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***In vivo* regulation of the heme oxygenase-1 gene in humanized transgenic mice**

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Abstract

Heme oxygenase-1 (HO-1) catalyzes the rate-limiting step in heme degradation producing equimolar amounts of carbon monoxide, iron, and biliverdin. Induction of HO-1 is a beneficial response to tissue injury in diverse animal models of diseases including acute kidney injury. *In vitro* analysis has shown that the human HO-1 gene is transcriptionally regulated by changes in chromatin conformation but whether such control occurs *in vivo* is not known. To enable such analysis, we generated transgenic mice, harboring an 87-kb bacterial artificial chromosome expressing human HO-1 mRNA and protein and bred these mice with HO-1 knockout mice to generate humanized BAC transgenic mice. This successfully rescued the phenotype of the knockout mice including reduced birth rates, tissue iron overload, splenomegaly, anemia, leukocytosis, dendritic cell abnormalities and survival after acute kidney injury induced by rhabdomyolysis or cisplatin nephrotoxicity. Transcription factors such as USF1/2, JunB, Sp1, and CTCF were found to associate with regulatory regions of the human HO-1 gene in the kidney following rhabdomyolysis. Chromosome Conformation Capture and CHIP-loop assays confirmed this in the formation of chromatin looping *in vivo*. Thus, these bacterial artificial chromosome humanized HO-1 mice are a valuable model to study the human HO-1 gene providing insight to the *in vivo* architecture of the gene in acute kidney injury and other diseases.

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The authors have nothing to disclose.

Introduction

Heme oxygenase (HO) is an enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism resulting in the production of carbon monoxide, iron, and biliverdin¹⁻³. Two isoforms of heme oxygenase have been characterized: an inducible enzyme, HO-1, and a constitutive isoform, HO-2. A third catalytically inactive isoform, HO-3, has been described but is suggested to be a pseudogene⁴. Besides its function in heme degradation and iron reutilization^{5,6}, HO-1 exhibits potent cytoprotective effects via anti-inflammatory, anti-apoptotic and anti-oxidant properties of its reaction products^{6,7}. Due to the beneficial roles of HO-1 in various disease states including renal injury from ischemia-reperfusion injury^{8,9}, glomerulonephritis^{10,11}, nephrotoxins¹², renal transplant rejection¹³⁻¹⁵, and rhabdomyolysis^{16,17}, a large number of studies have explored the mechanisms of HO-1 transcription, translation, and its multiple functions using *in vitro* and *in vivo* model systems. Among them, a global HO-1 knock out mouse (HO-1^{-/-}) has been used widely as a model for studying the functions of HO-1 *in vivo*^{5,18}. HO-1^{-/-} mice show several phenotypical alterations including reduced live birth rates, tissue iron deposition, splenomegaly, increased sensitivity to oxidative stress and acute kidney injury, anemia and leukocytosis^{5,18}.

There have been studies using mouse models overexpressing HO-1 generated with cDNA constructs¹⁹⁻²⁵. Organ-specific HO-1 overexpressing mice were generated to study protective functions of HO-1 in cardiac diseases²² and global HO-1 overexpressing rats have also been characterized²³. Gene delivery via viral vectors to transfer the HO-1 gene has been tested in animal models²⁶. However, despite the fact that the main function of HO-1, heme degradation and cytoprotection, is the same in both human and rodents, it is difficult to utilize these models for mechanistic analysis of the human HO-1 gene because of major differences between the molecular regulation of the mouse and human HO-1 genes²⁷. For instance, the human HO-1 gene contains a potential cadmium response element, which has been identified at -4.0 kb of the 5' flanking region of the HO-1 gene^{28,29}. However, the cadmium response element in the mouse HO-1 gene is immediately 3' to this region, and is a nuclear factor (erythroid-derived 2)-like 2 sequence³⁰. Heat shock and cytokines such as interferon gamma- γ and hypoxia are potent inducers of HO-1 in rodent cells, but repress HO-1 expression in human cells^{28,31,32}. Moreover, a 220-bp intronic enhancer that we recently identified in the human HO-1 gene^{33,34} is not present in the mouse HO-1 gene. Therefore, generating a relevant *in vivo* model is a fundamental prerequisite to study human HO-1 regulation and expression.

We have shown that multiple regulatory regions within the HO-1 gene interact with each other via chromatin looping and a transcription factor, specificity protein 1 (Sp1), mediates changes in chromatin conformation and initiation of human HO-1 transcription in renal epithelial cells *in vitro*³³. Both positive and negative regulatory sequences in the human HO-1 promoter that bind to specific transcription factors have been described to control HO-1 gene transcription *in vitro*. For example, JunB and upstream transcription factor (USF1/2) activate HO-1 gene expression while JunD acts as a repressor.^{35,36} In order to investigate these findings including changes in chromosome conformation and associated transcription factors *in vivo*, an animal model of the human HO-1 gene along with critical regulatory regions was developed. HO-1 BAC mice were used to generate "humanized"

HO-1 (hHO-1) BAC mice containing the human HO-1 gene but not the mouse HO-1 gene and studied in a rhabdomyolysis model of acute kidney injury *in vivo*.

Results

Generation of HO-1 BAC transgenic mice

HO-1 BAC transgenic mice were generated by microinjection of purified BAC DNA containing the human HO-1 gene (GenBank accession #: Z82244) depicted in Figure 1A into C57BL/6 oocytes. Two founders were obtained and genotyped by PCR using four primer pairs that amplify different regions within the BAC DNA (Table 1). As shown in Figure 1B, both founders carried the transgene and the copy number of the transgene was estimated by Taqman real-time PCR as described in the Methods. Estimated copy number of the two founders was 3 to 6. However, after the 3rd generation (F3), copy number of all transgenic mice was between 1 and 2. Southern blot analysis on tail genomic DNA digested with EcoRI and probed with a 320 bp fragment within the HO-1 gene, revealed a single band at 8 kb (Figure 1C). FISH analysis using HO-1 BAC as a probe showed a single integration site in peripheral blood (Figure 1D, right panel) and bone marrow cells (Figure 1E). Sequential metaphase FISH analysis localized the site of integration into chromosome 5 band qF (Figure 1E).

Characterization of HO-1 BAC transgenic mice

Monitoring body weight (Figure 2A) and blood pressure (data not shown) in the HO-1 BAC transgenic mice showed no significant differences compared to HO-1^{+/+} mice. Furthermore, no difference in terms of growth, development and survival were noted between HO-1 BAC compared to HO-1^{+/+} mice. Tissues from HO-1 BAC transgenic and HO-1^{+/+} mice were tested for HO-1 mRNA and protein expression. HO-1 mRNA levels were significantly higher (15–40 fold) in the heart, lung, liver, spleen, and kidney of the HO-1 BAC transgenic mice compared to the HO-1^{+/+} tissues (Figure 2B). HO enzyme activity in tissues from HO-1 BAC mice was also increased compared to HO-1^{+/+} mice (Figure 2C). In addition, HO-1 protein was overexpressed (2–3 fold higher) in aorta, brain, heart, lung, liver, spleen, intestine, skin, and kidney of HO-1 BAC transgenic mice compared to tissues from HO-1^{+/+} mice (Figure 2D). HO-2 levels in the HO-1 BAC mice were similar to HO-1^{+/+} mice (Figure 2D). These results indicate that HO-1 mRNA and protein are globally overexpressed in HO-1 BAC transgenic mice in the basal state and associated with increased HO enzyme activity indicating that the overexpressed HO-1 protein in HO-1 BAC transgenic mice was functionally active.

To examine the localization of HO-1 expression in HO-1 BAC transgenic mice, immunohistochemistry was performed (Figure 2E). As expected, higher levels of HO-1 expression were observed in tissues (brain, heart, lung, liver, spleen, and kidney) of HO-1 BAC transgenic mice compared to HO-1^{+/+} mice. The expected high basal HO-1 expression in the spleen was even higher in the HO-1 BAC transgenic mice. The staining pattern in the kidney showed the presence of HO-1 predominantly in renal proximal tubules, which was confirmed by co-localization with lotus lectin (Figure 2F).

Generation and characterization of humanized HO-1 BAC transgenic mice

While the HO-1 BAC mice showed global overexpression of HO-1, the endogenous mouse HO-1 gene is still intact in these mice. To overcome this limitation, HO-1 BAC mice were bred with HO-1^{-/-} mice. Due to low number of live births from female HO-1^{-/-} mice, we initially crossed female HO-1 BAC mice with male HO-1^{-/-} mice to obtain HO-1^{+/-} mice, which would carry the human HO-1 BAC transgene (BAC positive and HO-1^{+/-}). Then these HO-1^{+/-}-BAC positive mice were crossed with each other to obtain HO-1^{-/-} mice expressing only the human HO-1 gene (BAC positive and HO-1^{-/-}). The initial “humanized” HO-1 BAC (hHO-1 BAC) transgenic mice were genotyped by using two primers that were used for genotyping HO-1 BAC transgenic mice (Primer a and b, Table 1) and mouse HO-1 primers to confirm that the transgenic mice are indeed both BAC positive and mouse HO-1 negative (Figure 3A). Among five transgenic mice, three were HO-1 BAC positive (hHO-1 BAC #101, 104, and 105) and three were mouse specific HO-1^{-/-} for the mouse gene (hHO-1 BAC #103, 104, 105). Thus, hHO-1 BAC #104 and #105 were determined to be both mouse specific HO-1^{-/-} and HO-1 BAC positive and referred to as “humanized” HO-1 BAC mice (hHO-1 BAC).

Using species-specific primers as listed in Table 1 further generations were genotyped to confirm the presence of human and absence of mouse HO-1 in hHO-1 BAC mice (Figure 3B). Next, the basal level of HO-1 protein expression in HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice were examined in microsomal fractions from the kidney and spleen (Figure 3C). As illustrated, HO-1 protein expression was significantly higher in both the kidney and spleen of hHO-1 BAC mice compared to HO-1^{+/+} mice and as expected no HO-1 expression was detected in the HO-1^{-/-} samples. No significant differences were noted between HO-1 and HO-2 protein levels in the liver, lung, kidney and spleen isolated from the HO-1 BAC and hHO-1 BAC (Supplemental Figure 1).

Human HO-1 in hHO-1 BAC transgenic mice rescues the phenotype of HO-1^{-/-} mice

Since hHO-1 BAC transgenic mice express human HO-1 in the HO-1^{-/-} mice background, it is important to determine if the integrated human HO-1 gene could rescue the phenotype of HO-1^{-/-} mice. The fertility and live birth rate was notably increased when we crossed hHO-1 BAC mice compared with HO-1^{-/-} breeding pairs from which no litters were obtained from at least 10 to 12 breeding pairs. Spleens from age-matched HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC transgenic mice were compared (Figure 4A and B). All HO-1^{-/-} mice had enlarged spleens with average length of 2.3±0.4 cm. On the contrary, spleens from hHO-1 BAC mice were within the same range of length as the HO-1^{+/+} mice (1.3±0.2 and 1.2±0.3 cm, respectively). Splenic weights were significantly higher in HO-1^{-/-} mice, but this was also reversed in hHO-1 BAC mice (Figure 4B).

Decreased hemoglobin levels, leukocytosis and reticulocytosis noted in HO-1^{-/-} mice were reversed in hHO-1 BAC mice, and were similar to HO-1^{+/+} mice (Figure 4C). Additionally, the hHO-1 BAC hematocrit was restored to normal levels (HO-1^{+/+}: 44±0.7, HO-1^{-/-}: 32.5±2.5 and hHO-1 BAC: 41±0.5%, $p < 0.05$ for HO-1^{+/+} and hHO-1 BAC vs HO-1^{-/-}). Kidneys of HO-1^{-/-} mice showed increased iron deposition as previously described^{5,37}, whereas iron was barely detected in tissues from HO-1^{+/+} and hHO-1 BAC transgenic mice

(Figure 4D). When compared to peripheral smears of HO-1^{+/+} mice, HO-1^{-/-} mice showed hypochromic microcytic anemia with considerable anisocytosis and fragmented RBC (Figure 4E). However, the smears of hHO-1 BAC transgenic mice showed near normal restoration of RBC morphology. Interestingly, RBCs from HO-1^{-/-} and hHO-1 BAC mice displayed inclusions that mimicked Howell-Jolly bodies. These were also detected in peripheral smears of HO-1 BAC transgenic mice. The number of these inclusions in the peripheral smears of hHO-1 BAC mice was 1.54 per 100 RBC compared to 0.1% in HO-1^{+/+} mice and 0.4% in HO-1^{-/-} mice.

We have recently reported that the proportion of splenic CD8⁺ DCs was significantly decreased in HO-1^{-/-} mice³⁸. To determine whether the human HO-1 gene product in hHO-1 BAC mice could alter this phenotype, flow cytometry analysis of splenocytes from HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice was performed. CD8⁺ DCs in HO-1^{-/-} mice were significantly decreased compared to HO-1^{+/+} mice, but splenocytes in hHO-1 BAC mice showed similar levels as HO-1^{+/+} mice (Figure 4F and G).

Induction of rhabdomyolysis and cisplatin-induced kidney injury in hHO-1 BAC transgenic mice

To examine the response of HO-1^{+/+} and hHO-1 BAC transgenic mice in heme-mediated renal injury, a model of glycerol-induced rhabdomyolysis was utilized as described in the Methods. The HO-1 mRNA expression was increased ~23 fold at 4 hours (data not shown) and ~30 fold at 8 hours following glycerol injection in HO-1^{+/+} kidneys (Figure 5A). In contrast, hHO-1 BAC kidneys revealed ~5 and ~14 fold increase in HO-1 mRNA expression at 4 (data not shown) and 8 hours, respectively, post glycerol administration (Figure 5B). HO-1 protein and HO enzyme activity showed higher basal levels in the hHO-1 BAC kidneys compared to the HO-1^{+/+} mice and this increase was even higher following glycerol-induced rhabdomyolysis at 16 h (Figure 5C and D). Survival rate and serum creatinine level of each group of mice were monitored to determine the protective effects of the human HO-1 gene in hHO-1 BAC mice after glycerol-induced rhabdomyolysis. All HO-1^{-/-} mice died within 4 days after glycerol injection with markedly increased serum creatinine. 90% of HO-1^{+/+} mice and 100% of hHO-1 BAC mice survived for 10 days post injection without significant changes in serum creatinine levels suggesting that the human HO-1 gene and product in hHO-1 BAC mice remains functional (Figure 5E and F).

To further examine the response of HO-1^{+/+} and hHO-1 BAC transgenic mice in a model of non-heme-mediated renal injury, we tested the effects of cisplatin nephrotoxicity in these mice. We have previously reported that HO-1 is induced in this model and is a protective response (12). As expected HO-1 mRNA, protein and enzyme activity were increased in HO-1^{+/+} mice after cisplatin administration (Supplemental Figure 2A, C and D). hHO-1 BAC displayed significant increases in HO-1 mRNA (~3.5 fold at 6 hours)(Supplemental Figure 2B), HO-1 protein (~2.2 fold at 12 h)(Supplemental Figure 2C), and HO enzyme activity (~2.2 fold at 24 h)(Supplemental Figure 2E), compared to basal levels. These data demonstrate that even though basal levels of HO-1 are elevated in the hHO-1 BAC mice, HO-1 is significantly inducible in another disease setting (e.g. cisplatin nephrotoxicity), consistent with the rhabdomyolysis model. In addition, renal function measured by serum

creatinine was markedly preserved in the hHO-1 BAC mice compared to HO-1^{+/+} mice following cisplatin administration (Supplemental Figure 2F).

Molecular mechanisms of the human HO-1 transcription *in vivo*

We have previously shown the importance of USF1/2 and Sp1 in regulating hemin-mediated HO-1 expression in renal epithelial cells *in vitro*^{33,35,36}. To examine the *in vivo* relevance of the *in vitro* findings, we performed ChIP assay to determine the association of the indicated transcription factors with regulatory regions in the HO-1 gene in kidneys of the hHO-1 BAC transgenic mice subjected to rhabdomyolysis (Figure 6). Upon induction of HO-1, JunB, Sp1, USF1, and USF2 were associated with the -4.5kb promoter region that contains the DNase I hypersensitive site, HS-2³⁵. However, JunD did not show any association to the promoter region (Figure 6A). For the 220-bp intronic enhancer region maximal binding was observed with Sp1, to a lesser extent with JunB, USF1 and USF2 and no binding with JunD (Figure 6B). The +2.2 kb region of the human HO-1 gene was used as a negative control and none of the tested transcription factors were associated with this region (Figure 6C).

CCCTC-binding factor (CTCF) is a multi-functional transcription factor that is a master controller of several genes by regulating activation, repression, insulation, and silencing³⁹⁻⁴⁴. Interestingly, one of major mechanisms of CTCF-mediated transcriptional regulation is the formation of chromatin looping^{41,45}. We tested if CTCF was bound to various regulatory regions in the kidneys of hHO-1 BAC mice following rhabdomyolysis. ChIP assay showed that CTCF associates with the proximal E-box, -4.5kb promoter, and 220bp intronic enhancer but not the +2.2kb region upon induction of the human HO-1 gene *in vivo* (Figure 6D).

***In vivo* chromatin looping occurs in hHO-1 BAC mice for induction of human HO-1 gene transcription**

Chromatin looping was studied in hHO-1 BAC mice using the 3C assay upon induction of HO-1 transcription after glycerol-induced rhabdomyolysis. Mice were injected with 50% glycerol or saline (negative control) and kidneys were removed after 3 h for the 3C assay. BglII restriction enzyme was used to determine the interaction of fragments containing the 220-bp enhancer and the -4.5 kb promoter as described in the map in Figure 6E. As shown in Figure 6F, a 7.5-fold increase of crosslinking efficiency was detected between the enhancer containing fragment with the -4.5 kb region after induction of rhabdomyolysis compared to saline-injected mice. These results demonstrate the pivotal role of USF1, CTCF, and Sp1 in regulating HO-1 gene expression *in vivo* via chromatin looping.

USF1/2, Sp1 and CTCF regulate chromatin loop formation

Overexpression of USF1 and/or USF2 as well as Sp1 induced endogenous HO-1 protein and mRNA *in vitro*. In addition, hemin-induction of HO-1, resulted in increased endogenous Sp1 expression but the levels of USF-1 and USF-2 were unchanged. ChIP assays showed that USF1 associated with the -4.5 kb HS-2, proximal E-box and 220-bp intronic enhancer regions upon induction of HO-1 by hemin (Supplemental Figure 3). 3C assay demonstrated that USF1 overexpression facilitated chromatin loop formation between the -4.5kb promoter region (HS-2) and the 220-bp intronic enhancer (Supplemental Figure 4A). Similarly, the

proximal E-box interacted with the -4.5kb promoter via chromatin looping (Supplemental Figure 4B). Further confirmation of chromatin loop formation was achieved using the ChIP-loop assay⁴⁶⁻⁴⁸. Cells were treated with 5μM hemin for 3 h to induce HO-1 transcription and ChIP-loop assay was performed using the BglIII restriction enzyme along with anti-Sp1 and anti-USF-1 antibodies to precipitate crosslinked DNA-protein complex. We demonstrate that for the interaction between the -4.5kb promoter (HS-2) and the 220-bp intronic enhancer, Sp1 and USF-1 are involved in the process of chromatin looping and subsequent HO-1 transcription (Supplemental Figure 4C).

In order to determine if CTCF is involved in HO-1 transcription and related with other transcription factors, we performed ChIP assay using anti-CTCF antibody for the proximal E-box, -4.5kb promoter, and 220-bp intronic enhancer regions. CTCF bound to all three regions tested upon induction of HO-1 by hemin treatment (Supplemental Figure 5A). Hemin-mediated HO-1 induction caused the binding of RNA PolIII to proximal E-box, -4.5kb promoter, and 220-bp intronic enhancer region, but no association was detectable with the +2.2 kb region that also contains an E-box sequence (Supplemental Figure 5A and 5B).

Discussion

In the present study, we report a novel *in vivo* model for studying the functional and regulatory mechanisms of the human HO-1 gene via the generation of “humanized” HO-1 (hHO-1) BAC transgenic mice. The integration of human HO-1 successfully rescued the phenotype of the HO-1^{-/-} mice demonstrating the integrity and functional role of the human HO-1 transgene and its gene product. Furthermore, the results of the current studies using rhabdomyolysis induced kidney injury in hHO-1 BAC mice demonstrate the complex changes in chromatin conformation and loop formation *in vivo*, providing important insights to the molecular regulation of human HO-1 gene expression. The studies have also identified for the first time, the involvement of CTCF, a master regulator in HO-1 gene expression *in vitro* and *in vivo*.

HO-1 mitigates cellular injury by exerting anti-oxidant, anti-apoptotic, and anti-inflammatory effects^{31,49-51}. Due to the beneficial and protective effects of HO-1 in several pathological states, developing an appropriate *in vivo* model for the study of the molecular mechanism of HO-1 expression is imperative. Unlike other currently available *in vivo* models to study the biological aspects of human HO-1, HO-1 BAC transgenic mice can be used to study the intricate molecular mechanisms that control human HO-1 gene transcription. HO-1 BAC mice showed global HO-1 mRNA and protein overexpression in multiple tissues. Interestingly, the pattern of expression closely mimicked the sites where HO-1 would otherwise be inducible following tissue injury. In the kidney, HO-1 expression was seen in the proximal tubules, a site where HO-1 is induced in models of acute kidney injury⁵². In the liver, HO-1 expression was detected in Kupffer cells. Consistent with this observation, is the finding that Kupffer cell depleted mice fail to show resection-induced increase of HO-1 suggesting that the major hepatic cell type which accounts for HO-1 expression in liver is the Kupffer cell^{53,54}. In the brain, astrocytes were the predominant cell type with increased HO-1 expression in the HO-1 BAC mice. Hypoxia-induced HO-1

expression in the brain results in increased HO-1 levels in astrocytes, but not in the microglia⁵⁵. Our results also demonstrate that increases in HO enzyme activity confirm the global overexpression and active nature of the inserted HO-1 gene in the BAC mice. In addition, the body weight and life span of HO-1 BAC mice and HO-1^{+/+} did not show any major differences, indicating that the integrated human HO-1 gene did not cause any significant physiological derangements in the transgenic mice.

More importantly, we demonstrate that the inserted human HO-1 gene is functional and rescues the pathological phenotype of the HO-1^{-/-} mice. HO-1^{-/-} mice have reduced live births and partial embryonic lethality, which were reversed in the hHO-1 BAC mice. Splenomegaly, anemia, dendritic cell abnormalities, and tissue iron deposition seen in HO-1^{-/-} mice were normalized in the hHO-1 BAC mice. HO-1^{-/-} mice are highly sensitive to rhabdomyolysis-induced acute kidney injury¹⁷ and hHO-1 BAC mice showed markedly improved survival and preserved renal function. hHO-1 BAC mice were also protected in a model of cisplatin-induced nephrotoxicity. Interestingly, RBC of the HO-1 BAC and the hHO-1 BAC mice contained increased number of inclusions that appeared similar to Howell-Jolly bodies. Despite the fact that Howell-Jolly bodies are generally considered a hallmark of asplenia, the physical appearance of the spleens of hHO-1 BAC mice as well as their weights were not significantly different from the HO-1^{+/+} spleens. This observation is intriguing and deserves further investigation.

Our studies using hHO-1 BAC mice support previous *in vitro* evidence for chromatin looping as a mechanism for regulation of the human HO-1 gene. Transcription factors, Sp1, USF1/2, and JunB, were identified as positive regulators for human HO-1 expression and our results indicate that they are associated with the same regulatory regions and involved in chromatin looping. These studies have also identified a pivotal role for the transcription factor, CTCF, in the regulation of human HO-1 gene expression. Sp1 is a transcription factor that plays an important role in regulation of a number of genes. It has a zinc finger protein motif, by which it may bind to DNA enhancing the transcription of the specific gene. In contrast, CTCF belongs to a family of proteins known as chromatin insulators. These proteins are suggested to form domains within which specific enhancer-gene interactions may occur. Mounting evidence suggests that CTCF may bind to distant sites or even different chromosomes, thereby allowing chromatin looping and transcriptional regulation.

“Humanized” HO-1 BAC transgenic mice are an invaluable *in vivo* model for studying not only the function but also the regulation of the human HO-1 gene. hHO-1 BAC mice can be used to better understand how the human HO-1 gene is induced and protects against various disease states such as acute kidney injury, atherosclerosis, sepsis, vascular restenosis, organ transplantation as well as others. Moreover, for the study of transcriptional regulation of human HO-1 gene, the hHO-1 BAC mouse containing the complete human HO-1 gene with all its regulatory elements would be a prerequisite for better elucidation of the mechanisms underlying HO-1 gene expression. Additionally, the absence of the mouse HO-1 gene in hHO-1 BAC mice removes any confounding effects from endogenous HO-1, allowing all outcomes to be attributed to the human HO-1 gene. It is important to note that the humanized HO-1 mouse described herein depends on transcription factors that are of murine origin. However, significant (>90%) homology exists between the transcription factors of

the two species and obviates this limitation. Another advantage of the humanized transgenic mouse model is the potential for testing of novel small molecular agents that could be evaluated in future therapeutic applications.

On the other hand, we acknowledge that there are certain limitations of this mouse model. Most importantly, the basal levels of HO-1 are significantly higher in hBAC mice compared to HO-1^{+/+} mice. This may be due to the insertion of the BAC DNA into a euchromatin region. Another possible explanation is the absence of human specific inhibitory regulators (e.g. micro-RNAs) in the mouse environment. These are currently being investigated in our laboratory. However, our results show that HO-1 mRNA is significantly induced (~5-fold and 14-fold at 4 and 8 hours, respectively) in hBAC mice in the rhabdomyolysis model. Moreover, HO-1 protein levels and HO enzyme activity are significantly higher (~3.5 fold and ~8 fold, respectively) than control levels in the glycerol model (Fig. 5D). Inducibility of HO-1 mRNA, protein and enzyme activity was also confirmed in cisplatin-induced nephrotoxicity, a non-heme dependent model of acute kidney injury (Supplemental Figure 2). These results indicate that although in the basal state hBAC mice express a higher level of HO-1, there is still capacity for significant inducibility and thereby allowing for investigation of the human HO-1 gene in pathological conditions.

In summary, humanized HO-1 BAC transgenic mice are able to rescue the phenotype of HO-1^{-/-} mice and transcription factors including JunB, Sp1, USF1/2, and CTCF regulate transcription of the human HO-1 gene via chromatin looping *in vivo*. The hHO-1 BAC transgenic mice can be utilized as an *in vivo* model to better elucidate the functional significance and molecular regulation of the human HO-1 gene.

Methods

Reagents

Tissue culture media and supplements were obtained from Mediatech Inc. (Manassas, VA). Rabbit or mouse polyclonal antibodies against USF1 (SC-229), USF2 (SC-862), Sp1 (SC-14027), JunB (SC-8051), and JunD (SC-74) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti HO-1 (SPA-895) and HO-2 (SPA-897) antibody was from Stressgen (Plymouth Meeting, PA). Restriction endonucleases for chromosome conformation capture were obtained from New England Biolabs (Beverly, MA). LipofectamineTM for transient transfection was purchased from Invitrogen (Carlsbad, CA).

Cell culture, plasmids, and transfection

Human embryonic kidney 293 (HEK293) cells (A.T.C.C., Manassas, VA) were grown in Dulbecco's modified Eagle's medium/F12 medium (Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 1.5 mg/ml sodium bicarbonate. Cells were grown at 37°C in 95% air and 5% CO₂.

The plasmid constructs for USF1 (psvUSF1), USF2 (psvUSF2), and pRc/CMV566 were gifts from Dr. M. Sawadogo (University of Texas at Houston, Houston, TX). The dominant-negative expression vector for USF, hemagglutinin-tagged A-USF, was kindly provided by

Dr. C. Vinson (National Cancer Institute, National Institutes of Health, Bethesda, MD). The Sp1 expression vector was kindly provided by Kun-Sang Chang (University of Texas M.D. Anderson Cancer Center) and has been described elsewhere⁵⁶.

HEK293 cells at a confluence of 60–70% were grown in 10 cm tissue culture dishes and were transiently transfected with equimolar amounts of plasmids using Lipofectamine™ 2000 (Invitrogen) according to manufacturer's instructions. Transfected cells were allowed to recuperate for indicated time periods before harvesting.

Generation of HO-1 BAC transgenic mice

The HO-1 BAC clone (GenBank accession #: Z82244) (87,869 bp) containing the human HO-1 gene along with the 3' end of the TOM1 gene and the MCM5 gene was obtained from Sanger Institute (Cambridge, UK). The HO-1 BAC was purified for injection using the QIAGEN® Large-construct kit (QIAGEN Inc., Valencia, CA). The BAC was quantified using agarose gel electrophoresis, and sets of 200 C57BL6/J oocytes were microinjected with 30 ng of the purified BAC DNA at the UAB Transgenic Mouse Core facility. Fertilized ova were subsequently implanted into pseudo-pregnant females and offspring were analyzed for the insertion of the HO-1 BAC. Two independent founder lines were produced that transmitted the transgene and genotyped with DNA extracted from tails, followed by subsequent PCR amplification of four different regions within the HO-1 BAC (Figure 1A) using primers shown in Table 1. Integration of HO-1 BAC was demonstrated by Southern blot analysis after digestion of tail DNA with EcoRI and hybridization with the probe generated from PCR amplicon (primer pair b), which resulted in a single band at 8 kb (Figure 1C). The inserted BAC DNA contains the entire human HO-1 gene along with all of its regulatory regions. Hence, all experiments that involved hHO-1 BAC mice examine the full-length human HO-1 gene. All animal studies were approved by our Institutional Animal Care and Use Committee.

Copy number estimation of transgenic mice

Copy number estimates were derived from Ct values for standard curve samples that mimic the genetic integration of the transgene. Serially diluted purified HO-1 BAC DNA were mixed with 200 ng of mouse genomic DNA and used as standards. In order to calculate Ct values, the average of duplicate Ct values generated with the HO-1 BAC probe (Table 1) was subtracted from the average Ct value of mouse prolactin gene (*Prl*), which represents 2 copies. Then, Ct values from genomic DNA of transgenic mice were calculated using BAC and *Prl* probes as in standard samples. Ct values were calculated by comparing two Ct values and copy number was calculated.

Conventional Cytogenetic and Fluorescence *in situ* hybridization (FISH) analyses

The HO-1 BAC was labeled in SpectrumOrange using the Abbott nick translation kit (Abbott Molecular, Des Plaines, IL) and used as a FISH probe. The probe was validated by metaphase FISH analysis on 20 human metaphase peripheral blood cells to confirm that it hybridizes to chromosome 22 band q12.3 (HMOX1 gene) with no cross-hybridization elsewhere in the human genome (Figure 1D, left panel). Interphase FISH analyses were then performed on peripheral blood cells from both the wild type (non-transgenic) (Figure 1D,

middle panel) and transgenic mice using the HO-1 probe (Figure 1D, right panel). Slide hybridization and washes were performed using standard FISH protocols. The slides were then counterstained with DAPI and analyzed with an Olympus BX61 microscope (Olympus America, Inc., Melville, NY) equipped with the appropriate filter combination and a CCD camera, and coupled to the CytoVision image analysis system (Applied Imaging, Santa Clara, CA). One hundred interphase cells were analyzed from each mouse. G-banded chromosome analysis was performed on metaphase spreads prepared from unstimulated bone marrow cell cultures from the transgenic mice using standard techniques. The chromosomes were analyzed and karyotyped using the CytoVision image analysis system (Applied Imaging, Santa Clara, CA)

Real-time PCR

Total RNA was isolated from cells or tissues by TRIzol (Invitrogen), and SYBR Green-based real-time PCR was performed on cDNA first strand synthesis products generated from total RNA (Invitrogen). The reactions were performed in triplicate. Relative mRNA expression was quantified using the Ct method and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Real-time primers are described in Table 1. For copy number estimation of transgenic mice, custom TaqMan probe for BAC and prolactin (*Prl*) gene (control) were used. The primers for BAC were same as the primers used for genotyping of transgenic mice (BAC b, c, and d. Table 1) and sequences of *Prl* primers and probes are listed in Table 1.

Western blot analysis

Harvested cells or collected tissues were lysed in RIPA buffer (50 mM Tris/HCl, 1% NP-40, 0.25% deoxycholic acid, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate and 1 mM sodium fluoride) with protease inhibitor (Roche Applied Science, Indianapolis, IN) and quantified using BCA protein assay (Thermo Scientific, Rockford, IL). Total protein (10–15 µg) was resolved on a 12% Tris-glycine SDS-PAGE gel. After transfer to a polyvinylidene fluoride membrane (Millipore, Billerica, MA), HO-1, HO-2, and actin were detected using 1:5000 dilutions of primary rabbit polyclonal antibodies, followed by a horseradish peroxidase-conjugated anti-rabbit IgG. The antibody used to detect HO-1 is a polyclonal antibody that recognizes HO-1 of both species with the same intensity. Protein was visualized using the enhanced chemiluminescence detection system (GE Healthcare)

Measurement of HO enzyme activity

Tissue was homogenized in 3 ml of buffer (200 mM KH₂PO₄, 135 mM KCl, 0.1 mM EDTA, pH 7.4), and then sonicated 3 times for 5 sec. at 4°C. The samples were centrifuged (10,000 × g, 4°C, 20 min). The supernatant was transferred to ultracentrifuge tubes, followed by an ultracentrifugation (100,000 × g, 4°C, 60 min, Beckman L7-35 Ultracentrifuge, 70 Ti rotor). The microsomal pellet was resuspended in 320 µl of HO enzyme activity buffer (100mM KH₂PO₄, 2 mM MgCl₂, pH 7.4). After sonication (3 times for 5 sec each at 4°C), the supernatant was used to determine HO enzyme activity in a reaction mixture containing rat liver cytosol (as a source of biliverdin reductase, 2 mg/assay), hemin (20 µM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase

(0.2 U), and NADPH (0.8 mM) in a total volume of 400 μ l. After the reaction was incubated at 37°C for 60 min in the dark, the formed bilirubin was extracted with 1 ml of chloroform by vigorous vortexing 3 times for 10 sec. After centrifugation (1,000 \times g, 5 min, room temperature), optical density at 464 nm and 530 nm of the organic phase was determined and HO enzyme activity was calculated and expressed as fold increase versus controls.

Immunohistochemistry

Tissues were removed and fixed in 10% formalin for 24 h and dehydrated in 70% ethanol before embedding in paraffin. Paraffin-embedded tissues were cut into 4 μ m sections then deparaffinized and rehydrated using Citrisolv (d-limonene based solvent) and isopropanol, respectively. Retrieval of antigen for anti-HO-1 was performed by steaming in Trilogy solution. Sections were blocked with 5% normal goat serum for 20 min then incubated for 16 h at 4°C with polyclonal rabbit anti-HO-1 antibody (SPA-896, Stressgen) diluted 1:1000 in PBS with 0.1% Tween-20. Peroxidase-conjugated goat anti-rabbit antibody was applied for 1 h at room temperature, followed by PBS washes. VIP substrate kit (Vector Laboratories, Inc.) was prepared following the manufacturer's protocol and applied for 3 min. Tissues were dehydrated using ethanol and xylene and mounted. For the study of HO-1 expression pattern in kidney, serial paraffin-embedded tissue sections were incubated with biotinylated lotus lectin (Vector Laboratories Inc.) or anti-HO-1 as described earlier⁵⁷. Images were captured using a Leica DM IRB microscope (Leica Microsystems Inc., Bannockburn, IL) and Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD).

Splenic DC preparation

Spleens from HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice were harvested, cut into small fragments, minced, and digested with 100 U/ml collagenase D (Roche Diagnostics, Indianapolis, IN)-containing Dulbecco's PBS (Invitrogen, Grand Island, NY) solution and incubated for 20 min at room temperature. EDTA was added (final concentration, 0.1 M) to disrupt DC-T cell complexes and incubated for 5 min. Cells were filtered through a 70- μ m stainless still sieve (BD Falcon; BD Biosciences, Bedford, MA) for removing undigested fibrogenous materials and centrifuged at 300 \times g for 10 min. Supernatants were removed, and red blood cells were lysed using buffered ammonium chloride lysis solution. After cells were washed in PBS, they were used for experiments.

Flow Cytometry

Cells were washed once with cold PBS and were incubated for 10 min. at room temperature with anti-mouse CD16/32 (eBioscience, San Diego, CA) to block nonspecific binding to FC γ 3 receptors. Cells were initially stained with fluorescein isothiocyanate-conjugated anti-mouse CD86 (clone GL-1), phycoerythrin -conjugated anti-mouse CD19 (clone 1D3), anti-mouse CD11c biotin (clone HL3), and APC-conjugated anti-mouse major histocompatibility complex class II (MHC II) (clone M5/114.15.2) for 30 min on ice. Cells were washed and stained with peridinin chlorophyll protein complex -conjugated anti-mouse streptavidin for additional 30 min on ice. PE-, fluorescein isothiocyanate-conjugated, and APC-conjugated isotype matched Abs of irrelevant specificity were used as controls. DC subsets were identified by incubation with fluorescein isothiocyanate-conjugated, PE-conjugated, peridinin chlorophyll protein complex -conjugated and APC-conjugated mAb specific for CD11c,

CD4, CD8, and MHC II, respectively. Data acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences), and results were analyzed using Winlist Software (Verity Software House, Topsham, MA)

Hematological Assays

Peripheral blood smears were prepared using a drop of blood from the mouse tail. Slides were submerged in methanol for 1 min followed by Wright-Giemsa Fuccillo stain (Harleco, Lawrence, KA) for 5 min as described⁵⁸. Then slides were transferred into 300 ml of the Giemsa solution containing 80 ml Wright-Giemsa stain, 50 ml phosphate buffer, pH 6.8, and 170 ml distilled water for 10 min. Slides were washed in phosphate buffer (pH 6.8) for 3 min followed by deionized water for 3 min. Then slides were dried at room temperature and covered with cover slips using mounting medium xylene (Fisher Scientific, Pittsburgh, PA)

For other hematologic indices, peripheral blood was collected from anesthetized mice into Microtainer® EDTA collection tubes (BD Biosciences, Franklin Lakes, NJ). Hemoglobin concentrations were determined after conversion to cyanmethemoglobin by lysing red blood cells (RBCs) in Drabkin reagent (Sigma-Aldrich, St. Louis, MO), removing insoluble RBC membranes by centrifugation, measuring the absorbance at 540 nm on a spectrophotometer, and comparing with hemoglobin standards. Reticulocyte counts were determined by flow cytometry after staining with thiazole orange as described elsewhere⁵⁹.

Chromosome Conformation Capture (3C)

3C assay was performed according to the method described earlier^{60,61}. Briefly, transiently transfected HEK293 cells were recovered for 24 h. Cells were washed with PBS twice and 2% formaldehyde was added to the cells for 10 min at room temperature for crosslinking. For *in vivo* 3C assay, kidneys from hHO-1 BAC transgenic mice injected with saline (control) or 50% glycerol to induce rhabdomyolysis at 3 h post-injection were collected and diced into small pieces (between 1–3 mm³). 1% formaldehyde was added to the tissue for 20 min at room temperature for crosslinking. The crosslinking was terminated by adding 2.5 M glycine to a final concentration of 0.125 M for 10 min at 4°C and then samples were centrifuged at 100 × g for 2 min. After washing, cells or tissues were disaggregated by using Dounce homogenizer. Cross-linked cells were washed with cold PBS and lysed for 1 h with ice-cold lysis buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl, 0.2% NP-40, and 1mM dithiothreitol. The nuclei of the cells were then harvested and suspended in the appropriate restriction enzyme buffer containing 0.3% SDS and incubated at 37°C for 1 h with gentle shaking. SDS was then sequestered from the samples by the addition of 1.8% Triton X-100 (Fisher Scientific) and the samples were incubated at 37°C for 1 h. The samples were digested with either *BglII* or *SphI* restriction enzyme (New England Biolabs) at 37°C for 16 h. Restriction enzymes were inactivated by the addition of 1.6% SDS and further incubation at 65°C for 20 min. Samples were diluted with T4 DNA ligase buffer (New England Biolabs) to achieve approximately 3 ng of DNA/μl, then 200 U T4 DNA ligase (New England Biolabs) added and incubated for 4 h at 16°C. Then samples were incubated with Triton X-100 followed by incubation with Proteinase K (200 μg/ml) at 65°C for 16 h to reverse the cross-linking. This was followed by the addition of 10 μg of RNase/ml and the DNA was purified by phenol-chloroform extraction. 300 ng of DNA was then analyzed by

quantitative real-time PCR was performed with SYBR GreenER qPCR Mastermix (Invitrogen) using primers listed in Table 1. Cycling was performed on a Applied Biosystems Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with the following parameters: 50°C for 2 min, *Taq* activation at 95°C for 10 min then 40 cycles of 95°C for 15 sec and 60°C for 1 min. Reactions were performed in triplicate and specificity monitored using melting curve analysis after cycling. Crosslinking efficiency was calculated as described previously³³.

Chromatin Immunoprecipitation (ChIP)

In vivo ChIP assay was performed by using ChIP-IT Express kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, kidneys from hHO-1 BAC transgenic mice were collected and sliced into small pieces (1–3 mm³) using a razor blade or scalpel. Then, tissues were transferred into 15 ml conical tubes containing 10 ml fixation solution (1% formaldehyde in minimal cell culture medium). Cells were resuspended completely and rotate tube at room temperature for 10 min. Crosslinking was stopped by adding 1 ml 10X Glycine Stop-Fix Solution, rotate 5 min at room temperature. Cells were pelleted by centrifuging for 5 minutes at 720 RCF followed by resuspension in 1.5 ml ice-cold Lysis Buffer. After homogenization, nuclear extract was digested with 3.3 U of Enzymatic Shearing Cocktail for 15 min at 37°C. The enzyme reaction was stopped by adding EDTA and then the sheared chromatin was collected after centrifugation for 10 min at 4°C. 2 µg of indicated antibodies as well as IgG control were used for pulling down in 7 µg of sheared chromatin. After 16 h incubation at 4°C, magnetic beads were washed and chromatin was eluted by adding Reverse Cross link Buffer. Quantitative real-time PCR was used for analysis as described earlier³³.

Rhabdomyolysis

Glycerol model of rhabdomyolysis was induced in age-matched hHO-1 BAC, HO-1^{+/+}, and HO-1^{-/-} mice deprived of water for 16 h as described previously⁶². Mice were anesthetized by isoflurane and were injected with 50% glycerol in water or saline as a control, 7.5 ml/kg body weight, half the volume delivered into each anterior thigh muscle. Mice were sacrificed 3 h after injection for the ChIP and 3C assay and at 4 and 8 h for HO-1 mRNA and at 16 h for HO-1 protein and enzyme activity measurements. Survival studies were performed in additional hHO-1 BAC, HO-1^{+/+}, and HO-1^{-/-} mice (n=10/group).

Cisplatin Injury Model

HO-1^{+/+} and hHO-1 BAC mice (8 to 14 weeks of age) were administered 20 mg/kg body weight cisplatin (1.0 mg/ml solution in sterile 0.9% NaCl) or normal saline by a single intraperitoneal injection. All animals were sacrificed at the indicated time points after cisplatin administration and kidneys were harvested for HO-1 mRNA, protein and enzyme activity measurements. Blood was collected via heart puncture and serum was isolated for creatinine determination.

Statistical Analysis

All the experiments were performed at least 3 times. Results are expressed as mean \pm SEM and are derived from 3 independent experiments. Student's t-test and ANOVA with Student-Newman-Keuls post-test was used for comparisons. All results are considered significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations used

3C	chromosome conformation capture
APC	allophycocyanin
BAC	bacterial artificial chromosome
ChIP	chromatin immunoprecipitation
CTCF	CCCTC-binding factor
DC	dendritic cell
FISH	fluorescence in situ hybridization
HEK293	human embryonic kidney 293
hHO-1 BAC	humanized HO-1 BAC
HO	heme-oxygenase
HS	hypersensitive site
MHCII	major histocompatibility complex class II
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Prl	prolactin gene
RBC	red blood cell
Sp1	Specificity Protein 1
USF1 and 2	Upstream Stimulatory Factor 1 and 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

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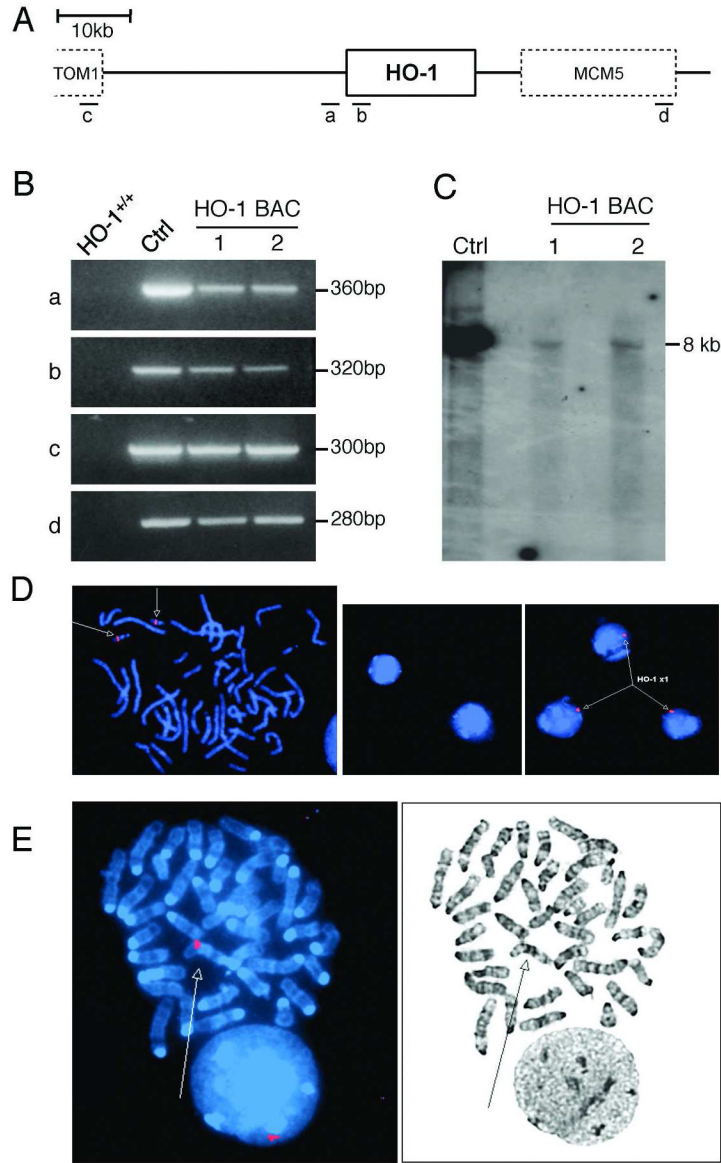


Figure 1. Generation of heme oxygenase-1 (HO-1) bacterial artificial chromosome (BAC) transgenic mice
 (A) Schematic of ~87 kb human BAC DNA (GenBank accession number: Z82244), a portion of human chromosome 22, containing the HO-1 gene along with the 3' end of TOM1, and MCM5 genes used for the generation of HO-1 BAC mice is shown. a, b, c and d represent the amplicons which were used to genotype the HO-1 BAC transgenic mice. Sequences of primers are listed in Table 1. (B) Four primer pairs were used to amplify regions a–d by PCR for genotyping the HO-1 BAC transgenic mice using tail DNA. DNA from wild-type C57BL6/J was used as a negative control (HO-1^{+/+}). Positive control was purified HO-1 BAC DNA used for the generation of HO-1 BAC transgenic mice. (C) Founder mice were identified by Southern blot analysis of tail DNA using a 320-bp PCR amplicon (region b) used as a probe after *EcoRI* digestion. Purified BAC DNA was used as a positive control (+Ctrl) and two founders (1 and 2) are shown. (D) FISH analyses were

performed on interphase peripheral blood cells from HO-1 BAC mice using the HO-1 BAC containing DNA as a probe (right panel). Human metaphase peripheral blood cells were used as positive control and arrows indicate the hybridization location of the HO-1 probe labeled in SpectrumOrange on chromosome 22 band q12.3 (left panel). Negative control using the HO-1 probe on peripheral blood cells from non-transgenic mouse (middle panel). (E) FISH analysis on HO-1 BAC transgenic bone marrow metaphase spread using the HO-1 BAC as a FISH probe labeled in SpectrumOrange (left panel). Note the single site of integration of the HO-1 BAC into chromosome 5 band qF (right panel).

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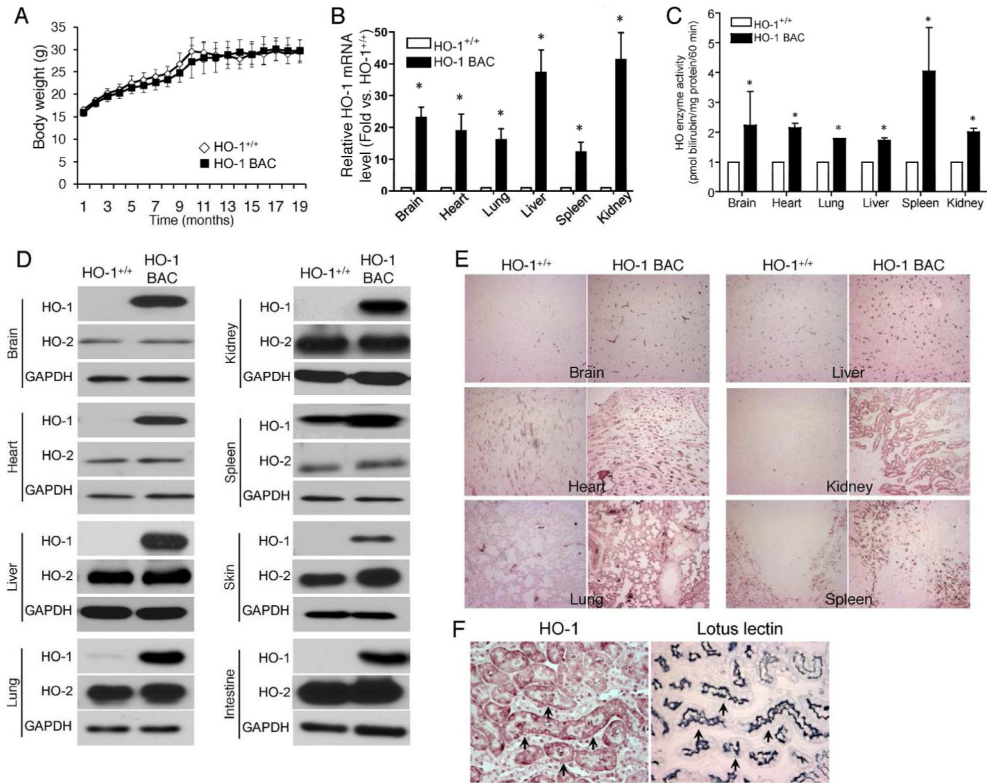


Figure 2. HO-1 protein and mRNA are overexpressed in organs of HO-1 BAC transgenic mice (A) Average body weights of HO-1 BAC mice ($n=5$, solid square) and HO-1^{+/+} mice ($n=4$, open diamond) that were monitored for 19 months. (B) Total RNA was isolated from indicated organs and HO-1 mRNA expression level was quantified using real-time PCR. GAPDH was used to normalize HO-1 mRNA levels. Results are shown as fold-increase versus HO-1^{+/+} from 3 independent experiments performed in triplicate each time (Mean \pm SEM). * $p<0.01$ (C) HO enzyme activity in indicated organs of HO-1^{+/+} and HO-1 BAC transgenic mice was compared. Results are shown as fold-increase versus HO-1^{+/+} from 3 independent experiments performed (Mean \pm SEM) * $p<0.05$. (D) Indicated tissues were harvested and HO-1 protein expression was determined by Western analysis. Anti-GAPDH antibody was used as a loading control. (E) HO-1 expression was determined by immunohistochemistry using HO-1 antibody (SPA-896, Stressgen) in brain, heart, lung, liver, spleen, and kidney tissues from HO-1^{+/+} and HO-1 BAC transgenic mice. (F) Localization of HO-1 protein was determined by staining with lotus lectin (proximal tubule marker) and HO-1 on serial sections of kidneys from HO-1 BAC transgenic mice. Arrows indicate co-localization of HO-1 and lotus lectin in proximal tubules.

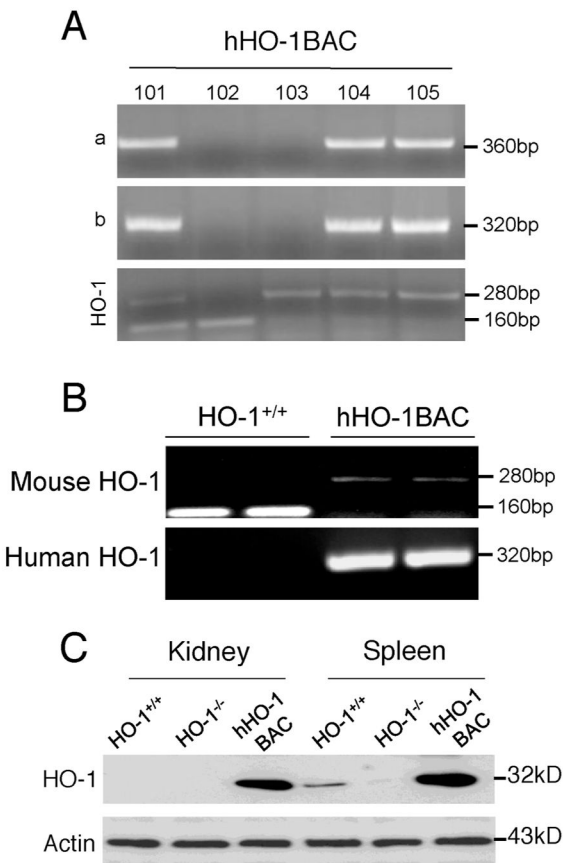


Figure 3. Generation and characterization of “humanized” HO-1 BAC (hHO-1 BAC) mice
 (A) Five transgenic mice were obtained from a pair of HO-1^{+/-} BAC positive mice. Primers for HO-1 BAC (a and b, Table 1, Figure 1A) were used to screen whether they were positive for HO-1 BAC. Mice were also genotyped using a combination of primers for the detection of mouse HO-1 gene (lower 160-bp band) and/or neomycin cassette (upper 280-bp band) inserted in order to generate HO-1^{-/-} mice (Table 1). (B) Genomic DNA was used to evaluate the presence of mouse and human HO-1. While the genotyping of HO-1^{+/+} mice reveals a single band at 160 bp for mouse HO-1, the absence of mouse HO-1 in hHO-1 BAC mice gives a single band at 280 bp denoting the presence of the neomycin cassette derived from the HO-1^{-/-} mice. A heterozygote would reveal two bands at 160 and 280 bp. The presence of human HO-1 in hHO-1 BAC mice is confirmed by using a human specific primer that yields a 320 bp band. (C) Microsomal fractions of kidney and spleen from HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice were isolated and analyzed by immunoblot for HO-1 expression. The blot was stripped and reprobbed with anti-actin antibody for a loading control.

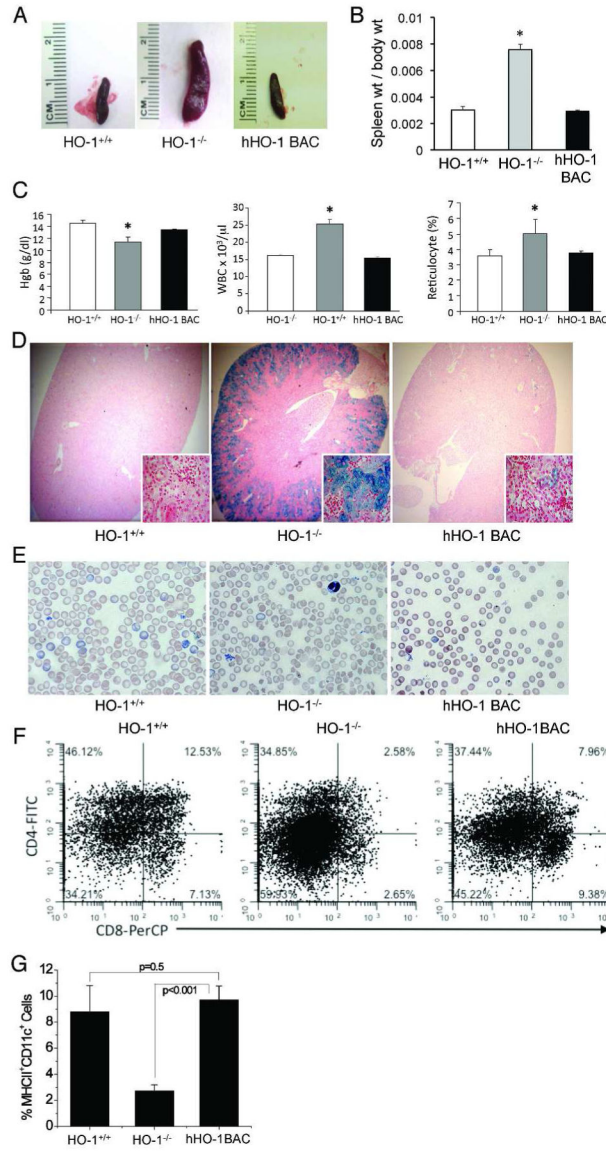


Figure 4. Human HO-1 in hHO-1 BAC transgenic mice rescues the pathological phenotype of HO-1^{-/-} mice

Spleens from 25–35 week-old HO-1^{+/+} (n=8), HO-1^{-/-} (n=7), and hHO-1 BAC (n=7) transgenic mice were collected and their lengths (A) and weights (B) were compared. Weights of spleen were normalized to body weight of each animal, and average normalized values were plotted for each group (Mean±SEM) * p<0.05 vs. HO-1^{+/+} and hHO-1 BAC mice. (C) Whole blood from 30–40 week-old HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice were collected and hemoglobin (Hgb), leukocyte (WBC) and reticulocyte counts were measured (n=4–5/group). * p<0.05 vs. HO-1^{+/+} and hHO-1 BAC mice. (D) Kidneys from 25–35 week-old HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice were collected and Prussian blue staining was performed to detect tissue iron in paraffin-embedded sections. (E) Peripheral blood smears were prepared from HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice and stained using Wright-Giemsa staining to determine RBC morphology. (F and G) Subsets of splenic DC from HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC transgenic mice were compared as described

in Methods. CD8 and CD4 expression is shown on CD11c and MHC II-gated cells from each group of mice (F). Numbers within graph quadrants depict the percentage of CD11c⁺MHC II⁺ positive cells. The histogram shown depicts the average from three animals in the percentage of CD8⁺ DC among the population of CD11c⁺MHC II⁺ DC.

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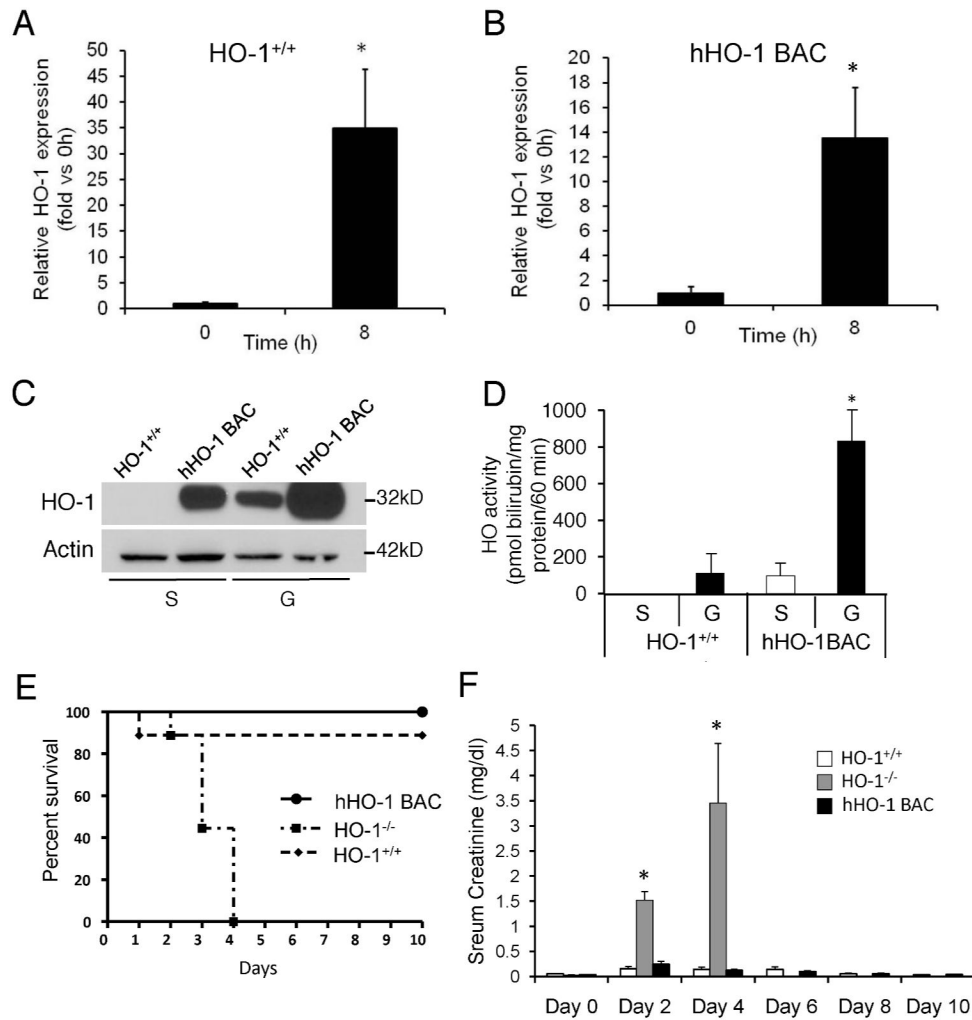


Figure 5. HO-1 expression is induced at higher levels after rhabdomyolysis in hHO-1 BAC mice Rhabdomyolysis was induced by injecting 50% glycerol (7.5 ml/kg body weight) in HO-1^{+/+} and hHO-1 BAC mice (A and B, respectively) (n=9/group). Kidneys from HO-1^{+/+} and hHO-1 BAC mice were collected and total RNA was isolated. At 8 hours post glycerol administration HO-1 was induced ~30 fold in HO-1^{+/+} (panel A) kidneys, while the hHO-1 BAC kidneys revealed ~14 fold HO-1 induction (panel B) *p<0.01. (C) Microsomal fractions from kidneys of HO-1^{+/+} and hHO-1 BAC mice after injection of saline (S) as a control and 50% glycerol (G) for 16 h were analyzed for HO-1 protein expression using Western analysis. Note that normalization of the band intensity to corresponding actin levels shows no significant difference between glycerol treated HO-1^{+/+} and hHO-1 BAC saline treated mice. (D) Kidneys of HO-1^{+/+} and hHO-1 BAC mice after injection of saline (S) and glycerol (G) were collected and HO enzyme activity was measured at 16 h after glycerol administration as described in the Methods. (n=3/group) *p<0.05 vs. HO-1^{+/+} (E) Survival rate was monitored after induction of rhabdomyolysis in HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice (n=10/group). Experiments were ceased 10 days after injection. (F) Serum creatinine was measured in HO-1^{+/+}, HO-1^{-/-}, and hHO-1 BAC mice after indicated days after induction of rhabdomyolysis.

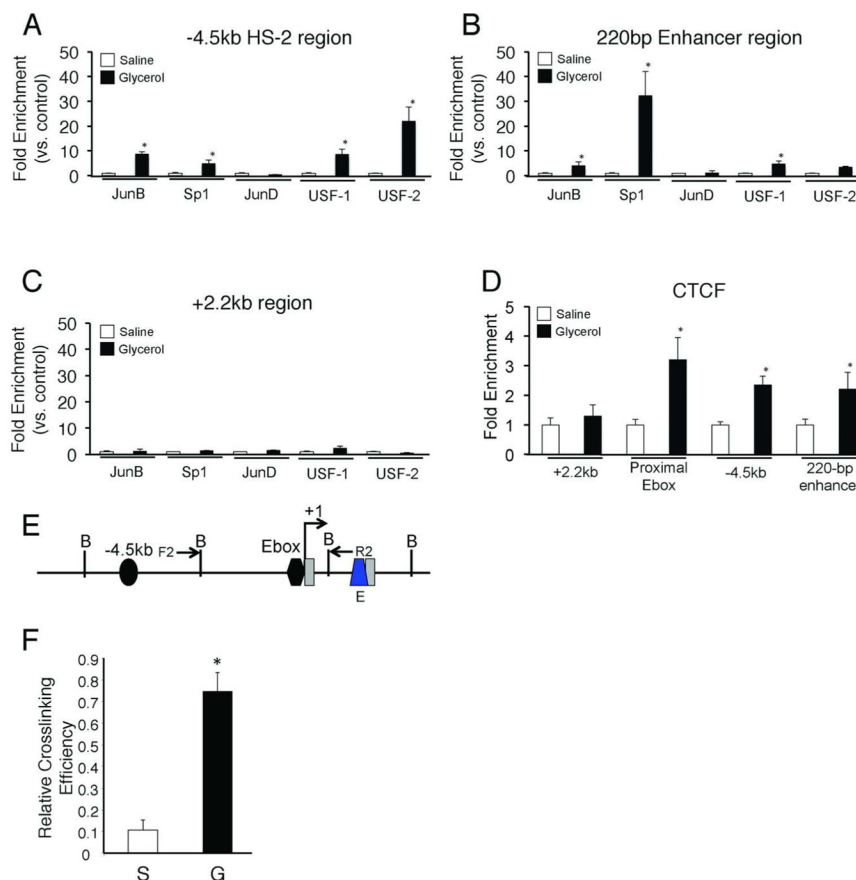


Figure 6. Glycerol-induced rhabdomyolysis induces chromatin-looping-mediated promoter-enhancer interaction and increases binding of JunB, Sp1, USF1/2, and CTCF to regulatory regions of the human HO-1 gene *in vivo*

(A–C) hHO-1 BAC transgenic mice were injected with 7.5 ml/kg body wt 50% glycerol (G) or saline (S) and kidneys were harvested after 3 h. An *in vivo* ChIP assay was performed to determine changes in association of indicated transcription factors within the –4.5kb human HO-1 promoter region (A), 220-bp intronic enhancer region (B), and the +2.2kb internal E-box region as a negative control (C) as described in Methods. Results represent three independent experiments and fold enrichment vs. saline-injected mice are shown (Mean \pm SEM). (D) Kidneys from hHO-1 BAC transgenic mice injected with 50% glycerol or saline were harvested and *in vivo* ChIP was performed to determine the association of CTCF with –4.5kb promoter, 220-bp enhancer, and proximal E-box regions. The +2.2kb E-box region was used as negative control. Results represent two independent experiments and relative fold enrichments vs. saline-injected mice are shown (Mean \pm SEM). * $p < 0.05$ vs. saline-treated hHO-1 BAC mice. (E) Map of the human HO-1 promoter, where B denotes BglII restriction sites flanking the –4.5kb promoter regions, proximal E-box elements, and 220-bp enhancer (E). Arrows denote primers used for 3C assay. Gray rectangles indicate exons in the human HO-1 gene. (F) hHO-1 BAC mice were injected with glycerol (G) and saline (S) for 3 h and kidneys were harvested. 3C assay was performed to determine the interaction between –4.5kb promoter and 220-bp enhancer (F2/R2) using BglII restriction enzyme. Interactions between promoter and enhancer regions were determined and quantified as

relative crosslinking efficiency. Results shown are from 3 independent experiments (Mean \pm SEM). * $p < 0.05$ vs. saline-injected hHO-1 BAC mice.

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Table 1

Primers for genotyping, 3C, ChIP-loop, and ChIP assays

Assay	Name	Primer sequence (5'-3')
Genotyping	BAC a For	gcctctgggctgtagtgg
	BAC a Rev	agtgcagggtgggaagattgctt
	BAC b For	catctatgtgccctggagga
	BAC b Rev	ccaggcctttctgggcaat
	BAC c For	gcagaccaagcgggtaag
	BAC c Rev	ccacttcctgtctgcacatttc
	BAC d For	ggaacaacagttgatgctctgcttcc
	BAC d Rev	cgggaccctgtgattgattgctc
Characterization of hHO-1 BAC mice	Human specific HO-1 For	catgacaccaaggaccagag
	Human specific HO-1 Rev	agtgtaaagaccatcggag
	Mouse specific HO-1 For	ggtgatggcttctgtacc
	Mouse specific HO-1 Rev	agtgagccataccagaag
3C and ChIP-loop	F1	cttgggcttgtcttcttctgctgaagaa
	F2	gaaagcaagcccagaccggcagc
	R1	cccttgggaaacaagtctggccata
	R2	gctagtgaggacagatgccaccaag
	Internal control For	gctaatagaggctgcacgatgttga
	Internal control Rev	gcctaatacacacagctcaccggg
ChIP	220bp enhancer For	acaagctgcacatctaaagc
	220bp enhancer Rev	tctctgcctgggtgtgcacc
	-4.5kb For	atatgactgctcctctccacc