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The liver in regulation of iron homeostasis

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Abstract

The liver is one of the largest and most functionally diverse organs in the human body. In addition to roles in detoxification of xenobiotics, digestion, synthesis of important plasma proteins, gluconeogenesis, lipid metabolism and storage, the liver also plays a significant role in iron homeostasis. Apart from being the storage site for excess body iron, it also plays a vital role in regulating the amount of iron released into the blood by enterocytes and macrophages. Since iron is essential for many important physiological and molecular processes, it increases the importance of liver in the proper functioning of the body's metabolism. This hepatic iron-regulatory function can be attributed to the expression of many liver-specific or liver-enriched proteins, all of which play an important role in the regulation of iron homeostasis. This review focuses on these proteins and their known roles in the regulation of body iron metabolism.

Liver and its functions

The liver performs an array of functions which include supporting lipid and carbohydrate homeostasis, detoxification of blood, removal of infectious agents via the Kupffer cells, and maintenance of metal homeostasis. The liver also regulates iron homeostasis. Iron is an essential element as it is either a part of molecules required for essential functions (e.g. hemoglobin) or is required as a cofactor for the functioning of enzymes regulating important physiological functions. The human body does not have a recognized mechanism for removing excess iron, and increased body iron can be detrimental to health. This makes it essential for the body to regulate the level of iron, a function performed by the liver.

Systemic iron homeostasis

As our understanding of iron homeostasis has increased, it has become evident that the liver is at the center of this regulation. In addition to the liver, other major organs/tissues involved in iron metabolism are the erythroid compartment the site of iron absorption (enterocytes) and site of iron recycling - the macrophages. Once iron is absorbed by enterocytes, it is transported through the body to other organs and tissues via the circulation. In the blood, iron is either bound to a protein called transferrin (transferrin-bound iron (TBI)) or present as non-transferrin bound iron (NTBI). Transferrin-bound iron forms the major part of iron transport under normal iron conditions and unless specified, the term iron mentioned in this review would be TBI.

Erythropoiesis uses a major portion of the body iron to produce new red blood cells (RBCs), and hence forms another critical component of the machinery. The iron from senescent RBCs is recycled by macrophages of the reticuloendothelial system. Iron is exported from the cells by ferroportin (FPN), which is the only known iron exporter.

Iron, in excess to the requirements of the organs and various metabolic processes, is stored in the hepatocytes. In addition to acting as the storehouse of excess iron, the liver acts as the central regulator of iron homeostasis. The liver produces a 25-amino acid peptide, hepcidin, also known as the iron-regulatory hormone. This peptide binds to and induces the internalization of FPN (69, 78), thus limiting the amount of iron released into the blood. Hepcidin expression is controlled by many stimuli including inflammation, erythropoiesis, hypoxia and body iron status (77). Although there have been reports of local hepcidin production in various other tissues and organs including the heart (54), alveolar macrophages (60) and splenic macrophages (52), hepatic hepcidin is thought to be the major contributor to systemic regulation of iron. The physiological stimuli and the mechanisms involved in the regulation of hepcidin have been discussed in detail in other recent reviews (77, 88, 94), and as such will not be described in detail here.

Liver proteins involved in iron homeostasis

The liver maintains systemic iron homeostasis by regulating the levels of hepcidin. One of the physiological stimuli that results in an increase in hepcidin production is body iron status. This iron-dependent regulation of hepcidin is mediated by the liver through several proteins which are either expressed exclusively or at very high levels in the liver. Our understanding of iron homeostasis has increased with the

progressive identification and analysis of the genes mutated in the iron overload disorder, hereditary hemochromatosis (HH), and subsequent studies on animal models of this disorder. This genetic iron overload disorder is characterized by excess iron entering into the blood, thus exceeding normal body iron requirements (57). This results in the gradual accumulation of iron in a number tissues and organs. Excess iron in patients with HH deposits mainly in the parenchymal cells of the liver, pancreas, heart and pituitary gland (2). Generation of reactive oxygen species by excess iron can result in tissue damage, ultimately leading to clinical complications like cirrhosis, diabetes, cardiomyopathy, hypogonadism and arthropathy (2). The analysis and observations made on patients suffering from this disorder have helped us identify the molecules which play an important role in the regulation of iron metabolism. The proteins involved in the iron-mediated regulation of hepcidin are highly expressed in the hepatocytes which are the predominant cell type in the liver. This review focuses on these proteins and their contribution to systemic iron homeostasis.

Hemochromatosis protein (HFE)

The *HFE* (High Fe) gene was first identified in 1996 using linkage disequilibrium and haplotype analysis (17) and is the gene responsible for or mutated in HH. HFE is a member of the major histocompatibility complex (MHC) class 1 family of proteins, which are involved in the presentation of antigens to T-cells. Similar to other members of the family, HFE is composed of three extracellular domains (α1, α2 and α3). It is highly expressed in tissues which are involved in iron metabolism, namely liver (hepatocytes and Kupffer cells) (3, 100), in the crypts of Leiberkuhn of the

duodenum (67) and in resident tissue macrophages and circulating monocytes (66). The development of iron overload in the *Hfe* knockout mouse confirmed that the product of this gene plays an important role in iron metabolism (103). HFE is believed to play an important role in the regulation of hepcidin.

HFE interacts with β2-microglobulin (β2M) through its α3 domain which is responsible for the proper cell surface expression of the protein in hepatocytes (19). The most common mutation in HFE, p.C282Y, disrupts this interaction resulting in the intracellular retention of the protein, leading to inappropriate hepcidin levels and consequently leading to iron overload (19, 89). HFE was also shown to interact with the ubiquitously expressed transferrin receptor 1 (TFR1) (18, 45, 66). The binding site of HFE with TFR1 overlaps with the site for transferrin (TF) binding, suggesting that HFE competes with TF for TFR1 binding and that this interaction plays a key role in iron homeostasis. An increase in iron levels increases the TF saturation which makes more holo-TF available to bind to TFR1, thus interrupting the HFE-TFR1-β2M complex (18). In agreement with the proposed importance of this interaction, it was shown that a constitutive binding of HFE to TFR1 in mice leads to an inability of HFE to regulate iron levels and development of iron overload (80). An interaction between HFE and bone morphogenetic protein (BMP) type I receptor, Alk3, has also been suggested to be another molecular mechanism of HFE-mediated regulation of hepcidin (97). It was proposed that HFE is required for the proper localization of the BMP receptor to the surface, in the absence of a disruption in the receptors in the BMP-SMAD (sma and mothers against decapentaplegic homologue) pathway mediated signalling of hepcidin (97).

Mice with a hepatocyte-specific deletion of *Hfe* develop iron overload similar to the global knockout (KO) mice demonstrating that hepatic Hfe is sufficient for

maintaining systemic iron homeostasis (87). Recent studies however have suggested that *Hfe* may have a function in maintaining iron homeostasis in erythroid cells (46, 73). In addition, using bone marrow transplants it was shown that macrophage *Hfe* expression can restore hepcidin expression levels in *Hfe*-deficient mice and decrease hepatic iron levels, suggesting a role for macrophage *Hfe* as well (53).

Transferrin receptor 2 (TFR2)

Transferrin receptor 2 (TFR2) was cloned independently by two groups. Glockner *et al*. (27) cloned a large portion of human chromosome 7 which contains *TFR2* and other important genes. A second study by Kawabata *et al*. (40) cloned *TFR2* and found that it coded for two transcripts, the α-and β-form (40). The α-form has 18 exons and encodes an 801-amino acid type II transmembrane protein. The protein has an amino-terminal cytoplasmic domain, a transmembrane domain and a carboxy-terminal extracellular domain (40). The β-form lacks exons 1-3 which encode the cytoplasmic, the transmembrane and a part of the extracellular domain of the protein, and is thought to be a soluble form of TFR2 protein, similar to the soluble form of TFR1 (40).

The extracellular domain of TFR2 has a high degree of homology with TFR1 (66% similarity and 45% identity) (40). Despite the structural similarity between the two transferrin receptors, they differ from each other in many ways. Similar to TFR1, TFR2 binds to holo-TF in a pH-dependent manner but with ~20-fold lower affinity (38, 96). It was suggested that TFR2 could be involved in the uptake of NTBI, as knockdown of TFR2 in Chinese hamster ovary (CHO) cells inhibited NTBI uptake

(30). The expression patterns of the two proteins are also significantly different, with TFR1 expressed in all tissues, while *TFR2* mRNA was found to be highly expressed in the liver and to a lesser extent in spleen, lung, muscle, prostate and peripheral blood mononuclear cells (PBMCs) (37-39). *Tfr1* knockout mice are embryonic lethal, dying of severe anemia and irregular development of the nervous system (48), whereas mice with a mutation in or knockout of *Tfr2* survive and develop significant iron overload (13, 22, 92).

TFR1 and TFR2 are also regulated differently; *TFR1* is regulated post-transcriptionally by intracellular iron levels through the iron-responsive element - iron-responsive element-binding protein (IRE-IRP) system due to the presence of IREs in its 3' UTR (42); *TFR2* does not have any IREs (40) and mice fed a high iron diet do not show any change in the mRNA levels of hepatic *Tfr2* (23). It has been shown that increased iron levels lead to an increase in the levels of TFR2 protein (35, 36) due to an increase in protein stability; the half-life of TFR2 in holo-TF treated cells increases from 4 to 14 hours (36).

The detection of *TFR2* mutations in patients with non-*HFE* iron overload (5) indicated that the product of this gene has an important role to play in the regulation of iron metabolism rather than the uptake of iron itself. Subsequent studies with mice: *Tfr2* mutant mice (13, 22), *Tfr2* knockout mice (92) and mice with a hepatocyte-specific knockout of *Tfr2* (93), confirmed that a disruption of *Tfr2* function leads to iron overload which can be attributed to inappropriate hepcidin levels

Recent studies from our laboratory (75, 91) and others (58, 59) have suggested an erythroid function for Tfr2 in anemic conditions. It was shown that in transgenic mouse models with either genetic (59, 91) or dietary (58, 75) iron-deficiency,

erythroid Tfr2 may be required for maintaining a proper number (58) or development of red blood cells (75, 91). The erythroid function of Tfr2 is independent of its hepatic function and as suggested may also be involved in erythropoiesis-mediated regulation of hepcidin (64). Hfe and Tfr2 form a vital component of the iron-mediated regulation of hepcidin, and the current hypotheses which predict their role are discussed in a later section.

Hemojuvelin (HJV/ HFE2)

The *HFE2* gene, which encodes for the protein hemojuvelin (HJV) was identified in 2004 as a result of positional cloning in patients with a severe form of iron overload known as juvenile hemochromatosis (65). The protein belongs to the family of repulsive guidance molecules (RGM) and shares 50-60% amino acid identity and key structural features with RGMA and DRAGON (RGMB), hence it is also known as RGMC (1). The protein product of this gene can either be cell membrane associated through a glycophosphosphatidylinositol (GPI) anchor or a soluble protein. A knockdown of *HJV* gene expression resulted in a corresponding decrease in hepcidin expression in the human hepatoma cell line Hep3B (49). Subsequent studies showed that HJV regulates hepcidin expression by acting as a co-receptor for the BMP receptors in the BMP-SMAD pathway (1, 43, 51, 98). The soluble and the GPI-anchored forms of HJV work in a reciprocal fashion. The GPI-anchored form provides a positive signal for the production of hepcidin through the BMP-SMAD pathway, whereas the soluble HJV competes with the BMP ligands and results in decreased hepcidin signalling (49, 50, 62).

Initially it was suggested that HJV is required for iron sensing but in a recent study (26) using *Hjv*$^{-/-}$ mice, which were fed a normal or an iron-rich diet, it was shown that the animals responded to dietary iron by increasing hepcidin irrespective of the presence or absence of HJV, suggesting that iron-dependent regulation of *Hamp* could function in the absence of HJV (26). Previous studies (71) where *Hfe*$^{-/-}$, *TfR2*$^{-/-}$, and *Hfe*$^{-/-}$/*TfR2*$^{-/-}$ mice were fed an iron-rich diet also showed that the animals respond to dietary iron changes by increasing *Hamp* (71). These results suggest that there appear to be more than one pathway involved in the regulation of hepcidin in response to iron levels (71) or that iron may be directly influencing the regulation of hepcidin. The *Hfe*/*Hjv* double knockout mice had a phenotype very similar to the *Hjv* KO mice as shown by similar transferrin saturation, hepatic and splenic iron concentrations (41), suggesting that both *Hfe* and *Hjv* may be involved in the same molecular pathways regulating hepcidin expression.

Although both *Hfe* and *Tfr2* are not regulated by iron levels at the mRNA level, a recent study has suggested that the same may not be true for *Hjv* (63), where two upstream open reading frames (ORF) in the 5' untranslated region of the mRNA have an AUG sequence and the ability to act as translation start sites. Under normal conditions, this alternate translation leads to repression of the main ORF. In conditions of iron overload, it was found that a hepatocyte-specific factor represses this alternate ORF and leads to an increase in the production of HJV protein through the main ORF (63).

In addition to the liver, HJV is also highly expressed in the skeletal muscle and promoter analyses revealed that it may be transcriptionally regulated at various stages of muscle development (82). This skeletal muscle *Hjv* does not appear to play

any role in the regulation of systemic iron homeostasis as shown by lack of iron loading in a muscle-specific KO mouse (9).

Matriptase-2 (TMPRSS6)

The gene encoding matriptase-2 (MT-2) was first identified as a result of *in silico* analysis performed in order to identify novel members of the type II transmembrane serine protease (TTSP) family of proteases (86). *Matriptase-2* also known as trans-membrane serine protease 6 (*Tmprss6)* was identified by positional cloning as a molecule affecting iron homeostasis in *mask* mutant mice. The *mask* phenotype is characterized by alopecia affecting the whole body except the face, and results from reduced absorption of dietary iron. It was also shown that mice with a disruption in the *Tmprss6* gene, including the *mask* mice, develop anemia (14, 21, 24). In adult human and mouse tissues, *Tmprss6* is primarily expressed in the liver, kidney and to some extent in the uterus as detected by *in situ* hybridization (33). It has been shown that *Tmprss6* mRNA expression increases in conditions of excess iron in both cells and mice (56). MT-2 protein was also shown to be stabilized in iron-deficient conditions in cells (102) and mice (99) suggesting a complicated iron-mediated regulation of MT-2.

MT-2 cleaves membrane HJV at an external site (83). There is some evidence that this interaction of HJV and MT-2 may be brought about by neogenin (16). The cleavage of HJV results in a disruption of BMP-SMAD signalling, leading to a

decrease in hepcidin transcription. Patients with mutations in *TMPRSS6* develop a rare form of anemia known as iron-refractory iron-deficiency anemia (IRIDA) which is characterized by high levels of hepcidin as the MT-2 protein is unable to cleave HJV. Similarly, mice with mutations in *Tmprss6* have increased hepcidin levels which are attributed to a constitutive activation of the BMP-SMAD pathway.

Hepcidin

Soon after it was discovered in 2000 by 3 separate groups, it was evident that hepcidin (encoded by *HAMP* gene) is central to systemic iron regulation. As mentioned earlier, hepcidin acts by binding to the only known iron exporter FPN. Given that hepcidin regulates iron, which is essential for the proper functioning of many molecules, it is not surprising that hepcidin itself is regulated by a wide variety of stimuli including but not limited to iron status, inflammation, erythropoiesis and hypoxia as reviewed in Rishi et al (77).

Hepcidin is expressed at very high levels in the liver and has also been shown to be expressed in the heart (54), adipose tissue (4), alveolar (60) and splenic (52) macrophages, retina (28) and also different regions of the rat brain (70). These studies suggest that in addition to the systemic regulation of iron by circulating hepatic hepcidin, there is a certain degree of local regulation as well in some tissues.

It is also apparent that several diverse signaling pathways can mediate hepcidin regulation. The two main signaling pathways known to regulate hepcidin are the BMP-SMAD pathway and the Janus activated kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) pathway. These two pathways regulate hepcidin

in response to two different stimuli, body iron levels and inflammation/infection, respectively. The stimulus for the JAK-STAT pathway comes from the various cytokines e.g. IL6, that are produced in response to an infection or inflammatory condition. The binding of a cytokine ligand, e.g. IL6, to its receptor, induces a signalling cascade which results in the phosphorylation of STAT3. The phosphorylated STAT3 then translocates to the nucleus where it binds to tissue-specific transcription factors and co-factors to mediate the transcriptional activation or repression of target genes.

The molecules discussed in this review are believed to be mainly involved in the iron-mediated regulation of hepcidin. The majority of the signaling in response to this stimulus (increased body iron stores) is through the BMP-SMAD pathway. So far transgenic mouse models lacking either one, two or three components of this pathway (as shown in Table 1) have been used to increase our understanding of how these molecules work. As seen in Table 1, most of the permutations and combinations have been tried in order to generate the transgenic mice to enhance our understanding. Using these, we have an idea how this pathway works, as explained below, but have yet to identify the molecular details of how the signaling works.

Iron-mediated regulation of Hepcidin: the role of BMP-SMAD signaling

Increased iron levels lead to an increase in the production of *Bmp6*. HFE and TFR2 are believed to be a part of the iron-sensing machinery which presumably relays the signal to the nucleus to increase *Bmp6* levels. The current theory is that in conditions of increased body iron levels TFR1 binds to saturated transferrin and is internalized,

297 thus releasing HFE (8, 25, 29). The free HFE then binds to TFR2 and this interaction
298 relays the iron-sensing signal (8, 25, 29). There have been studies which have both
299 supported or questioned (74, 79, 96) this interaction. The *Bmp6* produced in
300 response to increased iron levels is then believed to result in an increase in BMP6
301 protein levels. BMP6 is secreted and then binds to its receptors, the BMPRs I and II.
302 At this step, HJV acts as a co-receptor in this pathway and aids in BMP signaling.
303 Recently it has been suggested that HFE, TFR2, TFR1 and HJV all form a multi-
304 protein complex on the membrane of the hepatocyte (12) and that this is the complex
305 that mediates hepcidin signaling in response to increased iron levels (12). The
306 binding of BMP6 to its receptors induces a phosphorylation of BMPRII, this in turn
307 phosphorylates the SMAD1/5/8 complex. The phosphorylated SMAD1/5/8 then binds
308 to SMAD4 and this complex is then translocated to the nucleus. Inside the nucleus,
309 the complex binds to the SMAD binding sites on the DNA and results in the
310 transcription of the downstream SMAD responsive genes. In the hepatocytes, one of
311 these genes is *Hamp*.

312 This model supports our observations seen in mouse models which have been used
313 so far. There are however, a few unanswered questions. Firstly, how is *Bmp6*
314 produced? How is the increase in iron levels relayed to the nucleus? Is the BMP6
315 produced in the hepatocytes or in the non-parenchymal cells, as has been
316 suggested recently (15, 20). Is it the increase in transferrin saturation or the hepatic
317 iron levels that result in an increase in *Bmp6* synthesis (101)? What is the role of
318 HFE and TFR2 in this pathway, do they both act as sensors of iron levels? What is
319 the molecular nature of the signal they produce in response to increased iron? Last
320 but not least, is the BMP-SMAD pathway the only pathway involved in iron-mediated

hepcidin regulation and are HFE, TFR2, HJV, BMP6, MT2 the only molecules involved upstream?

The answer to the last question seems to be negative, as we have seen that when any of the mouse models of hemochromatosis are fed a diet rich in iron, their *Hamp* levels increase, suggesting that either iron can directly influence *Hamp* levels in the absence of either *Hfe* (10)*, Tfr2* (10)or *Hjv* (26) or the iron-mediated regulation of *Hamp* can utilize other molecules to induce the signal.

Using tissue-specific *Bmp6* KO mice it has been recently shown that *Bmp6* is produced in the liver sinusoidal endothelial cells (6). Mice lacking *Bmp6* in the endothelial cells developed iron-overload similar to the *Bmp6* global-knockout mice, whereas mice lacking *Bmp6* in the hepatocytes or the macrophages did not (6). As shown in Table 1, most of the molecules involved in the proposed sensing of iron status in the body are essentially performing their function in the hepatocytes, as shown by the hepatocyte-specific KO mice of *Tfr2*, *Hfe* or *Hjv*. These results suggest that the current iron-sensing hypothesis (as explained above) is incomplete and have added more weight to the view that iron-mediated regulation of hepcidin may involve inter-cellular cross talk, in which the molecular details are yet to be elucidated.

Several novel therapeutic interventions to ameliorate iron overload are being developed based on the molecules and signaling pathways discussed above. Exogenous BMP6 administration to *Hfe*$^{-/-}$ mice had decreased serum iron levels and transferrin saturation (11), demonstrating that BMP6 therapy may be effective in HH. *Tmprss6* has also been targeted effectively in mouse models of HH (31, 81) using either RNAi (81) or antisense oligonucleotides (31). A reduction in *Tmprss6* levels led to an increase in *Hamp* and reduction in transferrin saturation and body iron

levels. These results suggest that modulation of the BMP-SMAD pathway may be a therapeutic option to reduce body iron levels in HH patients.

There has also been an interest in targeting the hepcidin-ferroportin axis itself using hepcidin mimics called 'minihepcidins' (7, 68, 72). Minihepcidins are short peptides and consist of up to 9 N-terminal amino acids of hepcidin (68) and can effectively reduce the expression of cell surface ferroportin similar to natural hepcidin by binding to the protein and inducing its internalization. These therapeutic strategies have been effective in mouse models and are currently being developed further for clinical trials. One of the concerns with the *Tmprss6* targeting and BMP6 therapy would be the undesirable non-specific effects and the ability to direct these molecules to the site of action i.e. the liver. The minihepcidins seem to be a promising strategy, but whether we can modify them to have increased stability for effective use as a drug while maintaining their efficacy has yet to be shown.

The development of next-generation DNA sequencing tools has increased our ability to identify novel polymorphisms and mutations that act as modifiers of disease severity or may cause the disease itself. For example, using exome sequencing a variant in the Glyceronephosphate O-Acyltransferase (GNPAT) gene (54) was found to be associated with increased iron overload in a cohort of C282Y HFE patients, suggesting that this gene may have a role itself in modulating iron regulatory pathways. These novel discoveries and ongoing studies will help us understand the variable penetrance in the severity of iron overload seen in HH patients. Our knowledge of these proteins suggests that although these proteins are expressed at lower levels in other tissues, their function in iron homeostasis is only evident in the liver, thus enforcing the central role of liver in iron homeostasis.

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Figure Captions

Figure 1: Iron Homeostasis: Intercellular crosstalk between different cells in the liver. The hemochromatosis protein (HFE) and transferrin receptors (TFR) are involved in the regulation of hepcidin in the hepatocyte. The hepatocyte produces hepcidin in response to various stimuli including bone morphogenetic proteins (BMPs) and interleukin-6 (IL6). The endothelial cells of the liver produce BMP6 which then binds to the BMP receptors (BMPR) and BMP co-receptor hemojuvelin (HJV) on the hepatocyte cell membrane to activate the BMP-SMAD pathway. This pathway can be inhibited by matriptase 2 (MT-2) which cleaves HJV as indicated by the scissors. The macrophages in the liver produce inflammatory cytokines including IL6 in response to antigens and infectious agents. IL6 then binds to the IL6 receptor on the hepatocyte membrane resulting in the activation of the JAK-STAT pathway and eventual induction of hepcidin expression.

735 Table 1: Transgenic and knockout mouse models used to understand the role of the
736 liver in iron-mediated regulation of hepcidin.

737

Model	Serum iron as compared to WT	Hepatic iron	Hepcidin levels	Reference
Hfe$^{-/-}$	High	High	Low	(103)
Tfr2$^{-/-}$	High	High	Low	(13, 22, 92)
Hjv$^{-/-}$	High	High	Low	(34, 61)
Bmp6$^{-/-}$	High	High	Low	(55, 84)
AlbCre$^{+/-}$*Smad4*$^{f/f}$	High	High	Low	(95)
Tmprss6$^{-/-}$	Low	Low	High	(14, 21, 24)
Hfe$^{-/-}$/*Tfr2*$^{-/-}$	High	High	Low	(90)
Hfe$^{-/-}$*Hjv*$^{-/-}$	High	High	Low	(41)
Hjv$^{-/-}$/*Tfr2*$^{-/-}$	High	High	Low	(32)
Hjv$^{-/-}$ /*Tmprss6*$^{-/-}$	High	High	Low	(85)
Bmp6$^{-/-}$/*Tmprss6*$^{-/-}$	High	High	Low	(47)
Hfe$^{-/-}$/*Tmprss6*$^{-/-}$	Low	Low	High	(46, 91)
Tfr2$^{-/-}$/*Tmprss6*$^{-/-}$	Low	Low	High	(46, 59, 91)
Hfe$^{-/-}$/*Tfr2*$^{-/-}$/*Tmprss6*$^{-/-}$	Low	Low	High	(91)
$B_2m^{-/-}$/*Bmp6*$^{-/-}$	High	Higher than *Bmp6*$^{-/-}$ in females	Higher than *Bmp6*$^{-/-}$ in females	(44)
Bmp6$^{-/-}$ *Tfr2*$^{-/-}$	High	Higher than *Bmp6*$^{-/-}$ in females	Higher than *Bmp6*$^{-/-}$ in females	(44)
AlbCre$^{+/-}$*Tfr2*$^{f/f}$	High	High	Low	(93)
LysMCre$^{+/-}$*Tfr2*$^{f/f}$	Normal	Normal	Normal	(76)
AlbCre$^{+/-}$*Hfe*$^{f/f}$	High	High	Low	(87)

LysMCre$^{+/-}$*Hfe*$^{f/f}$	Normal	Normal	Normal	(87)
HSACre$^{+/-}$*Hjv*$^{f/f}$	Normal	Normal	Normal	(9)
AlbCre$^{+/-}$*Hjv*$^{f/f}$	High	High	Low	(9)
AlbCre$^{+/-}$*Bmp6*$^{f/f}$	Normal	Normal	Normal	(6)
LysMCre$^{+/-}$*Bmp6*$^{f/f}$	Normal	Normal	Normal	(6)
TekCre$^{+/-}$*Bmp6*$^{f/f}$	High	High	Low	(6)

738

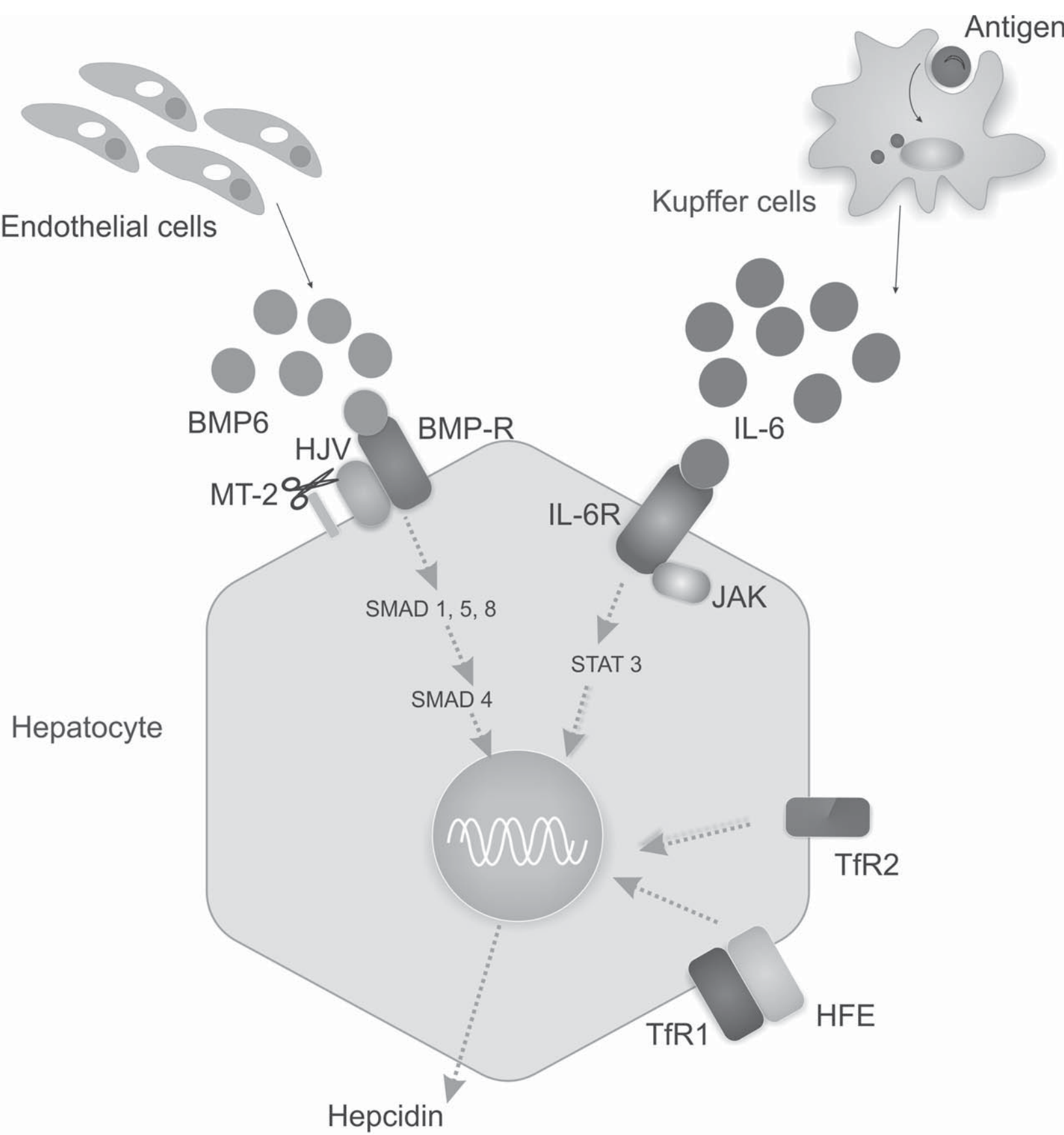

Figure 1. Rishi and Subramaniam