Chapter 1

Introduction
INTRODUCTION

1. Heme oxygenase

The search for the physiological mechanism of heme and hemoglobin degradation in the 1960s led to the discovery of the heme oxygenase system. The pioneering work was carried out in rats by Rudi Schmid’s group and reported between 1965 to 1972. They showed that the microsomal fraction of the liver, spleen and kidney had an enzyme system for degrading heme to bilirubin that required molecular oxygen and NADPH (Tenhunen et al., 1968). Their 1968 report was based on their findings that labeled hemoglobin or hemin injected into rats was converted into bile pigment mainly in the liver and the hemin accumulated in the microsomal fraction of the liver (references in Tenhunen et al., 1968). With biochemical characterization of the enzyme system they established that the heme is oxidized at the \( \alpha \)-methene bridge to form biliverdin and this in turn is reduced to bilirubin by the NADPH-dependent biliverdin reductase (Tenhunen et al., 1969, 1970). Biliverdin IX\( \alpha \) was the isomer produced in equimolar amounts to CO. They reported this enzyme system as a mixed function oxygenase which included a cytochrome P450 like hemoprotein as a terminal oxidase (Tenhunen et al., 1972).

The dependence of heme oxygenase activity on the microsomal NADPH-cytochrome c reductase was demonstrated by using an antibody to the enzyme (Schacter et al., 1972). The differences between the biochemical properties of the heme oxygenase system and the mixed function oxidase system observed by Schmid’s group (Tenhunen et al., 1969) were resolved when Maines and Kappas showed that cobalt induced heme degradation occurred independent of cytochrome P450 (Maines & Kappas, 1974, 1975). Their work established that the heme oxygenase system was a separate microsomal system. Maines and Kappas proposed that heme oxygenase and its substrate form a “transitory” hemoprotein and the substrate also functions as a prosthetic group for the enzyme (Maines & Kappas, 1977). Other groups (Hino & Minakami, 1979; Noguchi et al., 1979; Yoshida et al., 1980) showed that this system had a specific requirement for NADPH cytochrome P450 (cytochrome c) reductase but both NADPH and NADH could serve as cofactors.
Several subsequent studies have established the mechanism of heme degradation by heme oxygenase (EC 1.14.99.3) the first and rate limiting enzyme in this catabolic pathway (Reviewed in Ryter and Tyrrell 2000). It cleaves heme to form equimolar quantities of CO, biliverdin IXα and Fe^{2+} via several intermediates (Figure 1). The reaction requires 3 moles of molecular oxygen and NADPH cytochrome P450 reductase. The water soluble biliverdin is reduced to the hydrophobic bilirubin IXα by biliverdin reductase.

Subsequently in 1986 Mahin Maines’s group characterized two constitutive forms of heme oxygenase from rat liver microsomes, designated them as Hmox1 and Hmox2, and reported that only one molecular species of the enzyme is inducible (Maines et al., 1986). This study further suggested that the newly characterized Hmox1 was the same enzyme characterized earlier as heme oxygenase from the induced rat liver.

Heme oxygenase (Hmox) has two genetically distinct major mammalian isoforms: Hmox1, a 32kDa inducible protein, which is expressed at a high level in spleen and in other tissues involved in degradation of senescent RBCs but low levels are detected in virtually all mammalian tissues under normal physiological conditions (Ryter et al., 2006) and Hmox2, a 36kDa protein which is constitutively expressed at high levels in brain, testis, endothelium, kidney, liver and myenteric plexus of gut (Maines et al., 1986; Trakshel et al., 1986; Zakhary et al., 1996; Ryter et al., 2006). The two isoforms are encoded by separate genes: *hmox1* and *hmox2*. The human, mouse and rat *hmox1* gene and the rat *hmox2* gene have similar organization of 5 exons and 4 introns (Cruse & Maines, 1988). Both the isoforms exhibit almost identical biochemical properties, substrate specificity and cofactor requirements (Ryter et al., 2006). However, significant differences with regard to molecular weight, electrophoretic mobility, *K_m* values, thermolability, tissue distribution, transcriptional regulation, immunoreactivity and chromatographic properties were reported between the two isoforms obtained from rat tissues (Maines et al., 1986; Trakshel et al., 1986) (Table 1). Both Hmox1 and Hmox2 possess a COOH-terminal hydrophobic domain segment apart from heme catalytic site, that suggests a general membrane compartmentalization (Reviewed in Ryter et al., 2006). A third isoform, Hmox3 was
Figure 1: Degradation of heme by Heme oxygenase to produce equimolar quantities of CO, Fe$^{2+}$ and biliverdin IX$\alpha$. This reaction requires 3 moles of molecular oxygen and NADPH cytochrome P450 reductase. The water soluble biliverdin is reduced to the hydrophobic bilirubin IX$\alpha$ by biliverdin reductase. Adapted from Ryter and Tyrrell 2000
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hmox1</th>
<th>Hmox2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular weight (KD)</strong></td>
<td>32</td>
<td>36</td>
</tr>
<tr>
<td><strong>Km\textsubscript{heme} (\mu M)</strong></td>
<td>0.24 (3.8±0.5)</td>
<td>0.67</td>
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<tr>
<td><strong>Chromosomal localization (mouse)</strong></td>
<td>8</td>
<td>16</td>
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<tr>
<td><strong>Expression</strong></td>
<td>Highly inducible</td>
<td>Constitutive</td>
</tr>
<tr>
<td><strong>Tissue distribution</strong></td>
<td>Ubiquitous low basal levels, highest in spleen, liver, kidneys</td>
<td>Mainly brain and testes</td>
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<tr>
<td><strong>Inducers</strong></td>
<td>Heme, heavy metals, cytokines, growth factors, lipid metabolites, oxygen tension, ROS, gases</td>
<td>Glucocorticoids, hypoxia</td>
</tr>
</tbody>
</table>

**Table 1:** Characteristics of the two isoforms – Hmox1 and Hmox2
Adapted from Elbirt & Bonkovsky, 1999; Agarwal & Nick, 2000 and Ryter et al., 2006
also reported but later identified to be a pseudogene originating from hmox2 gene present in rat tissues (McCoubrey et al., 1997; Hayashi et al., 2004).

The biological significance of Hmox is related to its function in degrading pro-oxidant heme generated by the turnover of the heme containing proteins under various physiological and pathological conditions. Apart from preventing accumulation of free heme and thereby exhibiting antioxidant functions, Hmox also plays an important metabolic role of maintaining iron homeostasis.

- **Heme oxygenase 1**

  High basal levels of Hmox1 occur in macrophages of spleen and specialized reticuloendothelial cells of liver and bone marrow which degrade senescent red blood cells (Reviewed in Ryter et al., 2006). The constitutive expression of Hmox1 has been reported in human CD4+/CD25+ regulatory T cells, Kupffer cells of liver, renal medulla, and Purkinje cell layer in rat cerebellum (Nakaso et al., 2000; Zou et al., 2000; Bauer et al., 2003; Pae et al., 2003). In most other tissues low to undetectable basal levels of Hmox1 are detected which in response to various stimuli exhibit rapid transcriptional upregulation (Ryter et al., 2006). Hmox1 is induced and modulates tissue responses to injury caused by various physiological, pathophysiological and nonphysiological agents and conditions to regain homeostasis. Few of the known inducers of Hmox1 are heme and other metalloporphyrins, transition metals, inflammatory cytokines like IL-1α/β, IL-6, IL-10, IL-11, TNF-α, TGF-β, PDGF, NGF, IFN-γ, prostaglandins, UV light, phorbol esters, hyperthermia, hydrogen peroxide, lipopolysaccharide, thiol scavengers, sodium arsenite, hypoxia and hyperoxia, in a tissue specific manner (Elbirt & Bonkovsky, 1999; Ryter et al., 2006).

  Several pathological conditions like acute renal failure, atherosclerosis, myocardial ischemia, hypertension, transplant rejection, spinal cord as well as cerebrovascular accidents and injury, Alzheimer’s disease, sepsis and endotoxemia, acute pancreatitis, acquired immunodeficiency disease, hyperoxia-induced lung injury, asthma, chronic obstructive lung disease, iron deficiency anemia and iron
overload disorders, pleuritis, keratitis and retinopathy of prematurity also upregulate Hmox1 (Reviewed in Agarwal & Nick, 2000)

Data accumulated over the years indicates that the increased Hmox1 levels under various pathological, pro-oxidant and xenobiotic stresses, exerts a protective role. Hmox1 itself or by its enzymatic products i.e. iron, biliverdin IXa and CO functions to establish tissue homeostasis via suppressing oxidative stress and maintaining cellular integrity (Abraham & Kappas, 2008). Hmox1 has been reported to be cytoprotective in various models of lung and vascular injury, cardiovascular diseases, atherosclerosis, hypertension, renal injury, organ transplantation, hepatic ischemia-reperfusion injury and cirrhotic livers, anemia etc (Tracz et al., 2007a; Morse et al., 2009). The protective role of Hmox1 in mammalian central nervous system, aging and diseases is being debated (Schipper et al., 2009). Hmox1 also affords protection against non-cerebral form of malaria by suppressing the pro-oxidant effects of free heme produced by the Plasmodium chabaudi chabaudi (Seixas et al., 2009)

- **Heme oxygenase 2**

Hmox2 gene is located on chromosome 16 in both human and mouse (Ryter et al., 2006). Highest expression of Hmox2 occurs in testes, brain, central nervous system, vasculature, liver, kidney, and gut (Ryter et al., 2006). Although Hmox2 catalysis also produces the same reaction products as Hmox1, the inducibility of Hmox1 causes the latter to be much appreciated. Hmox2 is constitutively expressed and is refractory to induction but Hmox2 gene studies show the presence of a functional glucocorticoid response element (Raju et al., 1997).

The first Hmox2 KO mouse was generated in 1995 before the generation of the Hmox1 KO mouse, to study the potential role of CO in hippocampal long-term potentiation. The mutant mice are morphologically indistinguishable from their WT littermates and are fertile and survive normally (Poss et al., 1995).
Physiological role of heme degradation products

It is widely accepted that the protective functions of Hmox1 are mediated through the end products of the heme degradation reaction (Figure 2). Although initially viewed as physiological wastes, the end products of Hmox1 catalyzed reaction have drawn much attention and are now studied widely due to the paradoxical function in terms of cytotoxicity as well as cytoprotection. The reaction products: iron, CO and bile pigments are considered to mediate important functions at physiological levels whereas at elevated concentrations these molecules are potentially toxic.

Iron - Many research groups have identified and reviewed that the iron released from the Hmox1 catalyzed reaction is a cytotoxic pro-oxidant molecule which produces free radicals through Fenton chemistry. On the other hand it has also been acknowledged widely that Hmox1 derived iron has potential cytoprotective functions mediated through indirect mechanisms such as stimulation of ferritin synthesis and induction of iron efflux pumps. These two mechanisms in turn sequester or neutralize the pro-oxidant free iron thus affording cytoprotection (Nath et al., 1992; Vile et al., 1994; Gozzelino et al., 2010)

Carbon monoxide - Initially considered only as a air pollutant, the CO is now known to have significant importance in neurotransmission, vascular dilation, anticoagulation, and also has anti-inflammatory, anti-apoptotic and anti-proliferative effects varying according to the cell type, at low levels both in vitro and in vivo (Loboda et al., 2008; Ryter & Choi, 2009). CO participates in cell signaling and maintaining vascular homeostasis by directly activating soluble guanylyl cyclase and K+ channel (in vascular smooth muscle cells); and by indirect effects on endothelial-derived vasoconstrictor molecules and myogenic factors expression (Reviewed in Maines & Gibbs, 2005; Ryter et al., 2006)

Bile pigments - Biliverdin is reduced by biliverdin reductase to bilirubin, which is a potent antioxidant. Both biliverdin and bilirubin confer protection against cell necrosis induced by NO, H₂O₂, and hypoxia. The cytoprotective effects of bile pigments are mediated by inhibition of immune effector mechanisms that contribute to protective
functions of Hmox1 in a variety of immune-mediated inflammatory disorders like, ischemia reperfusion injury, transplant rejection, severe sepsis and vascular lesions due to hyperplasia (Reviewed in Maines & Gibbs, 2005; Gozzelino et al., 2010). Biliverdin also exhibits free radical quenching activity (Maines & Gibbs, 2005).

- The need for Hmox1 knockout mouse model

Chemical inhibitors of enzymatic activity have been used in the past as chemical probes in establishing the physiological role of specific enzymes (Appleton et al., 1999). On similar lines metalloporphyrins have been used to inhibit Hmox activity to variable degrees depending on the metal cations associated with the porphyrin ring and by the different ring substituents in vitro and in vivo (Vreman et al., 1993; Appleton et al., 1999). But several groups of researchers have criticized the use of metalloporphyrins to investigate the physiological role of Hmox as these metalloporphyrins not only inhibit Hmox activity but also inhibit nitric oxide synthase and soluble guanylyl cyclase (Grundemar & Ny, 1997; Appleton et al., 1999; Józkowicz & Dulak, 2003). Even chromium mesoporphyrin IX and zinc deuteroporphyrin IX 2,4-bis-ethylene glycol which have been reported to selectively inhibit Hmox activity, could cause a maximum inhibition of 89% and 80% of Hmox activity respectively (Appleton et al., 1999). Moreover the metalloporphyrins inhibit total Hmox activity without any specificity for the two isoforms.

Hence the development of Hmox1 gene-targeted mice and its subsequent use to elucidate the various developmental and physiological roles of Hmox1 was indispensable.

2. The Hmox1 knockout mouse

- The first mouse

The first Hmox1 gene-targeted mouse was developed by KD Poss and S Tonegawa on C57BL/6 x 129/Sv hybrid genetic background to describe a mouse
Figure 2: Heme oxygenase the protective enzyme. Iron released by Hmox activity exerts cytoprotective effects via ferritin synthesis. CO contributes to anti-inflammatory, anti-apoptotic and anti-proliferative pathways. Biliverdin –IXα and bilirubin – IXα are potent antioxidants which have anti-proliferative and anti-apoptotic effects.
Adapted from Ryter and Choi, 2009
model of human iron metabolic disorders (Poss & Tonegawa, 1997a). They observed 31% WT, 64% HET and only 5% KO mice at weaning by genotyping 223 progeny obtained from HET matings of Hmox1 mice. A lower than expected percentage of KO indicated partial prenatal lethality of the KO mice. This was also observed in KO x HET matings which yielded 11% KOs from 242 progeny mice genotyped at weaning. The survival percentage was approximately 20% to 22% of the expected KO in both types of mating. They further characterized the Hmox1 gene-targeted mice and reported observed phenotypes of the mutation.

They observed that the knockout mating pairs were sterile. At around 20 weeks of age all the KO mice suffered anemia, reduction in erythrocyte number, size and volume with anisocytosis, hypoferremia and tissue iron-loading disorders. Conspicuous iron loading was observed in renal proximal cortical tubules, Kupffer cells, hepatocytes and hepatic vascular tissues leading to increased protein and lipid peroxidation; hepatic and renal injury and fibrosis amongst the aging KO.

The phenotype included progressive chronic inflammatory disease characterized by enlarged spleen and lymph nodes, high peripheral WBC counts, increased splenic and lymph node CD4+:CD8+ T-cell ratios with many activated CD4+ T-cell and hepatic inflammatory cell infiltrates. With progressing age, all the KO mice at 20-24 weeks age exhibited mild and at 40-55 weeks displayed more severe phenotypes.

External morphological analysis revealed that the KO mice were slightly smaller as compared to the WT or HET littermates from birth to early adulthood and suffered late-onset weight loss, decreased mobility and premature mortality due to various phenotypic manifestations of Hmox1 gene loss as compared to the healthy HET or WT littermates. By 25 weeks of age, most KO mice became thin, poorly groomed, bred poorly, and appeared less active as compared to the WT or HET mice. Most of the KO mice which survived till adulthood, died after 25 weeks of age. The size of testes in the KO males was reduced to nearly 25% as compared to the HET littermates.

Challenging the KO cells with various oxidants both in vitro and in vivo revealed the cellular antioxidant properties of Hmox1 (Poss & Tonegawa, 1997b). Initially the authors demonstrated that free radical production increases and survival
of murine embryonic fibroblasts reduces upon exposure to oxidants like hemin, hydrogen peroxide, paraquat and cadmium chloride in vitro. Subsequently it was found that the KO mice were similarly more prone to hepatic injury and increased mortality when challenged with endotoxin as compared to the HET and WT littermates.

- The mice produced subsequently

Almost at the same time when KD Poss and S Tonegawa published their report, another Hmox1 gene-targeted mouse was developed by SF Yet and co-workers, with an aim to study the Hmox1 mediated adaptation of the cardiovascular system to chronic hypoxia in vivo (Yet et al., 1999). From the genotype analysis of the progeny of the heterozygous matings, the authors reported partial embryonic or neonatal lethality of the mutants. They observed that all the surviving KO mice were grossly normal.

In 2001, another Hmox1 gene-targeted mouse was developed by T Yoshida and his group to study the definitive physiological role of Hmox1 induction during myocardial ischemia/ reperfusion injury. They reported complete prenatal lethality of the KO. HET mice used for the experiments exhibited more susceptibility to ischemia/ reperfusion injury. (Poss & Tonegawa, 1997b; Yoshida et al., 2001).

Subsequently other research groups used Hmox1 gene-targeted mice generated by Poss and Tonegawa or Yet et al and confirmed the original reports of prenatal lethality (Zhuang et al., 2010; Zenclussen et al., 2011a). Apart from these reports, few other investigators (Kapturczak et al., 2004) used Hmox1 gene-targeted mice on other hybrid genetic backgrounds developed by backcrossing with same or other wild type parental strain and also reported prenatal lethality. A Agarwal’s group characterized and reported that the phenotype of the Hmox1 gene-targeted mouse on the new background was similar to originally reported characteristics by Poss and Tonegawa (Poss & Tonegawa, 1997a; Kapturczak et al., 2004).
Contrary to reports of extensive prenatal lethality H Matsumoto and his group backcrossed the Hmox1 gene-targeted mouse generated by KD Poss et al with the same parental strain and reported complete lethality of the KOs (Matsumoto et al., 2006).

- **Current understanding of Hmox1 knockout mouse phenotype**

After the availability of Hmox1 KO mice many groups of investigators further characterized the mutant mice and reported the observed phenotypes.

Contradicting Poss and Tonegawa’s observation of iron-loading disorders in older (20-25 weeks) KO mice, CD Ferris et al reported significant iron accumulation in livers of young KO mice of 10 weeks of age as compared to WT mice by atomic emission spectrometry (AES), and concluded that iron accumulation occurs at early age before it can be detected by Prussian blue staining, thus indicating that iron homeostasis was disrupted in these mice (Poss & Tonegawa, 1997a; Ferris et al., 1999).

Subsequently a Mg-ATP requiring iron transporter induced by iron was identified in mammalian cells, which causes transmembrane efflux of free ferrous ions generated by Hmox catalyzed heme degradation (Barañano et al., 2000). The ATP-dependent iron transport was strikingly augmented in the liver and kidney microsomes of KO as compared to HET and WT mice at 40 weeks age but was undetectable in younger mice of 10 weeks age. The increased iron accumulation in these tissues of KO mice could be the reason for augmented ATP-dependent iron transport. In contrast to liver and kidney, the spleen microsomes of mutant mice had a dramatic reduction in ATP-dependent iron transport as compared to HET and WT mice possibly due to anemia in mutant mice and resultant decrease in total erythrocyte turnover.

In a recent publication G Kovtunovych and co-workers attempted to provide an explanation for the abnormal tissue iron deposition observed in Hmox1 gene-targeted mice. In the spleen of mutant mice, initial splenic enlargement progressed to red pulp fibrosis, atrophy and functional hyposplenia as the KO mice ages, recapitulating the asplenia in Hmox1 human deficiency. The disruption of
phagocytosis of erythrocytes by spleen and liver in the KO mice caused due to reduced function and viability of erythrophagocytosing macrophages leads to the KO phenotypes of functional hyposplenism, intravascular hemolysis, endothelial and liver injury, pathological iron loading and resultant tissue damage. With in vitro experiments they showed that splenic macrophages from the mutants died after erythrophagocytoses due to inefficient metabolism of heme and subsequent heme intoxication. Thus senescent RBCs were no longer efficiently removed from circulation, leading to intravascular hemolysis and elevated circulating hemoglobin and heme. Hepatocytes of the mutants synthesized heme-scavenger protein hemopexin in response to increased hemolysis and the heme- hemopexin complexes are taken up and cleared in liver, which explained the observed iron loading in the KO mice hepatocytes. Hemoglobin and other heme-binding proteins are also taken up by proximal tubule cells and heme is metabolized by Hmox2 leading to kidney iron deposition (Kovtunovych et al., 2010).

The increased free iron in tissues of KO mice was speculated to modulate the expression of vascular cell adhesion molecule 1 which increased significantly by 3 to 4 folds in the liver of the KO mutants as compared to WT mice as quantified by real-time PCR. (Seldon et al., 2007).

Another group of investigators working on preeclampsia in human subjects reported soluble vascular endothelial growth factor receptor-1 and soluble endoglin are negatively regulated by Hmox1 in the deficient mice (Cudmore et al., 2007).

The postnatal lung development in the Hmox1 KO mouse model was characterized by T Zhuang and PA Dennery (Zhuang et al., 2010). They suggested that the defects in embryonic vasculature might significantly contribute to the lethality, although the exact mechanism underlying the lethality was not determined. The surviving KO mice displayed disorganized and simplified alveolar structure with less secondary septae and enlarged alveolar spaces, thinning of alveolar wall and thickening of interstitial section as compared to WT littermates at 10 days postnatal. Radial alveolar counts were also significantly decreased in mutant mice at 10 days postnatal. Thus they concluded that these functional defects of the mutant accounts
for reduced gas exchange surface area of the lung, leading to compromised pulmonary functions hence the partial postnatal lethality.

Similar to Poss and Tonegawa’s observation another group (Bak et al., 2003) reported that the KO mice were slightly lighter than the WT or HET mice. Most Hmox1 KO mice became thin, suffered weight loss, were poorly groomed, less active and died prematurely by the age of 22 weeks as compared to WT or HET mice. They also reported low survival percentage (~18%) of the KO mice obtained from HET x KO matings and that no viable litters are obtained from the KO mice parings.

The immune phenotype of the KO mice has also been characterized and the deficiency of Hmox1 is associated with pro-inflammatory tendency along with Th1 type cytokine responses. The number of CD3$^+$ and B220$^+$ cells was relatively low in the KO lymph nodes and IgM levels were significantly elevated in the KO as compared to the WT suggesting a possible abnormal B cell activation with defective immunoglobulin isotype switching. The splenocytes from the KO mice secrete disproportionately higher levels of pro-inflammatory Th1 cytokines as compared to the WT mice when stimulated with lipopolysaccharide or anti-CD3/anti-CD28 mitogen (Kapturczak et al., 2004).

Another group (George et al., 2008) reported significantly higher Foxp3-expressing cells among total CD4$^+$ and CD4$^+$CD25$^+$ cell population in the KO as compared to WT mice. Also, the expression of lymphocyte activation gene-3 (LAG-3), a protein thought to be correlated with increased suppression of T-regulatory cells was significantly lower in the KO mice suggesting that T-regulatory cell function could be abnormal.

As predicted from the immune phenotype of these mice, they are more susceptible to infections. Seixas E and the group reported 100% lethality of the KO mice after infection with Plasmodium chabaudi chabaudi causing non-cerebral malaria as compared to WT mice (Seixas et al., 2009).

The effect of Hmox1 deficiency on the reproductive physiology and pregnancy has been widely studied using the Hmox1 gene-targeted mouse as a model. Significant differences in the breeding patterns of WT and HET mice have been
reported. During gestation the weights of the placenta and embryos obtained from HET timed matings vary more than those obtained from WT timed matings. Comparison of placental weights obtained from the WT and HET matings at three gestational ages: 12.5 dpc, 15.5 dpc and 18.5 dpc, revealed that the placenta from the HET matings weighed less and were smaller than those obtained from the WT matings, though the standard deviations were high. At 12.5 dpc and 15.5 dpc differences in placental weights were found, which diminished as the gestation progressed to 18.5 dpc. It was speculated that the partial Hmox1 deficiency in HET mice resulted in delayed placental and embryonic development (Zhao et al., 2009).

Another group recently published two reports related to reproductive phenotype of Hmox1 KO mice and the fetal survival of KO (Zenclussen et al., 2011a; Zenclussen et al., 2011b). They reported that in Hmox1 deficient mice lesser oocytes are released and are also poorly fertilized as compared to WT controls (Zenclussen et al., 2011b). Although the number of follicles was comparable between KO and WT mice, the former had lower number of corpus luteum with increased apoptosis. It was suggested that since the corpus luteum synthesizes and releases hormones to cause pregnancy establishment and maintenance, Hmox1 emerges as a central figure in reproduction. They further compared the total implantations in the different combinations of matings of the three genotypes and concluded that although the total implantations did not differ significantly, fetal loss was significantly increased when KO males and females were paired with total fetal loss at 14 dpc. In vitro studies revealed that the KO blastocysts failed to attach to uterine endothelial cells. They also analyzed the histopathology of placentas obtained from different mating combinations and reported morphological abnormalities in the placenta of KO and HET embryos (Zenclussen et al., 2011a). They found that in the placenta of KO and HET embryos giant cells were significantly reduced, labyrinth areas were enlarged and junctional zone was either reduced or absent, revealing morphological abnormalities as compared to WT placentas. These placentas also exhibit fibrosis and haemorrhage. At 14 dpc the KO and HET placentas were significantly smaller than the WT. KO and HET embryos and whole implantation sites at 10 dpc were also significantly smaller than the WT. These observations led to the conclusion that the deletion of Hmox1
leads to insufficient nutrient and oxygen supply to the fetus leading to intrauterine growth restriction and fetal loss.

- **What we have learnt about the function of Hmox1**

  **Oxidative stress** - The antioxidant potential of Hmox1 was revealed by exposing the KO mice to various oxidative challenges (Poss & Tonegawa, 1997b). It was concluded that the KO mice were more prone to oxidative stress as compared to the HET and WT littermates. Many group of investigators have reviewed the antioxidative functions of Hmox1 (Elbirt & Bonkovsky, 1999; Agarwal & Nick, 2000; Ryter et al., 2006; Abraham & Kappas, 2008). Hmox1 also protects against oxidant damage in vascular system (True et al., 2007).

  **Protection against heme molecule toxicity** - Hmox1 provides protection against heme induced toxicity *in vivo*. The heme which is released from the destabilized hemoproteins during stress is lipophilic and can damage cell membrane, cytoskeleton, mitochondria and other intracellular molecules. Therefore degradation of heme by Hmox1 protects against the cellular toxic effects of heme itself. By using glycerol model of heme protein toxicity in KO mice, KA Nath and colleagues demonstrated the protective functions of Hmox1 against hemoglobin (Nath et al., 2000).

  **Kidney injury** - Nephrotoxicity caused by chemotherapeutic agents like cisplatin and glycerol (Nath et al., 2000), and in ischemia reperfusion injury (Wiesel et al., 2001) in the KO mouse suggests that Hmox1 plays a protective role in renal injury. The renal toxicity in KO was thought to be mediated by oxidant mechanisms brought about by free heme. Wiesel et al subjected the Hmox1 mice to reduced renal perfusion pressures by using one kidney-one clip model which led to volume retention and hypertension. The KO mice in absence of Hmox1 exhibit increased plasma creatinine levels, ischemic damage and mortality. These defects in the KO mice were related to vasoconstriction due to absence of Hmox1 derived CO and increased endothelin-1 levels.

  **Hepatic injury** - The liver plays crucial roles in iron homeostasis as well as in systemic inflammation, and Hmox1 is intricately involved in both the processes as
defined by the study of KO mice. Hmox1 is critically involved in physiological trafficking of iron both intra and extracellularly thereby regulating blood iron levels and relative tissue iron distribution (Immenschuh et al., 2010). The specific role of Hmox1 in liver is elucidated by phenotypic studies which revealed pathological hepatic iron loading in Kupffer cells, hepatocytes and hepatic vascular tissues leading to hepatic injury and dysfunction as well as chronic inflammatory disease in aging mutants (Poss & Tonegawa, 1997a). Hmox1 in the liver is also involved in phagocytosis of senescent RBCs as indicated by reduced viability of hepatic macrophages involved in erythrophagocytosis in KO mice (Kovtunovych et al., 2010).

Protection against sepsis or endotoxic shock - Hmox1 provides protection against endotoxemia and KD Poss and S Tonegawa first reported the in vivo protective role of Hmox1 during endotoxic shock (Poss & Tonegawa, 1997b). Administration of endotoxin leads to more liver injury and mortality in KO mice due to inflammation and resulting oxidative stress as well as tissue injury indicating that Hmox1 has a major antioxidant defense role. The protective role of Hmox1 during endotoxic shock caused by lipopolysaccharide (LPS) was demonstrated in WT, HET and KO mice at lower doses by another group (Wiesel et al., 2000). The Absence of Hmox1 leads to end organ damage of liver and kidney in KO mice mainly due to absence of CO which is a potent vasodilator and maintained levels of vasoconstrictor endothelin-1 causing reduced tissue perfusion.

Cardiovascular system protection - Study of blood parameters revealed that all KO mice had anemia, reduced erythrocyte number, size and volume as well as hypoferremia (Poss & Tonegawa, 1997a). A few group of investigators reviewed the involvement of Hmox1 in vasculogenesis as well as angiogenesis; and that impaired Hmox1 activity leads to decreased revascularization in ischemic or damaged tissues (Loboda et al., 2008; Grochot-Przeczek et al., 2010). Complete deficiency of Hmox1 leads to impairment of vascular endothelial growth factor (VEGF) mediated wound healing and the protective function of Hmox1 in vascular system is mainly mediated by CO (Reviewed in Loboda et al., 2008). Also, stromal cell-derived factor-1 (SDF-1) induced signaling in endothelial progenitor cells was decreased in the KO mice
leading to decreased blood vessel formation during regeneration of skin tissues and retinal neovascularization (Grochot-Przeczek et al., 2010). The bone marrow cells from KO mice formed fewer endothelial colony forming cells in vitro suggesting that Hmox1 contributes to endothelial progenitor cell (EPC) function and mobilization during wound repair (Wu et al., 2009). Hmox1 also plays an essential protective role against atherosclerosis and vein graft stenosis (Yet et al., 2003) and protects against arterial thrombosis and vascular damage during oxidative stress (True et al., 2007).

**Inflammation** - The KO mice had progressive chronic inflammatory disease and were more prone to hepatic injury and mortality caused due to inflammatory stress of endotoxin treatment (Poss & Tonegawa, 1997b). Hmox1 also protects against oxidative stress and end organ failure during inflammation caused by sepsis (Wiesel et al., 2000). Another group reported that cecal ligation and puncture caused sepsis in WT, HET and KO mice with complete loss of ileal villi and colon mucosal surface followed by higher mortality in KO as compared to others (Chung et al., 2008). High level of bacteremia was observed in KO mice indicating the importance of Hmox1 in protection against polymicrobial sepsis induced lethality. Infection of KO mice with experimental cerebral malaria indicated that Hmox1 has an important preventive role (Pamplona et al., 2007).

**Ischemia reperfusion injury** - To determine the role of Hmox1 in myocardial ischemia reperfusion injury, KO mouse was independently developed by Yoshida and colleagues (Yoshida et al., 2001). HET mice display reduced ventricular recovery, increased creatine kinase release, and increased infarct size after ischemia followed by reperfusion. It is now known that the Hmox1 cardioprotection against ischemia reperfusion is mediated by erythropoietin (Burger et al., 2009). Similar role of Hmox1 has been reported in hepatic, renal and cerebral ischemia reperfusion injury (Tsuchihashi et al., 2006; Tracz et al., 2007b; Zeynalov et al., 2009)

**Hypoxia** - Hmox1 plays a significant role in the adaptation of cardiomyocytes and cardiovascular system to chronic hypoxia and pulmonary hypertension (Yet et al., 1999). The heart of the KO mice suffered severe ventricular dilatation and infarcts with high percentage of mural thrombi and the cardiomyocytes had increased reactive
oxygen species causing cardiomyocyte death in absence of Hmox1. Thus it was concluded that Hmox1 is essential to adapt to hypoxia and pulmonary hypertension.

**Pregnancy and placental development** - In the mice, placental and decidual cells are important sources of Hmox1 and 2 during pregnancy and participate in the heme catabolism (Zenclussen *et al.*, 2005). High Hmox1 expression and activity in the trophoblast cells, located along the junction of maternal and fetal circulation suggests that the placental Hmox1 might be needed to supply iron, an essential element for cellular growth to the developing fetus and also for the removal of pro-oxidant free heme from the cells (Watanabe *et al.*, 2004). CO, produced by Hmox in placenta plays a significant role in the down regulation of uterine contraction and maintains a quiescent state of pregnant uterus. This is consistent with the observation that Hmox1 mRNA levels in placenta decline towards the end of pregnancy, when spontaneous labor occurs. The third product of the Hmox reaction, biliverdin IXα, is reduced to bilirubin IXα by biliverdin reductase A in both pregnant uterus and labyrinth and trophoblastic giant cells in placenta. Bilirubin IXα may then undergo facilitated transfer across the placental membrane and might serve as an antioxidant for the fetus (Stocker *et al.*, 1987).

Significant increase in Hmox activity has been reported in major maternal organs of mice such as liver, spleen and heart for coping with the physiological challenges of pregnancy. Although the blood volume increases during pregnancy, the systemic blood pressure in a healthy mother does not change significantly (Zhao *et al.*, 2008). This is brought about by significant dilation of afferent arterioles in pregnant as compared to non-pregnant mice, due to an increased Hmox activity leading to the subsequent production of carbon monoxide and resultant vasodilation.

Hmox1 is important for normal embryonic and placental development (Watanabe *et al.*, 2004). The Hmox1 is detected in early mouse embryos, as early as 6.5 dpc. Tin mesoporphyrin (SnMP), a potent competitive Hmox inhibitor when administrated to pregnant females leads to significantly increased maternal blood pressures but no effect was observed before 11.5 dpc (Zhao *et al.*, 2008). Histological studies of the 13.5 dpc placenta post-SnMP treatment by the authors suggested that the fetal circulation increased in order to compensate for the poor maternal-fetal
exchange, supported by the observation that there was a decrease in maternal blood cells in the maternal vascular compartment and an increase of fetal blood cells in fetal capillary space in the placenta.

Supporting the fact that Hmox1 has positive effect on pregnancy, Co-PP induced upregulation of Hmox1 protein at feto-maternal interface prevents fetal rejection caused by *Brucella abortus* infection (B. abortus) (Tachibana et al., 2008). Also, decreased Hmox1 in trophoblast giant cells due to knockdown by siRNA, served to induce cell death caused by B. abortus infection, supporting, the protective role of Hmox1 during pregnancy. It was also reported that T-regulatory cells are augmented during murine gestation, and mediate the maternal-fetal tolerance via local molecules such as Hmox1 among others, indicated by increased Hmox1 levels in placenta (Shou et al., 2009). Thus it is evident that Hmox1 plays a critical role in the maintenance of healthy pregnancy.

The placenta from the HET timed matings are lighter and smaller than those obtained from the WT timed matings and significant morphological differences are evident between the placentas of HET and WT matings at mid gestation. Histological analysis of 14.5 dpc placentas revealed morphological differences between HET and WT placentas. Thus, it was concluded that increasing degree of deficiency of Hmox1 protein in WT, HET and KO embryos obtained from heterozygous matings, exacerbates the vulnerability to placional defects and deaths. The variations were normalized at the later stages of pregnancy or after birth, implying that the state of Hmox1 deficiency may be substantially compensated by upregulation of other factors like Hmox2, iNOS, eNOS, soluble vascular endothelial growth factor receptor-1 and/or Mash-2 in placentas as speculated by the authors (Zhao et al., 2009)

In their subsequent publication (Zhao et al., 2011) they reported malformed feto-placental interface characterized by insufficient spiral artery remodeling, alterations of uterine natural killer cell differentiation and maturation; and abnormal changes in angiogenic profiles. These changes are independent of fetal genotype and are governed by maternal Hmox1 levels. They analyzed and compared the PCR array of angiogenic factors and their associated factors in decidual/ mesometrial lymphoid aggregates of pregnancy from wild type and heterozygous pregnancies at 10.5 dpc
thus concluding that of 84 genes involved in angiogenesis, 20 showed significant differences between the 2 genotypes, including Growth Factor and Receptors group, Cytokines and Chemokines group and Matrix Proteins and Transcriptional Factors group of genes. They concluded that growth restriction in placenta and fetus and the resulting placental vascular defects are induced by partial maternal Hmox1 deficiency.

Another group (Zenclussen et al., 2011a) has also characterized the KO and HET placentas and revealed morphological abnormalities in comparison to WT placenta.

Hmox1 seems to play an important role in placentation and maintenance of healthy pregnancy. It is also an important component in the regulation of maternal vascular tone during pregnancy and slight decrease in the enzyme’s expression and activity may lead to pregnancy-induced hypertension, preeclampsia, recurrent miscarriages, and spontaneous abortions.

3. Phenotype of the knockout embryos: Many unanswered questions

Mutation of the Hmox1 gene shows incomplete penetrance as is evident from the variable prenatal lethality reported by different groups. This is also reflected in the gestational age of embryonic lethality which was reported to be 10.5 dpc to 11.5 dpc (Poss, 1998; Zhao et al., 2009). Recently (Zhuang et al., 2010) lethality was reported in late gestation embryos (18.5 dpc) although it was significant only at P10. Taking into consideration the role of Hmox1 in placentation (Reviewed in the previous section), I think that the answer to basic question of gestational age of embryonic lethality is incomplete till date. The reasons being the type of mating and the depth of analysis. The only detailed analysis (Poss, 1998) was of mid to late gestation embryos (9.5 dpc to 13.5 dpc; 16.5 dpc and 19 dpc) from HET x KO matings. The other two reports were from HET x HET matings, but were too brief (Zhao et al., 2009) or for two late gestation embryo (Zhuang et al., 2010).

The initial report of phenotype analysis during development (Poss, 1998) was a general description of growth retardation and pallor in KO embryos which was
attributed to anemia. No abnormalities were observed in histological analysis. As the embryos analyzed were from HET x KO mating, there were no WT embryos for comparison. This is a major lacuna as comparison with WT littermates and embryos from WT matings is essential for phenotype analysis. In addition, determination of the developmental stages at any gestational age is required because it is not uncommon to observe a range of developmental stages within a litter even during normal development.

It was obvious to me that there were many unanswered questions in the phenotype analysis of Hmox1 mutant embryos. A systematic study would fill many lacunae and throw more light on the defect that causes embryonic lethality. Extensive literature survey till date indicates that such a comprehensive study has not been attempted or findings reported so far. Thus I set the aim of my study which are defined in the following objectives –

1. Establishment of Hmox1 gene-targeted mouse colony

To obtain the Hmox1 experimental mice from a colony which showed the known phenotype of the mutation this was a prerequisite for my study. Analysis of the colony parameters like the breeding pattern and the phenotype of Hmox1 mice provided important information that was used to define the criteria for experimental mice.

2. Phenotype analysis during mouse embryogenesis

To determine the role of Hmox1 during mouse development, the phenotype analysis of embryos from heterozygous matings was done with the following two approaches –

- Determination of gestational age of lethality
- Comparative morphological characterization of the embryos from the heterozygous and wild type timed matings.

3. Investigation into the cause of prenatal lethality

To determine the role of placentation in prenatal lethality, the placentas from the heterozygous and wild type timed matings were compared.