8. SUMMARY

As previously described, Icsbp deletion leads to changed differentiation program of myeloid progenitors reflected in disproportional high production of granulocytes and low production of macrophages. In order to study the underlying mechanism of this switch, the gene expression profiles of isolated bipotent granulocyte-macrophage progenitors (GMP) from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice were compared by Affymetrix technology. This analysis revealed deregulation of several other hematopoietic transcription factors, which led us to hypothesize that defective myelopiesis of Icsbp$^{-/-}$ mice is caused by deregulation of multiple transcription factors, rather than Icsbp deletion alone. The goal of this work was to investigate the contribution of one of these additional deregulations to the observed Icsbp$^{-/-}$ mouse phenotype, namely the down-regulation of the transcription factor Klf4.

Klf4 was chosen for this analysis as one of most strongly down-regulated factors (10 fold) in Icsbp$^{-/-}$ progenitors. At the time this study began, the expression of Klf4 in the hematopoietic system has not yet been reported. However, its essential role in the differentiation of epithelial tissues and important roles of its analogues Klf1 and Klf2 in erythroid i.e. lymphoid cells, prompted us to analyze the role of Klf4 in the development of myeloid cells.

The experiments described in this work identify Klf4 as a factor which has a strong influence on myeloid progenitors, directing them to differentiate along the macrophage lineage, as shown by significantly higher percentage of macrophage colonies developing from Klf4-ER^{T2} over-expressing myeloid progenitors. Klf4-ER^{T2} led to the macrophage maturation enhancement even when the progenitor cells were cultured with granulocyte-colony stimulating factor (G-CSF) or when it was expressed in the Icsbp$^{-/-}$ cells (confering genetic misbalance which results in a propensity to form granulocytes), suggesting the ability of Klf4 over-expression to override granulocyte-promoting effects of both extracellular cytokine signals and intracellular factors and reprogram the cell fate to the macrophage lineage. Analysis of the early gene expression changes caused by Klf4-ER^{T2} over-expression in myeloid progenitors revealed up-regulation of several putative macrophage genes and down-regulation of genes involved in granulocyte proliferation, suggesting that Klf4 exerts it macrophage promoting effect by coordinating molecular changes which precede the commitment to this lineage.

Another feature of the Klf4-ER^{T2} over-expression is its strong cytostatic effect on developing myeloid cells, identified by the significant reduction in the total colony formation. The proliferation inhibition coupled with the macrophage maturation promoting activity of Klf4-ER^{T2} suggest that Klf4 acts as a proliferation/differentiation switch in the myeloid development.

Klf4 effect on macrophage maturation is independent from Icsbp, as shown by uncompromised Klf4 function in Icsbp$^{-/-}$ cells and uncompromised Icsbp function in Klf4$^{-/-}$ cells.

Unlike Icsbp$^{-/-}$ mice, the mice with a conditional Klf4 deletion showed no overt deregulation in the hematopoietic development, suggesting that the role of Klf4 in the steady-state hematopoiesis is redundant. Alternatively, Klf4 could play a role in stress-induced conditions like infections (since it

up-regulates macrophage-activation markers) or in the protection from oncogenic processes (since it leads to $p21^{Waf1}$ up-regulation, cell-cycle arrest and maturation).

Klf4 effects are based on its transcriptional activity and interaction with other nuclear factors. Deletion of the protein-interaction responsible domain rendered Klf4 inactive, while deletion of the DNA-binding zinc-finger region resulted in a maturation block and an extensive proliferation of immature myeloid cells. This result suggests the oncogenic potential of Klf4 in the myeloid system.

One of the Klf4 transcriptional targets (confirmed by our study) is $p21^{Waf1}$. The $p21^{Waf1}$ contribution to the proliferation inhibition and macrophage maturation enhancement caused by Klf4 over-expression was analyzed by testing the effect of $p21^{Waf1}$ over-expression in $Klf4^{+/+}$ and $Klf4^{-/-}$ myeloid progenitors. In addition to the full-length $p21^{Waf1}$ over-expression, the compartmentalized $p21^{Waf1}$ expression was simulated by the ability of the p21-ER^{T2} fusion construct to translocate in the nucleus in the presence of the ER^{T2}-ligand (4-OHT) or to stay sequestered in the cytosol in the ligand absence. Enhanced macrophage formation was observed in all cases of forced $p21^{Waf1}$ expression (constitutive, cytosolic or nuclear), in wild type and $Klf4^{-/-}$ progenitor cells, suggesting that forced expression of $p21^{Waf1}$ itself enhances macrophage maturation. Gene expression analysis of p21-ER^{T2} expressing progenitors in early differentiation stages showed the same molecular changes as Klf4 over-expression, suggesting that $p21^{Waf1}$ mediates Klf4 macrophage maturation effect. However, the intensity of $p21^{Waf1}$ effect on the maturation tended to be milder than that of Klf4, indicating that Klf4 activates other, $p21^{Waf1}$ independent factors which contribute to the macrophage maturation.

The cytostatic effect of Klf4-ER^{T2} over-expression was largely reproduced by $p21^{Waf1}$ over-expression, suggesting that this aspect of Klf4 activity could also be mediated through $p21^{Waf1}$. Since the reduction in the colony formation was observed only when $p21^{Waf1}$ was allowed to enter the nucleus, we conclude that the roles of $p21^{Waf1}$ in the differentiation of myeloid cells and cell cycle control can be decoupled and depend on the subcellular localization of $p21^{Waf1}$.

Apart from analyzing the factors that govern the development of macrophages and neutrophilic granulocytes, another aspect of myelopoiesis, development of eosinophilic granulocytes, was analyzed in this work. The gene profiling of $Icsbp^{+/+}$ and $Icsbp^{-/-}$ GMPs described above identified strong down-regulation of several eosinophil-specific genes in the absence of Icsbp, suggesting perturbed eosinophilopoiesis in $Icsbp^{-/-}$ mice. Indeed, experiments described here confirmed that $Icsbp^{-/-}$ mice have reduced number of eosinophil progenitors and reduced capability to elicit eosinophilia in response to parasite infection or sterile inflammation. This defective eosinophil proliferation is based on reduced expression of the main eosinophil growth factor receptor, Il-5Rα and reduced expression of an important intrinsic factor in defining the eosinophilic lineage, Gata1.

Summarized, this work identifies Klf4 as novel macrophage maturation promoting factor, whose effects in myelopoiesis are partially mediated by its downstream target $p21^{Waf}$. Additionally, this work revealed previously undetected myeloipoietic defect in $Icsbp^{-/-}$ mice, namely perturbed development of eosinophils, identifying thereby Icsbp as an important factor in eosinophilopoiesis.