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Hepcidin Downregulation by Repeated Bleeding Is Not Mediated by Soluble Hemojuvelin

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Summary

Hepcidin is a key regulator of iron homeostasis, while hemojuvelin is an important component of the hepcidin regulation pathway. It has been recently proposed that soluble hemojuvelin, produced from hemojuvelin by the protease furin, decreases hepcidin expression. The aim of the presented study was to examine the downregulation of hepcidin by chronic bleeding in hemojuvelin-mutant mice. Male mice with targeted disruption of the hemojuvelin gene (*Hjv*-/- mice) and wild-type littermates were maintained on an iron-deficient diet and subjected to weekly phlebotomies for 7 weeks. Gene expression was examined by real-time PCR. In wild type mice, repeated bleeding decreased hepcidin mRNA by two orders of magnitude. In *Hjv*-/- mice, basal hepcidin expression was low; however, repeated bleeding also decreased hepcidin mRNA content by an order of magnitude. Phlebotomies reduced hepatic iron overload in *Hjv*-/- mice by 80 %. Liver and muscle furin mRNA content was not significantly changed. No effect on hepatic *Tmprss6* mRNA content was observed. Results from the study indicate that soluble hemojuvelin is not the sole factor responsible for hepcidin downregulation. In addition, the presented data suggest that, under *in vivo* conditions, tissue hypoxia does not transcriptionally regulate the activity of furin or TMPRSS6 proteases.

Key words

Hepcidin • Hemojuvelin • Furin • Tmprss6 • Matriptase-2

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Introduction

Hepcidin (gene symbol *HAMP*) is a key regulator of iron metabolism. It is produced in the liver, and controls degradation of the iron export protein ferroportin in macrophages and enterocytes. Iron overload increases hepcidin expression and decreases macrophage and enterocyte iron export, while dietary iron deficiency downregulates hepcidin expression (Muckenthaler 2008).

In addition to iron levels, hepcidin expression is also modulated by erythropoietic activity. It has been demonstrated by several groups that enhanced erythropoiesis lowers liver *Hamp* mRNA content (Nicolas *et al.* 2002, Vokurka *et al.* 2006, Pak *et al.* 2006); however, the exact signal mediating this decrease is at present unknown. Increased iron demand for erythropoiesis results in a decrease in the amount of diferric transferrin, which could function as an iron sensor by its interactions with hepatocyte membrane proteins (Frazer and Anderson, 2003). It has also been proposed that hepcidin transcription is regulated directly by hypoxia (Peyssonnaux *et al.* 2007), or erythropoietin (Pinto *et al.* 2008). Alternatively, hepcidin expression could be downregulated by circulating proteins secreted by tissues involved in iron metabolism. Possible candidate proteins include GDF15, secreted by erythroblasts (Tanno *et al.* 2007), or soluble hemojuvelin, secreted by skeletal muscle.

Soluble hemojuvelin (sHjv) represents a proteolytically processed, circulating form of hemojuvelin (Hjv, gene symbol *HFE2*), a GPI-anchored membrane protein whose mutations in humans cause

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juvenile hemochromatosis (Papanikolaou *et al.* 2004). Recently, it has been demonstrated that soluble hemojuvelin is produced from the membrane-bound hemojuvelin protein by proteolytic activity of the proprotein convertase furin (Lin *et al.* 2008, Silvestri *et al.* 2008), while expression of furin is reported to be under the control of hypoxia (McMahon *et al.* 2005). In one possible model of hepcidin downregulation (Silvestri *et al.* 2008), hypoxia or lack of iron increase furin transcription, increased furin activity produces more soluble hemojuvelin, and secreted soluble hemojuvelin subsequently decreases hepcidin expression by interaction with the bone morphogenetic protein (BMP)/ hemojuvelin signaling pathway, a well established component of the hepcidin regulatory cascade (Babitt *et al.* 2006, Truksa *et al.* 2006, Kautz *et al.* 2008).

Very recently, another protein with a potentially important function in hepcidin regulation has been discovered. A mutated form of the serine protease TMPRSS6 has been reported to cause inappropriately high hepcidin expression, coupled with symptoms of iron deficiency (Du *et al.* 2008, Finberg *et al.* 2008, Folgueras *et al.* 2008). TMPRSS6 therefore represents an important negative regulator of hepcidin expression; however, it has not yet been reported whether TMPRSS6 proteolytic activity could be modulated by hypoxia or enhanced erythropoiesis.

The critical importance of hemojuvelin in the hepcidin regulation pathway is evident from the fact that juvenile hemochromatosis caused by hemojuvelin mutations is clinically as severe as juvenile hemochromatosis caused by hepcidin mutations (Papanikolaou *et al.* 2004). In addition, male mice with targeted disruption of the hemojuvelin gene have very low hepcidin levels, and are unable to upregulate hepcidin expression following iron overload (Huang *et al.* 2005, Niederkofler *et al.* 2005). Since the cleaved form of hemojuvelin is proposed to participate in hepcidin downregulation following hypoxia or iron deficiency (Lin *et al.* 2005, Zhang *et al.* 2005, Babitt *et al.* 2006), it could be of interest to examine the response of hepcidin to severe iron deficiency in mice with targeted disruption of the hemojuvelin gene. To this purpose, the presented study examines liver hepcidin mRNA following repeated phlebotomies in both wild-type and hemojuvelin-deficient mice.

The results show that repeated phlebotomies decrease hepcidin expression even in the absence of hemojuvelin, and therefore demonstrate that soluble hemojuvelin is not the sole factor responsible for hepcidin downregulation. The amount of mRNAs coding for furin and Tmprss6 was not significantly changed, indicating that the biological activity of these components of the hepcidin regulatory pathway is not regulated at the level of transcription.

Materials and methods

Male 129SvJ mice with targeted disruption of exon 2 of the *Hfe2* gene (*Hjv*-/- mice), average age 7 months, were a generous gift from Prof. Silvia Arber, Basel, Switzerland (for animal details, see Niederkofler *et al.* 2005). Throughout the experiment, mice were maintained on an iron-deficient diet (C1038, iron content less than 10 μg/g, Altromin GmbH, Lage, Germany). In experiments with wild type mice, approximately 0.5 ml of blood was withdrawn once weekly for five weeks by retrobulbar puncture under light ether anesthesia; in *Hjv*-/- mice experiments, 0.7 ml of blood was withdrawn once weekly for 7 weeks. Iron was administered as iron polyisomaltosate (Ferrum Lek, Lek Ljubljana, Slovenia) by subcutaneous injection. All animal experiments were approved by the Ethics Committee of the First Faculty of Medicine.

Four days after last phlebotomy, mice were euthanized by cervical dislocation, and tissue samples were dissected and stored in RNALater (Sigma Aldrich, Prague, Czech Republic). Total mRNA was isolated with Qiagen RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany), or, in the case of skeletal muscle, by phenol-chloroform extraction as previously described (Vokurka *et al.* 2006). Reverse transcription was performed using Bio-Rad iScript (Bio-Rad Laboratories, Hercules, CA, USA). Target mRNA levels were determined by real-time PCR using SYBR Green protocol. Target mRNA content was calculated relatively to β-actin (*Actb*) mRNA content, assuming exact doubling of amplified DNA in each PCR cycle (Vokurka *et al.* 2006). *Hamp* and *Hamp2* specific primer sequences were: CTGAGCAGCACCA CCTATCTC (common forward primer); *Hamp* reverse primer TGGCTCTAGGCTATGTTTTGC, *Hamp2* reverse primer GGCTCTAGGCTCTCTATTCTTCA. Primer pairs for other genes were (forward and reverse respectively): *Actb*, GACATGGAGAAGATCTGGCA and GGTCTTTACGGATGTCAACG; *Slc40a1*, ATCG GTCTTTGGTCCTTTGAT and ATTGCCACAAAGG AGACTGAA; *Furin*, GTGCATTGTTGAAATCCTGGT and TCCCATAGTTGTTGGCTTCAC; ceruloplasmin (*Cp)*, TCACTACACAGGTGGCATGAA and GTCTTCT

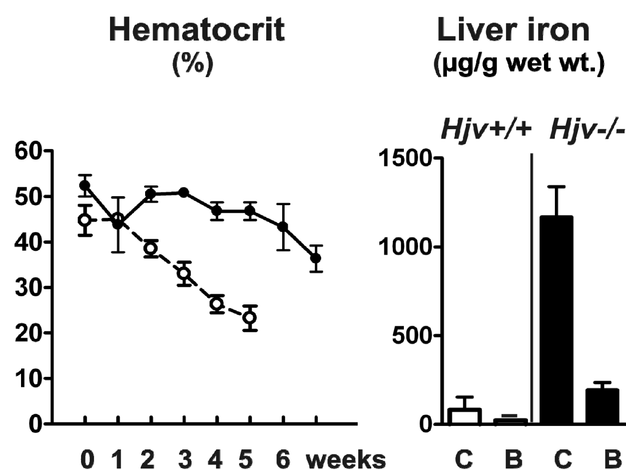

Fig. 1. Effect of repeated bleeding on hematocrit and liver iron content in male *Hjv*+/+ and *Hjv*-/- mice. *Hjv*+/+ mice were bled (0.5 ml) once weekly for 5 weeks, *Hjv*-/- mice were bled (0.7 ml) once weekly for 7 weeks. Open symbols: *Hjv*+/+ mice, closed symbols *Hjv*-/- mice. C = control, B= bled. Results are expressed as mean ± SEM.

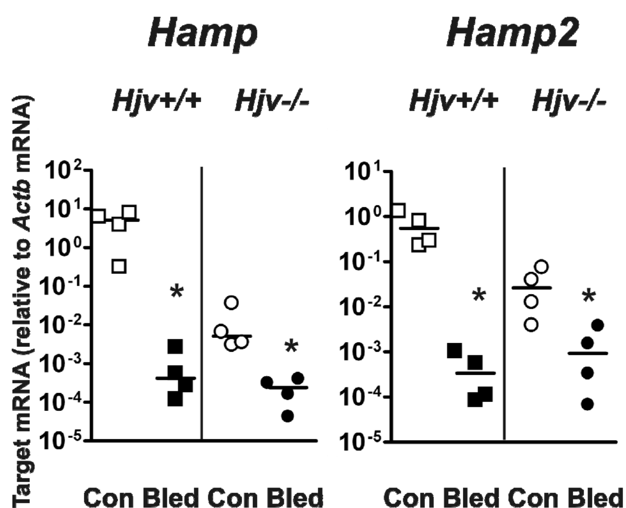

Fig. 2. Effect of repeated bleeding on liver *Hamp* and Hamp2 mRNA content. Male *Hjv*+/+ mice were bled (0.5 ml) once weekly for 5 weeks, *Hjv*-/- mice were bled (0.7 ml) once weekly for 7 weeks. Asterisks denote statistically significant differences ($p<0.05$) by Mann-Whitney test.

TCGGCTCGTCTCTT. Hepatic iron concentration was determined according to Torrance and Bothwell (1980). Statistical significance of real-time PCR results was determined by Mann-Whitney test, graphed data include medians. Other data are expressed as means ± SEM.

Results

Hjv-/- mice downregulate hepcidin mRNA levels following repeated phlebotomies

As expected, repeated phlebotomies in wild type mice resulted in a gradual decrease of hematocrit and a drop in liver iron concentration (Fig. 1). Hepatic hepcidin mRNA decreased by more than two orders of magnitude (Fig. 2). This decrease was much more pronounced than in acutely bled mice, where hepcidin mRNA content decreased only two- to fourfold (Krijt *et al.* 2007). In *Hjv*-/- mice, hematocrit decreased less rapidly than in wild-type mice; however, hepatic hepcidin mRNA content also decreased by an order of magnitude (Fig. 2), clearly demonstrating that tissue hypoxia and/or enhanced erythropoiesis downregulate *Hamp* mRNA even in the absence of functional hemojuvelin protein. The response of *Hamp2* mRNA was similar to *Hamp* mRNA in all experimental groups, indicating similar regulatory mechanisms for the two genes (Fig. 2).

Hjv-/- mice effectively mobilize liver iron

Hjv-/- mice accumulate large amount of iron in hepatocytes, while their Kupffer cell iron content is low (Huang *et al.* 2005). During the seven weeks of phlebotomy treatment, *Hjv*-/- mice lost approximately 1.8 mg of iron in removed red blood cells, while their dietary iron intake was limited by feeding of an iron-deficient diet. At the end of treatment, liver iron content was decreased by about 80 % (Fig. 1), suggesting efficient iron mobilization from hepatocytes. In total, about 1.4 mg of iron was mobilized from the liver, averaging approximately 25 µg/day. Although iron export from hepatocytes is not yet completely elucidated (Ganz 2006, Viatte *et al.* 2006), it is probably mediated by the transmembrane iron exporter ferroportin (gene symbol *Slc40a1*) in combination with the ferroxidase

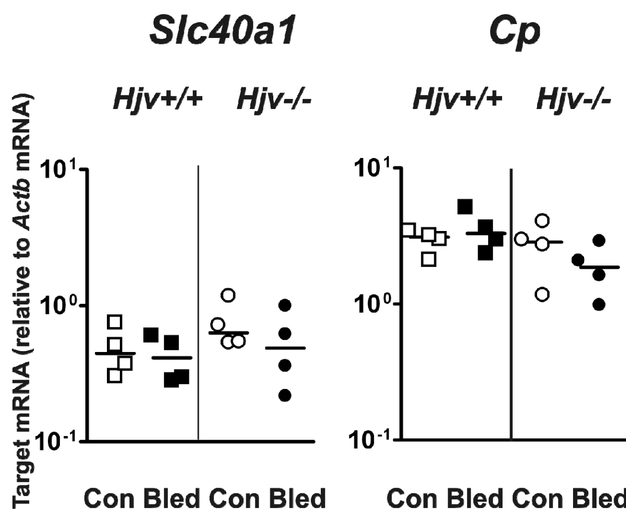

Fig. 3. Lack of effect of repeated bleeding on liver ferroportin (*Slc40a1*) and ceruloplasmin (*Cp*) mRNA content. Male *Hjv*+/+ mice were bled (0.5 ml) once weekly for 5 weeks, *Hjv*-/- mice were bled (0.7 ml) once weekly for 7 weeks.

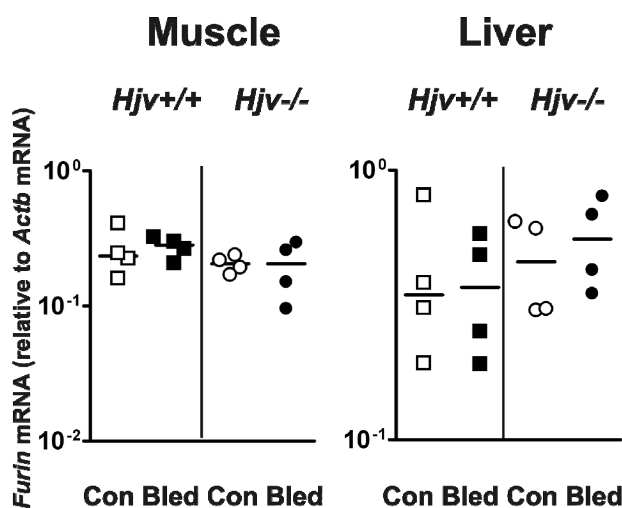

Fig. 4. Lack of effect of repeated bleeding on muscle and liver *Furin* mRNA content. Male *Hjv*+/+ mice were bled (0.5 ml) once weekly for 5 weeks, *Hjv*-/- mice were bled (0.7 ml) once weekly for 7 weeks.

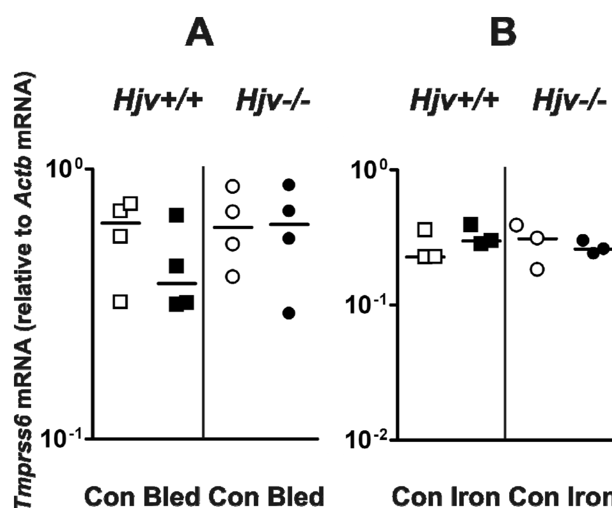

Fig. 5. Lack of effect of repeated bleeding **(A)** or iron administration **(B)** on liver *Tmprss6* mRNA content. Male *Hjv*+/+ mice were bled (0.5 ml) once weekly for 5 weeks, *Hjv*-/- mice were bled (0.7 ml) once weekly for 7 weeks. Iron was administered at 300 mg/kg as iron polyisomaltosate one week prior to sacrifice.

ceruloplasmin (*Cp*). As can be seen in Figure 3, liver *Slc40a1* mRNA and *Cp* mRNA levels were not significantly changed by phlebotomy treatment (Fig. 3).

Repeated phlebotomies do not influence liver or muscle Furin mRNA content

It has been proposed that the expression of the proprotein convertase furin is transcriptionally controlled by hypoxia (McMahon *et al.* 2005, Silvestri *et al.* 2008). In the presented experiments, prolonged decrease of hematocrit did not significantly decrease muscle or liver *Furin* mRNA levels, arguing against significant hypoxia-dependent *Furin* gene regulation *in vivo* (Fig. 4). Tissue hypoxia in bled mice was verified by an increase in kidney erythropoietin mRNA (relative to *Actb* mRNA), which increased from 0.0009±0.0007 in the wild-type control group to 0.0145±0.0053 in the wild-type phlebotomy group (n=3, data not shown).

Hepatic Tmprss6 mRNA content is not influenced by phlebotomies or iron overload

Very recently, the serine protease TMPRSS6 has been identified as a liver-specific factor capable of decreasing hepcidin gene expression. In the presented experiments, no change in hepatic *Tmprss6* mRNA level was observed following repeated phlebotomies (Fig. 5). In addition, there was no difference in *Tmprss6* mRNA content between wild-type and *Hjv*-/-mice, despite marked iron overload in the latter. The lack of response of *Tmprss6* mRNA to iron overload was verified in an additional experiment, in which mice were injected with iron polyisomaltosate (300 mg iron/kg). Iron injection did not change *Tmprss6* mRNA (Fig. 5), while *Hamp* mRNA content increased (relative to *Actb* mRNA) from 5.4±1.9 to 16.0±2.0 in wild type mice and from 0.037±0.038 to 0.072±0.049 in *Hjv*-/- mice (n=3, data not shown).

Discussion

Research conducted during the last few years indicates that the regulation of hepcidin expression is a complex process (Muckenthaler *et al.* 2008). A critical protein in the pathway regulating hepcidin response to excess iron is hemojuvelin. The importance of hemojuvelin for hepcidin regulation is demonstrated by the fact that *Hjv*-/- mice fail to significantly increase *Hamp* expression following iron overload (Huang *et al.* 2005, Niederkofler *et al.* 2005), while mice with targeted disruption of other iron regulatory genes – *Hfe*, *Trfr2* or *B2m* - still display the expected increase in *Hamp* mRNA after iron administration (Constante *et al.* 2006). Hemojuvelin exists in both membrane-bound and soluble forms, the soluble form probably originating by cleavage of membrane hemojuvelin by the proprotein convertase furin (Lin *et al.* 2008). According to present models of hepcidin downregulation, increased erythropoiesis or iron deficiency increases furin transcription (Silvestri *et al.* 2008), and the resulting increase in plasma sHjv content decreases hepcidin expression by interaction with the BMP/SMAD pathway.

Results presented in this study show that the absence of functional hemojuvelin protein does not prevent the very marked decrease in *Hamp* expression following repeated phlebotomies. Although male *Hjv*-/- mice have much lower basal *Hamp* mRNA levels than wild-type mice, they clearly respond to both acute (Krijt *et al.* 2007) and chronic (Fig. 2) bleeding. It is therefore evident that soluble hemojuvelin is not the sole mediator which downregulates *Hamp* expression *in vivo*.

In addition to *Hamp* mRNA levels, phlebotomies significantly decreased *Hamp2* mRNA levels as well. Although the existence of two hepcidin genes has been described only in mice, and although the Hamp2 protein does not regulate iron export (Lou *et al.* 2004), the *Hamp2* gene retains the iron-dependent regulation. The downregulation of *Hamp2* in *Hjv*-/- mice can therefore be viewed as a confirmation of the observed *Hamp* mRNA response. It can be concluded that although the presence of functional hemojuvelin protein is obligatory for the maintenance of physiological hepcidin expression levels, downregulation of hepcidin expression occurs even in the absence of hemojuvelin. These results suggest that the pathways mediating hepcidin upregulation and down-regulation might not be completely identical; in addition, they support the concept of a circulating negative regulator of hepcidin expression, which is probably related to erythropoietic activity (Tanno *et al.* 2007).

Deletion of the hemojuvelin gene results in marked accumulation of iron in hepatocytes (Huang *et al.* 2005). Figure 1 shows that a substantial part of excess hepatocyte iron in *Hjv*-/- mice was efficiently mobilized by repeated phlebotomies. The regulatory mechanism underlying this mobilization of hepatocyte iron is at present unclear. It is well established that the levels of the iron exporter protein ferroportin are regulated by hepcidin, which targets ferroportin protein for degradation (Nemeth *et al.* 2004). *Hjv*-/- animals have markedly depressed hepcidin expression, and their ferroportin-mediated iron export activity is therefore probably at the near maximum level. Even so, untreated *Hjv*-/- mice accumulate large amounts of iron in hepatocytes, apparently as a result of high transferrin saturation, high plasma iron content and high hepatocyte non-transferrin bound iron uptake. However, when transferrin saturation and plasma iron levels are reduced by phlebotomy, iron is efficiently removed from the liver. These data indicate that, in *Hjv*-/- mice, hepatocyte iron has a relatively high turnover. In addition, they also suggest that the liver iron content in these animals is mainly determined by the net balance between hepatocyte iron uptake and iron export. A similar situation occurs in patients with juvenile hemochromatosis due to hepcidin mutations, which are also reported to respond to phlebotomy (Matthes *et al.* 2004), although the hepcidin-ferroportin regulation in these patients is not functional. As can be seen in Figure 3, repeated phlebotomies did not change ferroportin or ceruloplasmin mRNA levels, excluding transcriptional regulation of hepatocyte iron export in *Hjv*-/- mice. Overall, the obtained results suggest that the therapeutical effect of phlebotomies in juvenile hemochromatosis probably depends primarily on the reduction of iron import into the hepatocyte, rather than on the modulation of hepatocyte ferroportin levels.

Soluble hemojuvelin is produced from membrane-bound hemojuvelin by the action of the proprotein convertase furin. An elegant hypothesis (Silvestri *et al.* 2008) proposes that furin mRNA levels, and therefore also furin activity and sHjv production, are upregulated by hypoxia inducible factor (HIF). In support of this hypothesis, *Furin* mRNA level was increased by hypoxia *in vitro* (Silvestri *et al.* 2008). However, in our *in vivo* experiments, hepatic or muscle *Furin* mRNA levels were not significantly changed by repeated phlebotomies, arguing against HIF-dependent regulation *in vivo*. Clearly, the effect of hypoxia on sHjv levels, as well as the precise role of sHjv in iron homeostasis and the determination of exact site of sHjv production, are still unresolved issues, which require further studies.

The putative negative regulators of hepcidin expression – sHjv or GDF15 – are proposed to interact with the HJV/BMP pathway. It is therefore intriguing that the downregulation of hepcidin expression remains functional in mice with targeted disruption of the *Hjv* gene. Apparently, low content of diferric transferrin, hypoxia, enhanced erythropoiesis, increased erythro-poietin levels or other physiological signals related to bleeding-induced anemia are capable to downregulate hepatic hepcidin expression even in *Hjv*-/- mice. A very recent development in the field of hepcidin regulation is the identification of the TMPRSS6 protein as a new negative regulator of hepcidin expression. TMPRSS6 is expressed almost solely in the liver, and, at present, there is little information on its regulation. Figure 5 indicates that hepatic *Tmprss6* mRNA is not influenced by chronic bleeding or iron overload. It is therefore evident that, if the TMPRSS6 protein participates in hepcidin downregulation following repeated phlebotomies, its activity must be mediated at posttranscriptional level.

In conclusion, result from our study demonstrate

significant downregulation of hepcidin expression in mice with targeted disruption of the hemojuvelin gene, indicating that soluble hemojuvelin is not the sole factor responsible for hepcidin downregulation during enhanced erythropoiesis. In addition, the presented data suggest that, under *in vivo* conditions, tissue hypoxia does not transcriptionally regulate the expression of *Furin* or *Tmprss6*.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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