the HFE gene and/or mutations in genes known to be involved in iron metabolism causing non-HFE hemochromatosis (Whitfield et al. 2000; Lee et al. 2004; Roetto et al. 2003; Camaschella et al. 2000). The risk of iron loading was higher when C282Y and H63D mutations coexist in HFE gene as a compound heterozygote (Beutler 2006; Aranda et al. 2010). A gender effect on iron loading phenotypes in HFE mutations was noted with male gender having higher risks (Aranda et al. 2010). Epigenetic factors and environmental factors such as dietary iron and alcohol also could play a role in determining the incidence of the disease. Alcohol consumption increased serum ferritin

Table 1 Genes that are differentially regulated in more than one group

4-week AKR HFE KO	8-week AKR HFE KO	8-week C57 HFE KO	Accession number	Unigene ID	Gene symbol	Title
4/8[a] Up	4/8 Up		AI840033	Mm.399660	Zdhhc14	Zinc finger, DHHC domain containing 14
5/8 Up	4/8 Up		AI841957	Mm.4915	Gabra6	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6
4/8 Up		6/8 Up	AI854769	Mm.329560	Nfatc1	Nuclear factor of activated t cells cytoplasmic calcineurin-dependent 1
	5/8 Up	5/8 Up	AI840407	Mm.396822		Transcribed locus, close to ubiquitin specific peptidase 34
5/8 Up	5/8 Down		AI838762	Mm.288567	Hbb-b1	Hemoglobin, beta adult major chain
4/8 Up	6/8 Down		AI847573	Mm.21880	Htra2	HtrA serine peptidase 2
4/8 Down		4/8 Up	AI852324	Mm.397284		Transcribed locus, strongly similar to NP_035897.1 finger RNA binding protein

[a] The frequency of significant detection of a gene in total eight independent microarray experiments

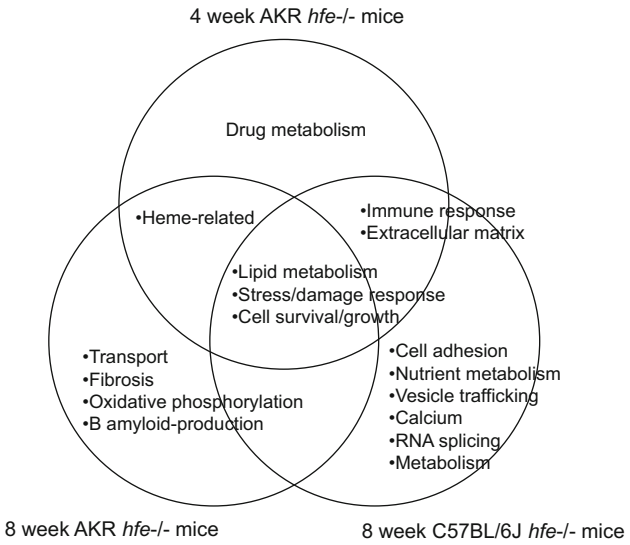

Fig. 4 Repartitioning of affected genes based on biological functions in *hfe*−/− mice groups

levels in C282Y homozygotes (Rossi et al. 2001; Scotet et al. 2003; Burke et al. 2001). An increase in dietary heme iron intake also adversely affected the iron status in people with HFE defects (Greenwood et al. 2005; Pedersen and Milman 2009; van der et al. 2006). On the contrary, fruit juice excluding citrus fruits was demonstrated to be protective in hindering serum ferritin levels (Milward et al. 2008). The presence of dietary calcium and fiber protected against iron loading (Aranda et al. 2010). Thus, environmental or genetic factors likely influence the development of HH-related clinical symptoms (Olynyk et al. 2005).

We demonstrated that regulation of hepatic gene expression is strongly influenced by mouse strain and iron loading phase (age) in *hfe*−/− mouse groups. The effect of strain differences was likewise noted in studies of duodenal gene expression in four mouse strains (Dupic et al. 2002). In AKR *hfe*−/− mice, inappropriate down-regulation of *hamp1* is consistent with the known role of hepcidin in regulating systemic iron metabolism. In C57 mice, hepcidin expression was not low, unless indexed to liver iron concentration. Inappropriately, low hepcidin relative to markers of iron status has also been reported in HH patients (Bridle et al. 2003; Piperno et al. 2007; van Dijk et al. 2008) and other *hfe*−/− mice models (Muckenthaler et al. 2003; Ahmad et al. 2002). Our results suggest that the milder phenotype of the C57 *hfe*−/− mice may be related to

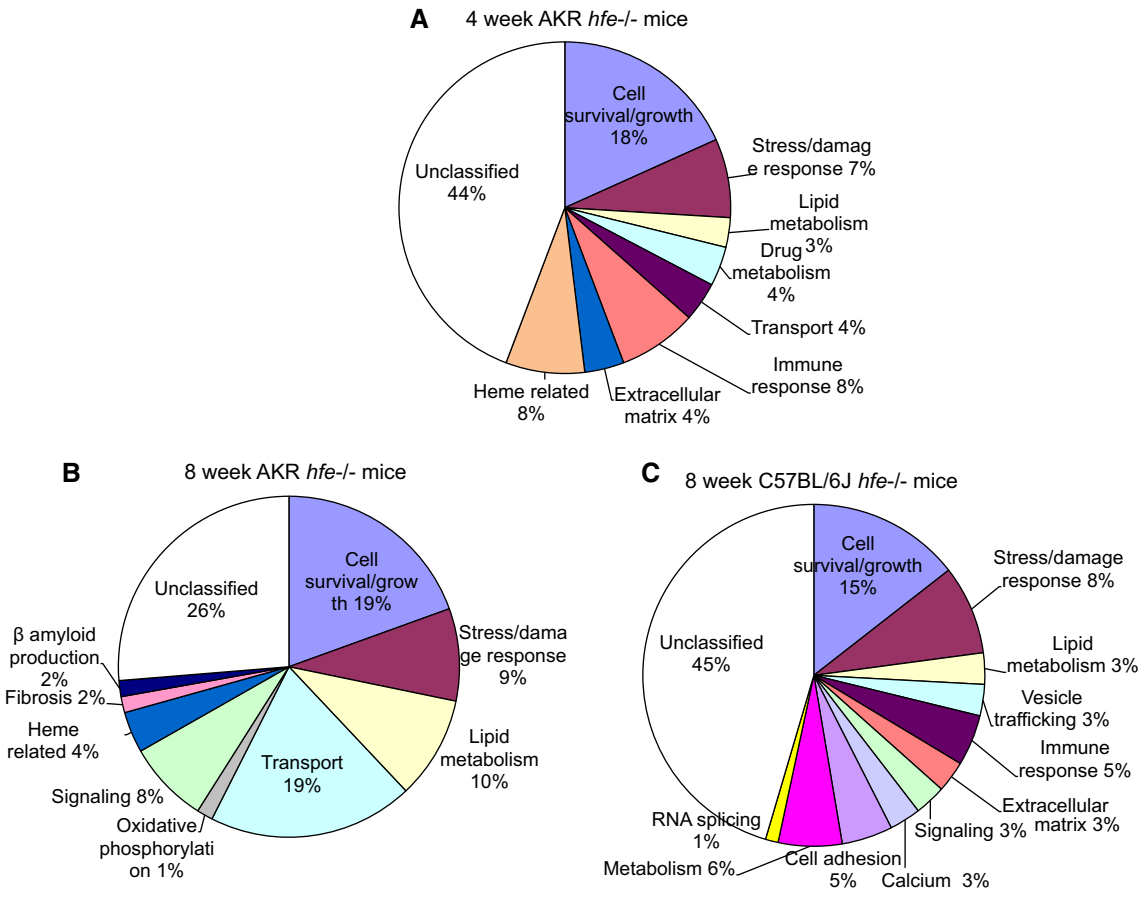

Fig. 5 Common biological processes between different *hfe*−/− mice groups

the relatively higher expression of hamp1 compared with AKR *hfe*−/− mice. Interestingly, however, the plateau phase observed in the AKR mice was not associated with up-regulation of liver hamp1 gene expression, and thus cannot be attributed to compensatory changes in signaling pathways regulating hepcidin. In the AKR mice, *tfr1* a gene known to be down-regulated by cellular iron, demonstrated suppressed expression compared with wild-type mice only during the "loading" phase. This observation raises the possibility that fluxes in cellular iron status have greater influence on expression of this gene than does cellular iron stores. The C57 mice and 4-week-old AKR mice demonstrated reduced mRNA expression of *Slc40a1*, which encodes ferroportin, a key iron export protein (Abboud and Haile 2000; Knutson et al. 2003). The strong posttranslational regulation of ferroportin makes it difficult to determine the significance of these findings.

Microarray analysis also revealed that only a few genes were found in common between *hfe*−/− mice with different genetic backgrounds and between different age groups. Genetic variation, the degree of iron loading, and age appear to strongly influence the differences in target genes affected by defective *hfe*. However, we may have filtered out genes with small changes in expression during data analysis. There is limited information on the biological function of the few genes with greatest commonality across the study groups. *Zdhhc14* and *Gabra6* were commonly up-regulated in 4- and 8-week-old AKR *hfe*−/− mice. The biological function of ZDHHC14 is not known, but has been annotated as a probable palmitoyltransferase. The GABA-A receptor is highly expressed in neurons, where it plays a key role in GABA-mediated responses, but is still expressed in other tissues including the liver. Differential expression of *Nfatc1* was commonly found in both 4-week-old AKR *hfe*−/− mice and 8-week-old C57 *hfe*−/− mice. NFATc1 is one of the several transcription factors that up-regulates proinflammatory cytokines and is involved in the development of fibrosis (Huang et al. 2002).

Although the lack of genes commonly affected in all *hfe* −/− mouse groups, biological functions related to cell survival, growth, and stress/damage response were commonly affected. These shared perturbations could contribute to the pathological conditions found in HH, such as hepatocellular carcinoma (Elmberg et al. 2003; Strohmeyer et al. 1988; Bradbear et al. 1985; Niederau et al. 1985). Our observation of up-regulation of cell proliferation genes in all *hfe*−/− mouse groups is in accordance with previous findings (Hann et al. 1990; Smith and Yeoh 1996; Pigeon et al. 1999). Increased cell growth was shown in an iron-treated human hepatoma cell line (Hann et al. 1990) and in animals grown on a carbonyl iron-supplemented diet (Smith and Yeoh 1996; Pigeon et al. 1999). This enhanced cell proliferation may play a role in the increased risk for hepatocellular carcinoma in patients with HH (Elmberg et al. 2003; Strohmeyer et al. 1988; Bradbear et al. 1985; Niederau et al. 1985). Up-regulation of genes in cell growth could be partly explained by cell damage caused by high hepatic iron content and the initiation of cell proliferation to compensate for the loss of damaged cells due to *Hfe* defects. Groups of genes in stress and damage response were altered in our study of *hfe*−/− mouse groups as compared to WT groups. These responses might be induced by high iron loading in *hfe*−/− mice, which can cause oxidative stress by reducing antioxidant levels and generating reactive oxygen species. Increased oxidative stress was reported in HH patients (Houglum et al. 1997) and in *hfe*−/− mice (Stevens et al. 2003).

Strain-specific differences in biological groups affected by *hfe*−/− could provide insight into the variable phenotypic manifestations of patients with HH. The iron plateau phase of AKR *hfe*−/− mice showed disturbed gene expression profiles in fibrosis, transport, oxidative phosphorylation, and β-amyloid production, whereas the iron plateau phase of C57 *hfe*−/−mice had differential expression of genes related to nutrient metabolism, cell adhesion, vesicle trafficking, RNA splicing, and calcium homeostasis. The effects of hepatic iron content on gene expression can be discerned by comparing different iron loading phases in *hfe*−/− mice. Unlike the iron plateau phase, the iron loading phase of AKR *hfe*−/− mice had dysregulated genes in drug metabolism, immune response, and extracellular matrix. Therefore, the differences in biological groups affected by differences in strains and/or iron loading status could be in part due to differences in hepatic iron content. Hepatic iron loading was greatest in 8-week-old C57BL/6J *hfe*−/− mice, followed by 4-week-old AKR/J *hfe* −/− mice, and was least in 8-week-old AKR/J *hfe*−/− mice. We suggest that the divergent phenotypes likely result from a complex interaction of the Hfe mutation with the different genetic backgrounds, consequent different rates of iron loading status, and the compensatory cellular response in the liver to the different levels of iron loading.

Acknowledgments This research was supported by an NIH Grant R01 DK095112.

Conflict of interest No conflict of interest.

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