4. DISCUSSION

4.1 Technical considerations of gene targeting experiments

Homologous recombination in ES cells is a rather low frequency event, and any procedure published so far must therefore include a selection step that reduces the number of clones to be screened. Cappechi and co-workers (Mansour, Thomas *et al.* 1988) introduced a strategy called the "positive/negative" selection to enrich for clones that underwent homologous recombination, as opposed to random integration. In brief, it is based on a targeting vector harbouring the sequence to be inserted, a positive selection marker (e.g. neo^r gene), and a thymidine kinase (TK) gene from herpes simplex virus (HSV) inserted at the end of the linearised targeting construct. Cells that undergo homologous recombination lose the TK gene, whereas cells into which the construct integrated randomly do not and can be eliminated using a toxic nucleoside analogue, such as gancyclovir or FIAU [1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil]. These drugs are efficiently recognised as a substrate by the viral thymidine kinase, but not by the endogenous cellular thymidine kinase. Subsequent integration of gancyclovir into the replicating DNA leads to termination of synthesis and cell death. Variations on the theme with increased enrichments consist in the use of two instead of one TK gene (Chisaka and Capecchi 1991), of a diphtheria toxin A (DT-A) fragment (Yagi, Ikawa *et al.* 1990), or, of an expression cassette encoding the interleukin-2 receptor α-subunit (IL-2-Rα) whose expression upon random integration can be negatively selected using a recombinant immunotoxin directed against IL-2-Rα. (Kobayashi, Ohye *et al.* 1996). For genes that are expressed in ES cells, conditional positive selection is another

option. In this case the targeting vector is designed in such a way that expression of a selectable marker, e.g. neo^r, depends on homologous recombination to supply a missing regulatory element, such as the promoter/enhancer, or a polyadenylation signal (Donehower, Harvey *et al.* 1992; Mansour, Goddard *et al.* 1993; Schwartzberg, Goff *et al.* 1989). Although the mechanism of homologous recombination is not clear, great concern should be taken with respect to the design of the targeting construct. First, the two isogenic DNA sequences in the construct should be homologous to those of ES cells to be targeted; second, the length of homologous DNA sequences should be maximized and repetitive sequences should be avoided. These will help to increase the probability of the homologous recombination event. In the present study, two homologous sequences of the $K_{Ca}3.1$ gene were amplified from ES cell genomic DNA and cloned into the pTV0 vector in the same orientation flanking the neo^r cassette. The TK cassette was placed downstream of the homologous sequences (Figure 3.1). In the present study, PCR was routinely used to detect positive ES cell clones in which the neo^r cassette had been homologously integrated into the genome. Following neomycin and gancyclovir double selection, only one of 238 of targeting vector-transfected ES cell clones showed homologous recombination.

ES cells are derived from the inner cell mass (ICM) of an embryo and can be cultured in vitro, maintaining their ability to differentiate to all kinds of cell lineages when reintroduced into a blastocyst (Bradley, Evans *et al.* 1984). The majority of targeted mouse mutants to date have been generated using ES cells derived from the 129 substrains (due to their higher frequency of germline transmission compared to C57Bl/6 derived ES cells). ES cells (E14.1) used in present study were subcloned from E14TG2a originated from 129/OlaHsd mice. Moreover, great care was taken to handle the ES cells in culture because their totipotency is only evaluated by their ability to generate

good chimeras which would be three months after the beginning of the experiment. Some typical steps were followed in ES cell culture. First, basic tissue culture rules were followed; second, ES cells were passaged as little as possible, third, ES cells in culture never reached confluency (at most 60%). In addition, ES cells in culture needed to grow on mitotically inactive feeder cells, in this study, i.e. mouse embryo fibroblasts (MEF). These cells were isolated from 13.5 d mouse embryos derived from a transgenic mouse strain harboring the neo resistant gene. Feeder cells kindly support the ES cells by providing leukemia inhibitory factor (LIF) which maintains ES cells' totipotency. Cultivation of MEFs was not as hard as ES cells although they were well prepared prior to plating ES cells on them.

The generation of mice containing defined mutations has contributed considerably to the understanding of gene function in mammals. Yet, this regionally and temporally unrestricted genetic deletion may lead to severe developmental defects or embryonic lethality (Joyner and Guillemot 1994). The application of site-specific recombinase systems along with gene targeting techniques in ES cells have now made it possible to modify the gene in a conditional manner (Lobe and Nagy 1998; Nagy 2000) so that experimenters can elucidate a complete picture of gene function in which the complete knockout leads to an early lethal phenotype preventing the study of its later roles and the phenotype affects multiple tissues preventing the detailed study of its function in a particular cell lineage. In present study, the *Cre-loxP* system was tried to conditionally inactivate the $K_{Ca}3.1$ gene because $K_{Ca}3.1$ channels are distributed in multiple tissues including endothelium, smooth muscle and T cells etc and $K_{Ca}3.1$ channels may play pivotal role in various cell proliferation (Grgic, Eichler *et al.* 2005; Kohler, Wulff *et al.* 2003). Targeting construct was produced as depicted in figure 2.1 B in a way that two *loxp* sites were introduced upstream and downstream of the exon 4 of the

$K_{Ca}3.1$ gene and additional *loxp* site flanked cassettes allowing positive and negative drug selection which was inserted downstream of exon 4. In this construct, the negative selection marker was floxed (flanked by *loxp* site) together with the positive selection marker, allowing only positive selection for enriching the targeted allele. This explains why none of ES cell clone from 998 clones which survived following neomycin selection for 6 days showed homologous recombination. This result also confirms that double selection is much more effective than the selection with only one selection marker. Since the conventional gene disruption of $K_{Ca}3.1$ did not lead to early lethal phenotype, the conditional gene targeting experiment was temporally discontinued and the targeting vector was stored for future experiment.

4.2 Complete knockout of $K_{Ca}3.1$ channel in homozygous mutant mice

Breeding of mice heterozygous for the $K_{Ca}3.1$ gene disruption yielded $K_{Ca}3.1^{-/-}$ progeny in expected Mendelian proportions and with normal sex ratios. The $K_{Ca}3.1^{-/-}$ mice survive to adulthood and all mutant mice behave normally. Furthermore, mutant mice showed normal fertility. By far $K_{Ca}3.1$ knockout mice stay healthy for 8 months. Whether or not other complications may occur with further aging or total lifespan is altered due to the inactivation of $K_{Ca}3.1$ remains to be explored.

To genotype gene-targeted mice, multiplex PCR was used as a routine method since it is fast and thus suitable for screening large populations of mice. The neo resistant gene left in the disrupted $K_{Ca}3.1$ gene was detected by PCR so that $K_{Ca}3.1^{-/-}$ mice can be distinguished from their wild-type

counterparts. Southern blot analysis was employed to further confirm the PCR results of genotyping. The probe used for southern blot analysis hybridized to complementary DNA fragments digested by endonuclease BglII. The result showed the expected different signals for $K_{Ca}3.1^{-/-}$ mice and wild-type mice. The PCR-genotyping results together with southern blot results clearly demonstrated that the $K_{Ca}3.1$ gene of both alleles has been disrupted by replacement of exon4 of the gene by a neo resistant cassette in $K_{Ca}3.1^{-/-}$ mice. To check whether or not the consequent gene expression of $K_{Ca}3.1$ was abolished, mRNAs extracted from mice spleen were investigated by RT-PCR. Primers used in this assay were designed to detect not only the loss of exon4 but also the remaining mRNA of the $K_{Ca}3.1$ (Figure 3.3). The results showed a lack of $K_{Ca}3.1$ specific transcripts in $K_{Ca}3.1^{-/-}$ mice, indicating that not only exon4 was not expressed but the whole mRNA of $K_{Ca}3.1$ protein was absent in homozygous mutant mice. This might be explained by the fact that any transcribed mRNA which can not be translated appropriately is rapidly degraded by ribonucleases.

Previous studies showed that $K_{Ca}3.1$ channels are functionally expressed in the membrane of endothelial cells, lymphocytes, neonatal smooth muscle cells, erythrocytes, etc (Ayabe, Wulff *et al.* 2002; Ghanshani, Coleman *et al.* 1998; Ghanshani, Wulff *et al.* 2000; Ishii, Silvia *et al.* 1997; Joiner, Wang *et al.* 1997; Logsdon, Kang *et al.* 1997; Meyer, Schonherr *et al.* 1999). To further check whether or not the functional $K_{Ca}3.1$ channel is absent in $K_{Ca}3.1^{-/-}$ mice, channel currents were examined in naive $CD4^+$ T-cells by the whole-cell patch-clamp technique. In $CD4^+$ T-cells, a Ca^{2+}-activated K^+-current with the electrophysiological properties of $K_{Ca}3.1$ was activated by cell dialysis with Ca^{2+}. This current was completely abolished by ChTX in T-cells from wild-type mice, while no K_{Ca} current was detected in $K_{Ca}3.1^{-/-}$ mice. This indicates that

functional $K_{Ca}3.1$ proteins are absent in the knockout mice. In endothelial cells, apamin and ChTX together abolished the Ca^{2+}-activated K^+ currents in $K_{Ca}3.1^{+/+}$ wild-type mice while apamin alone blocked the Ca^{2+}-activated K^+ channels in $K_{Ca}3.1^{-/-}$ mice. Likewise, the combination of TRAM-34 and UCL1684 abolished the Ca^{2+}-activated K^+ currents in ECs from $K_{Ca}3.1^{+/+}$ wild-type mice, while UCL1684 alone abolished the K_{Ca} currents in ECs from $K_{Ca}3.1^{-/-}$ mice. These results further confirmed that knockout mice lack the $K_{Ca}3.1$ channel current. In addition, the results in T-cells and ECs are also consistent with the published expression pattern of K_{Ca} channels, i.e. $K_{Ca}3.1$ is the exclusive K_{Ca} channel expressed in T-cells (Ghanshani, Wulff *et al.* 2000), and both $K_{Ca}2.3$ and $K_{Ca}3.1$ are expressed in endothelium (Eichler, Wibawa *et al.* 2003; Gutman, Chandy *et al.* 2003; Hosseini, Benton *et al.* 2001; Kohler, Hirschberg *et al.* 1996; Kohler, Brakemeier *et al.* 2001; Kohler, Degenhardt *et al.* 2000; Stocker 2000; Tacconi, Carletti *et al.* 2001). Unfortunately, western blot analysis of $K_{Ca}3.1$ could not be performed as a specific antibody against this protein is not commercially available so far. However, showing the lack of protein function is also a suitable approach to prove the absence of the $K_{Ca}3.1$ channel. Taken together, southern blot analysis of the targeted gene locus together with PCR and RT-PCR analysis as well as functional analysis of the channel protein clearly demonstrate that gene targeting of $K_{Ca}3.1$ was successful.

4.3 Phenotypes of female and male $K_{Ca}3.1^{-/-}$ mice

4.3.1 Morphological and histological changes of major organs in $K_{Ca}3.1^{-/-}$ mice

Female $K_{Ca}3.1^{-/-}$ mice showed enlarged internal organs, such as spleen, heart

and kidney, which were significantly enlarged by 51%, 12% and 13% respectively, compared to their wild-type littermates. Male $K_{Ca}3.1^{-/-}$ mice spleens were also 38% heavier and were also visibly larger than those of male wild-type mice. Kidneys from male $K_{Ca}3.1^{-/-}$ mice showed also a tendency to be larger although the difference did not reach statistical significance. In contrast to female $K_{Ca}3.1^{-/-}$ mice, heart weight in male $K_{Ca}3.1^{-/-}$ mice was not different and no changes in heart size were apparent compared to their wild-type littermates. Histological examination revealed no gross pathological changes in spleen as well as the kidney of both male and female $K_{Ca}3.1^{-/-}$ mice. In kidney from $K_{Ca}3.1^{-/-}$ mice, glomeruli and tubular structures were intact with no sign of inflammation, proliferative malignancies or fibrosis. In contrast, hearts of female but not males showed signs of left ventricle hypertrophy (LVH) as judged by the larger size and increased wall thickness of the left but not right ventricle.

The spleen is an organ which is known to be involved in the production and turnover of red blood cells, the production of certain circulating white blood cells especially in the adult animal, and is therefore a part of the lymph system and the immune system. Because of its wide variety of functions, the spleen may be affected by many conditions involving the blood or lymph system, and by infection, malignancies, liver disease, and parasites. Since $K_{Ca}3.1$ is expressed in both erythrocytes and T-cells (Gardos 1958; Ghanshani, Wulff ***et al.*** 2000), a possible reason would be excessive degradation of aged and/or defective red blood cells caused by some kinds of anemia due to the lack of $K_{Ca}3.1$. At this stage it should be pointed out again that $K_{Ca}3.1$ is critically involved in erythrocyte volume regulation. So the lack of $K_{Ca}3.1$ may accelerate erythrocyte aging, although this must be investigated in more detail in future studies. Other possible causes would be a certain lymphoid cancer or some kind of infection.

For the enlargement of heart in female mice, the elevated blood pressure would be a good explaination, i.e. the adaption of the elevated blood pressure leads to excessive growth of myocytes in heart. For example, left ventricular hypertrophy (LVH) is thought to be a structural adaptation of the heart, at least in part, as a maladaptive mechanism to systemic arterial hypertension (Bielen, Fagard *et al.* 1991). Furthermore, high blood pressure can damage blood vessels over time throughout mice body, including vessels in kidneys. This would lead to the enlargement of the kidney.

4.3.2 Composition of K_{Ca} currents in female and male mice

Previous studies including studies from our group demonstrated that both $K_{Ca}3.1$ and $K_{Ca}2.3$ are expressed in vascular endothelium in mice and rats (Bond, Sprengel *et al.* 2000; Kohler, Brakemeier *et al.* 2001; Kohler, Degenhardt *et al.* 2000; Latorre, Oberhauser *et al.* 1989; Latorre, Vergara *et al.* 1983; Mark S. Taylor, Adrian D. Bonev *et al.* 2003; Vergara, Latorre *et al.* 1998). In ECs from rat carotid artery, the K_{Ca}-currents conducted by $K_{Ca}3.1$ and $K_{Ca}2.3$ were reduced to 60-70% of the maximal current amplitude in the presence of the selective $K_{Ca}3.1$-blocker TRAM-34 (100 nmol/L), and the remaining TRAM-34-insensitive K_{Ca}-current was almost completely abolished by the $K_{Ca}2.3$-blocker apamin (200 nmol/L) (Eichler, Wibawa *et al.* 2003). This shows that a substantial contribution of $K_{Ca}2.3$ to K_{Ca}-currents in rat EC cells. In the present study, K_{Ca} currents were characterized by patch-clamping technique and pharmacological tools. In brief, dialysis with Ca^{2+} in ECs induced a hyperpolarizing outward current with slight inward rectification at positive membrane voltages, the reversal potentials extrapolated from current-voltage relations were around -80 mV which is near the K^+ equilibrium potential, such outward current was completely abolished by the combination

of selective blockers of $K_{Ca}3.1$ and $K_{Ca}2.3$, such as TRAM-34 and UCL1684. All these features fit well to the published electrophysiological properties and pharmacological profiles of $K_{Ca}3.1$ and $K_{Ca}2.3$ in ECs (Kohler, Brakemeier *et al.* 2001; Kohler, Degenhardt *et al.* 2000).

Furthermore, compositions of K_{Ca} currents in mice aortic ECs were quantitatively determined. Interestingly, $K_{Ca}3.1$ currents contribute about 44% to the total K_{Ca} currents in female wild-type mice, whereas only about 32% in male wild-type mice (Figure 3.6). Moreover, total K_{Ca} current was significantly reduced in female $K_{Ca}3.1^{-/-}$ mice but not in male $K_{Ca}3.1^{-/-}$ mice. Thus, female $K_{Ca}3.1^{-/-}$ mice could lose approximately 12% more of K_{Ca} currents than male $K_{Ca}3.1^{-/-}$ mice. This may account for any different phenotype between female and male knockout mice. In fact, as described in last section, although both male and female $K_{Ca}3.1^{-/-}$ mice had an enlarged spleen, the comparison between female $K_{Ca}3.1^{-/-}$ mice and their wild-type littermates showed a much more significant difference than that between males. In addition, female $K_{Ca}3.1^{-/-}$ mice showed around 12% enlarged heart than wild-type female mice, while male $K_{Ca}3.1^{-/-}$ mice just showed a tendency of increased heart weight without statistical worth.

4.3.3 Probable compensation of K_{Ca} currents in male $K_{Ca}3.1^{-/-}$ mice

The $K_{Ca}3.1$ gene mutation may prompt compensatory adaptations during development that may overcome the effect of the alteration. These compensation, most likely from the co-expressed $K_{Ca}2.3$ channels in mice ECs, can therefore influence the phenotype of $K_{Ca}3.1^{-/-}$ mice. Thus, in this study, apamin-sensitive $K_{Ca}2.3$ currents in ECs of $K_{Ca}3.1^{-/-}$ mice were compared with those in ECs of wild-type mice (Figure 3.7). The results showed the amplitude of $K_{Ca}2.3$ currents, the exclusive source of K_{Ca} currents in ECs from $K_{Ca}3.1^{-/-}$ mice, did not have a significant change between $K_{Ca}3.1^{-/-}$

mice and their wild-type littermates, suggesting no obvious compensatory effect due to the lack of $K_{Ca}3.1$ in $K_{Ca}3.1^{-/-}$ mice. In addition, total K_{Ca} currents in ECs of $K_{Ca}3.1^{-/-}$ and wild-type mice were also determined. Figure 3.6 E shows that the total K_{Ca} currents in female $K_{Ca}3.1^{-/-}$ mice were about 58% of those in female wild-type mice, while the total K_{Ca} currents in male $K_{Ca}3.1^{-/-}$ mice were about 80% of those in male wild-type mice. Since the $K_{Ca}2.3$ currents are the exclusive source of K_{Ca} currents in ECs of $K_{Ca}3.1^{-/-}$ mice, and $K_{Ca}2.3$ currents contribute 55% and 67% to the total K_{Ca} currents in wild-type female and male mice respectively (Figure 3.6 F, G), this result showed around 13% increase of the $K_{Ca}2.3$ currents in male $K_{Ca}3.1^{-/-}$ mice, whereas no alteration of $K_{Ca}2.3$ current in female $K_{Ca}3.1^{-/-}$ mice, suggesting there might be a low-level elevation of the $K_{Ca}2.3$ currents as a compensatory adaptation in male $K_{Ca}3.1^{-/-}$ mice, but no such effect in female $K_{Ca}3.1^{-/-}$ mice.

4.3.4 Impaired EDHF-type vasodilation in female $K_{Ca}3.1^{-/-}$ mice

In the 1980s, it was found that the endothelium regulates the contractile state of vascular smooth muscle by releasing nitric oxide (NO) and prostacyclin (PGI_2) (Furchgott and Vanhoutte 1989; Furchgott and Zawadzki 1980; Ignarro, Buga *et al.* 1987; Palmer, Ferrige *et al.* 1987). Later, evidence began to emerge that besides NO and PGI_2 a third endothelium-derived relaxing factor must exist since blockers of NO and PGI_2 synthesis did not abolish endothelium-dependent vasodilation completely. This was termed EDHF as this factor causes hyperpolarization of the VSMC and thereby induces vasodilation (Chen, Suzuki *et al.* 1988; Golding, Marrelli *et al.* 2002; Taylor and Weston 1988). This implies the activation of potassium channels in vascular cells and in fact, EDHF-mediated relaxation of the smooth muscle is inhibited by blockers of potassium channels. Previous studies in our group

demonstrated that EDHF-mediated vasodilation involves the activation of $K_{Ca}2.3$ and/or $K_{Ca}3.1$ channels in endothelial cells of rat carotid artery and is blocked by the $K_{Ca}2.3$ blocker apamin in combination with the $K_{Ca}3.1$ blocker charybdotoxin or TRAM-34 (Eichler, Wibawa *et al.* 2003; Kohler, Eichler *et al.* 2005). Thus, EDHF-signaling depends on the activation of $K_{Ca}3.1$ and/or $K_{Ca}2.3$ in the endothelium, which is then most likely transmitted to the VSMC via myoendothelial gap-junction as shown previously by other scientists (Griffith 2004). Since $K_{Ca}3.1$ plays a pivotal role in EDHF-meditated vasodilation, I tested whether or not EDHF-signaling is impaired in $K_{Ca}3.1^{-/-}$ mice. In the pressure-myograph experiments of the present study, acetylcholine (ACh)-induced NO- and PGI_2-independent diameter changes of the freshly isolated carotid artery (CA) of $K_{Ca}3.1^{-/-}$ mice and their wild-type littermates were determined and compared. CA of female $K_{Ca}3.1^{-/-}$ mice showed an about 50% reduction of vasodilation at a physiologically relevant concentration of ACh (100 nmol/L) compared with wild-type littermates (Figure 3.9 A). Thus, under physiological condition $K_{Ca}3.1$ may contribute by 50% to the total ACh-induced EDHF-mediated vasodilation and $K_{Ca}2.3$ may be responsible for the rest of the dilation effect in mice CA. Moreover, direct activation of K_{Ca} channels by K_{Ca} channel opener instead of ACh induction also resulted in about 20% less vasodilation in female $K_{Ca}3.1^{-/-}$ mice than that in female wild-type mice (Figure 3.9 C). All these results clearly indicate that $K_{Ca}3.1$ channel plays a pivotal role in the EDHF-mediated vasodilation in female mice.

This reduced EDHF-mediated vasodilation might be well explained by the significantly decreased K_{Ca} currents in female $K_{Ca}3.1^{-/-}$ mice. This result fits well with the K_{Ca} currents composition result discussed above that $K_{Ca}3.1$ currents contribute approximately 44% to the total K_{Ca} currents. By the loss of 44% of K_{Ca} currents, almost commensurate EDHF-type vasodilation is

missing.

ACh-induced EDHF-type vasodilation in CAs of male $K_{Ca}3.1^{-/-}$ mice only showed a tendency to be decreased, without statistical significance when compared to $K_{Ca}3.1^{+/+}$ male mice (Figure 3.9 B&D). This might be explained by the fact that male $K_{Ca}3.1^{-/-}$ mice retain more K_{Ca} currents than female $K_{Ca}3.1^{-/-}$ mice (Figure 3.6 E), therefore, male $K_{Ca}3.1^{-/-}$ mice did not show severely impaired EDHF-mediated vasodilation.

The endothelial production of NO is impaired in a variety of pathological conditions, such as hypertension, diabetes, heart failure, and hyperlipidemia (Cohen 1995). In contrast, it remains unclear whether these diseases cause similar impairments in EDHF-mediated vasodilation. Endothelium-dependent hyperpolarization is present in coronary arteries form patients with different cardiac diseases (Nakashima, Mombouli *et al.* 1993). Carotid arteries in rabbits with a high-cholesterol diet suggest an increased EDHF-mediated vasodilation in the presence of reduced production of NO (Najibi, Cowan *et al.* 1994). Other studies have shown that hypertension results in a compensatory increase in the activity of potassium channels (Liu, Hudetz *et al.* 1998; Paterno, Heistad *et al.* 1997), and possibly, increased synthesis/release of the putative EDHF (Asano, Masuzawa-Ito *et al.* 1993; Cachofeiro and Nasjletti 1991). These studies suggest that in cardiovascular disease states, the EDHF-mediated response is present or even augmented when the endothelial function is impaired due to the attenuated NO production or availability.

In the present study, it was found in female $K_{Ca}3.1^{-/-}$ mice that the loss of the $K_{Ca}3.1$ led to a significant reduction of the endothelial-dependent vasodilation due to an impaired EDHF-signaling. Thus, it is clear that the impairment of the $K_{Ca}3.1$-mediated EDHF-signaling can directly induce endothelial dysfunction.

4.3.5 Blood pressure and EDHF-mediated vasodilation

The release of paracrine vasodilators from endothelial cells is a critical determinant of vascular tone and, hence, an integral regulatory mechanism involved in the maintenance of local blood flow and systemic blood pressure. Endothelium-dependent vasodilators (such as NO, PGI_2 and EDHF) induced by agonist such as ACh increase the size of blood vessels, thereby decreasing blood pressure (Feletou and Vanhoutte 2000; Furchgott and Zawadzki 1980; Garland, Plane *et al.* 1995). Previous studies showed endothelial NO-synthase (eNOS)-deficient mice are hypertensive (Huang, Huang *et al.* 1995), thus, it could be hypothesized that EDHF-mediated vasodilation might have some influence on the animal blood pressure.

Tail-cuff blood pressure measurements in female $K_{Ca}3.1^{-/-}$ mice showed an about 12 mmHg elevated systolic pressure than their wild-type littermates. By telemetry blood pressure experiments, female $K_{Ca}3.1^{-/-}$ mice showed an about 10 mmHg elevated systolic pressure and 8 mmHg higher MAP than their wild-type littermates (Figure 3.10 A and 3.11). Male $K_{Ca}3.1^{-/-}$ mice and their wild-type littermates did not show obvious difference of systolic blood pressure by tail-cuff blood pressure measurements (Figure 3.10 B). Thus, these results indicate that female $K_{Ca}3.1^{-/-}$ mice have a modest but significantly elevated blood pressure due to the loss of $K_{Ca}3.1$ and reduced EDHF-type vasodilation. Therefore, the endothelial $K_{Ca}3.1$-mediated EDHF-signaling contributes to the control of blood pressure *in vivo*. Interestingly, previous study showed that the lack of $K_{Ca}2.3$ led to an elevated blood pressure in mice (Mark S. Taylor, Adrian D. Bonev *et al.* 2003). However, whether the loss of $K_{Ca}2.3$ may result in an impaired EDHF-mediated vasodilation has not been shown so far.

Previously, it was not known whether an impaired EDHF-system will result in

an endothelial dysfunction and in turn a higher systemic blood pressure. In the present study, $K_{Ca}3.1^{-/-}$ female mice showed an impaired EDHF-signaling caused by the loss of the endothelial $K_{Ca}3.1$. This impaired EDHF-signaling led to an endothelial dysfunction, which in turn resulted in an elevated systemic blood pressure in female mice.

Recently, gene-targeted mice lacking both eNOS and cyclooxygenase (COX, PGI_2 synthase) were found to have elevated blood pressure of male but not of female mice and to maintain significant vasodilator endothelial function in female but not male (Scotland, Madhani *et al.* 2005), indicating EDHF is the predominant endothelium-derived relaxing factor in female mice. Thus, it suggests that female mice may be more susceptible to an impaired EDHF-type vasodilation than male mice. This might be an explaination why only female $K_{Ca}3.1^{-/-}$ mice showed an elevated blood pressure but male $K_{Ca}3.1^{-/-}$ mice not in this study.

In conclusion, $K_{Ca}3.1$ gene expression and functional protein were completely absent in $K_{Ca}3.1^{-/-}$ mice, showing the gene targeting experiment is successful. Although the mechanism of enlarged kidney and spleen remains to be determined, the heart of female $K_{Ca}3.1^{-/-}$ mice showed sign of left ventricular hypertrophy (LVH). In endothelium, K_{Ca} currents of female $K_{Ca}3.1^{-/-}$ mice were largely reduced but males not when compared to those from their wild-type littermates. Consequently, the EDHF-mediated vasodilation in response to ACh or DC-EBIO was significantly reduced in CAs only from female $K_{Ca}3.1^{-/-}$ mice, leading to the elevated systemic blood pressure in female but not in male $K_{Ca}3.1^{-/-}$ mice. Thus, the $K_{Ca}3.1$ is an important component of the EDHF-system and the loss of $K_{Ca}3.1$ leads to an endothelial dysfunction and in turn an elevated systemic blood pressure in a gender-specific manner.