Chapter 6
General Discussion
GENERAL DISCUSSION

Hmox1 is the inducible isoform of heme oxygenase. It catalyses the degradation of heme to ferrous iron, biliverdin IXα and carbon monoxide. Hmox1 plays an important metabolic role in maintaining iron homeostasis by preventing accumulation of free heme. The first Hmox1 gene-targeted mouse generated to study the role of this isoform in iron homeostasis led to the discovery of partial prenatal lethality in the mutants (Poss & Tonegawa, 1997a). Subsequently other groups (Reviewed in Chapter 1) confirmed prenatal lethality but the extent of lethality observed was variable which has been attributed to the difference in genetic background (Montagutelli, 2000; Yoshiki & Moriwaki, 2006). Extensive prenatal lethality of the Hmox1 KO mice suggests that the gene plays an important role in embryogenesis. The analysis of the phenotype during embryogenesis was preliminary and incomplete. Keeping these in view, the aim of my study was to analyze the phenotype of Hmox1 deficiency during mouse embryogenesis and attempt to ascertain the cause of lethality in the KO embryos.

As a prerequisite for this study, Hmox1 mouse colony was established and partial prenatal lethality was also observed in our colony. The breeding profile of the wild type and heterozygous matings indicated similar pattern of reproduction with similar number of litters /MP though this is a contradiction to an earlier report (Zhao et al., 2009). The gender distribution was as expected with equal number of males and females in both type of matings. The phenotype analysis of the colony confirmed previous reports of lower survival of KO mice as compared to WT and HET littermates (Poss & Tonegawa, 1997a; Bak et al., 2003). A gender bias in survival was observed, (which has not been reported so far) with males surviving longer than the females.

The first step in phenotype analysis of the embryos obtained from the heterozygous timed matings was to carry out a systematic morphology analysis of the embryos from the gestational age 9.5 dpc to 18.5 dpc. Based on colony data it was clear that the females from 2 to 6 mo and males from 2 to 8 mo could be used for experiments and use of primiparous females would not introduce any bias in the
study. The genotype analysis revealed that most KO embryos start dying at 16.5 dpc which is inconsistent with the few published reports (Reviewed in Chapter 1). The prenatal lethality observed at late gestation (18.5 dpc) was much more than that observed in the colony and this was probably due to the difference in the breeding and experimental set up. Due to limitation of time and availability of experimental mice I could not investigate this.

To determine the phenotype of the mutation a systematic morphological analysis was carried out on the embryos from the heterozygous matings. In the embryos of the three genotypes, analysis of the embryo weights, developmental staging and external morphology was done between gestational ages 9.5 dpc to 18.5 dpc. Comparison of embryo weights revealed a trend of lower body weights in the KO embryos, similar to that reported earlier (Reviewed in Chapter 1). It is speculated that lighter KO embryos could be due to either growth or developmental retardation. Developmental staging analysis revealed marked developmental delay in the three genotypes at 9.5 dpc and 10.5 dpc. This delay disappeared in the WT and KO embryos from 11.5 dpc and 13.5 dpc onwards but persisted in the HET embryos throughout development. The analysis of the CR lengths taken together with developmental staging revealed that embryos of the three genotypes exhibit variation in size and/or growth retardation from 12.5 dpc onwards. Growth retardation was more pronounced in KO embryos only at 11.5 dpc and 16.5 dpc. Growth retardation of the mid-gestation embryos from heterozygous matings has been reported (Zhao et al., 2009) earlier. As my results show growth and developmental retardation in the three genotypes, it was most likely a consequence of partial deficiency of the maternal Hmox1. Furthermore, due to the relatively small sample size of the KO embryos in my analysis, it is not clear whether the KO embryos are more susceptible to growth retardation.

External examination also revealed reduced vasculature in KO, HET and WT embryos between 12.5 dpc and 16.5 dpc. This defect is a phenotype of the Hmox1 mutation as many KO embryos show the reduced vasculature whereas none of the WT embryos had this defect. Normal vasculature observed in few of the KO embryos could be the reason for survival of a small percentage to adulthood. It is
speculated that the appearance of the reduced vasculature could be due to poorly
developed blood vessels or due to reduced number of red blood cells. During
embryogenesis, the vascular development occurs by vasculogenesis and angiogenesis,
both stimulated by VEGF (Loboda et al., 2008; Grochot-Przeczek et al., 2010) and it
is known that Hmox1 promotes vascular development by induction of VEGF and
SDF-1 (Dulak et al., 2004; Grochot-Przeczek et al., 2010). It is thus speculated that
the deficiency of Hmox1 in KO embryos leads to a more pronounced reduction in
vasculature by affecting the function of VEGF and SDF-1. Gene expression analysis
is required to find this correlation and also to understand the less severe phenotype
observed in the WT and HET embryos. Similarly the occurrence of reduced
vasculature due to reduced red blood cells also needs to be investigated.

Many groups of investigators have observed phenotype of Hmox1 deficiency
in adult mice (Reviewed in chapter 1) as well as in human. Similar to the Hmox1 KO
mouse, the human Hmox1 deficiency cases displayed vascular system pathologies
including anemia, tissue iron deposition, elevated levels of haptoglobin, ferritin and
heme in serum, severe enhanced endothelial cell injury and intravascular hemolysis
(Kawashima et al., 2002; Koizumi, 2007). Taken together, these studies indicate a
critical role of Hmox1 in vascular system and its functioning in adults as well.

The role of placentation in the prenatal lethality of the KO embryos was
determined by analysis of the placentas from the heterozygous matings. This study
revealed that these placentas exhibit suboptimal developmental and placental defects,
indicated by wide variation in weights as well as defects in tissue organization (in
placentas from WT and KO embryos) as compared WT embryos. These placental
defects could be attributed to decreased Hmox1 levels in heterozygous maternal
background, confirming that maternal Hmox1 level is an important factor in the
normal development of placenta. It is important to note that placental defects were
observed in WT and KO embryos but only the KO embryos die prenatally. Thus
defective placentation cannot be the reason of prenatal lethality of the KO embryos.
Our speculation is supported by a recent publication (Zhao et al., 2011) reporting that
the malformation of the fetoplacental unit occurs in case of HET maternal background
is independent of the genotype of the embryos. We thus infer that defects in placental
development lead to disruption of its functions to some extent and is possibly the cause of growth retardation of these embryos but not prenatal lethality of the KO embryos. Highlighting the role of Hmox1 in the human pregnancy, it has been reported that Hmox1 is important for maintenance of healthy pregnancy and suboptimal levels due to microsatellite polymorphism leads to idiopathic recurrent miscarriages (Denschlag et al., 2004). Recently (Zenclussen et al., 2011a) the role of Hmox1 in successful pregnancy has been demonstrated elegantly using the Hmox1 gene-targeted mouse model.

The results presented in this thesis show for the first time the defective vasculature phenotype in the Hmox1 embryos. This phenotype was more severe in the KO embryos. Taken together with the embryonic lethality results, it is speculated that the KO embryos with the most severe vasculature phenotype die prenatally. The less severe vasculature phenotype observed in some WT and HET embryos were not affecting the survival of these two genotypes. This work has set the direction in our laboratory for further investigation into the cause of embryonic lethality of the Hmox1 KO embryos.