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Identification of a functional mRNA-miRNA network in mTEC development

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Hiermit erkläre ich, dass ich die vorgelegte Dissertation selbstständig verfasst und mich dabei keiner anderen, als der von mir ausdrücklich bezeichneten Quellen bedient habe.

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This thesis was completed in the
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Zusammenfassung

Das adaptive Immunsystem gewährleistet mit seinem breiten Spektrum an Immunzellen und deren Rezeptoren eine effektive Bekämpfung von einer Vielzahl von Pathogenen. Die T-Zell Generierung, welche im Thymus stattfindet, durchläuft einer zweistufigen Selektion, welche für die immunologische Selbsttoleranz essentiell ist. Für diese strikte Kontrolle der beiden Selektionen spielen vor allem zwei große Zellpopulationen eine wichtige Rolle, die kortikalen thymischen-Epithel-Zellen (kTEZ) und die medullären thymischen-Epithel-Zellen (mTEZ). Für die Vorbeugung von Autoimmunreaktionen spielt die Einbindung der mTEZ eine entscheidende Rolle, da diese periphere Selbst-Antigene exprimieren und diese den reifenden T-Zellen präsentieren. Die bisherigen Kenntnisse beschreiben die jeweiligen Funktionen der kTEZ und mTEZ, die Entwicklung einer Stammzelle bis zur Entwicklung einer mTEZ, bis hin zu welche molekularen Mechanismen die Entwicklung beeinflussen, aber bislang nicht in kleinsten Details. Bisher wurde der Sprung einer nicht unreifen mTEZ zu einer maturierten mTEZ nicht hinzureichend charakterisiert. Vorausgegangene Forschungen haben gezeigt, dass in fötalen und embryonalen unreifen mTEZ vier Subpopulationen zu finden sind, welche in adulten mTEZ bisher nicht beschrieben wurden. Bisher wurde die Existenz einer Handvoll microRNAs (miRNA), die eine Rolle in mTEZ spielen, entdeckt, aber keine miRNAs entdeckt, die für die Entwicklung der unreifen mTEZ zu reifen mTEZ eine entscheidende Rolle spielen. Im Rahmen der Doktorarbeit wurden die vier Subpopulationen in adulten mTEZ mit Hilfe von miRNA und mRNA Genexpressionsanalysen charakterisiert. Dabei wurde folgende Entwicklungssequenz der vier Subpopulationen aus den unreifen mTEZ anhand der mRNA Genexpression ermittelt Sca-1 < DP < CD24 < DN. DN war laut den Analysen die Vorstufe von reifen mTEZ. Daraufhin wurden mit Hilfe verschiedener bioinformatischen Methoden miRNAs herausgefiltert, die für die Entwicklung der vier Subpopulationen eine entscheidende Rolle spielen. Diese miRNA wurden mit Hilfe eines anderen Mausmodels verifiziert, welche die dynamische Entwicklung der vier

Subpopulationen nachahmt. Darauffolgend wurden die potentiellen miRNAs in frisch isolierten unreifen mTEZ inhibiert oder überexprimiert um deren Einfluss bei der Entwicklung der vier Subpopulationen zu untersuchen. Am Ende haben wir 11 miRNAs gefunden, die eine funktionelle Rolle bei der Reifung der 4 Subgruppen spielen. Mit dieser Erkenntnis haben wir mRNA-Interaktionspartner der miRNAs gesucht, die für diesen Entwicklungsprozess wichtig sind. Nachdem 35 mRNAs untersucht wurden, stachen am Ende vier miRNA-mRNA-Interaktionspaare hervor, die bisher nicht in mTEZ beschrieben wurden. Folgendes Interaktionspartner wurden gefunden: miR-34a mit Foxp1, Mir-125b-5p mit Bak1 und Mapk14, miR-125a-3p mit Fam83e. Die Arbeit vervollständig das Wissen über die Entwicklungslinie der zuvor gefundenen vier Subpopulationen in adulten Mäusen und gibt Ausschluss über neue miRNA und mRNA- Interaktionspartnern, die bei der Entwicklung der vier Subpopulationen von Wichtigkeit sind. Darüberhinaus können diese Erkenntnisse auf den humanen Thymus und deren MTEZ Entwicklung übertragen werden und im Hinblick auf Thymus-Regeneration eine äußerst wichtige Rolle spielen, vor allem nach Chemotherapie und Organtransplantationen.

Summary

The adaptive immune system with its diversity of immune cells and their receptors allows an effective fight against a plethora of pathogens. The development of T cells is taking place in the thymus, where it undergoes two selections that is immensely important to ensure self-tolerance. For those two strictly regulated selection processes there are two major cell components being responsible for that called cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC). MTECs are playing an important role for the prevention of autoimmune diseases as they express self-antigens to the maturing T cells. The current knowledge describes basically the function of cTECs and mTECs, the development of a stem cell to mTEC, the involvement of molecular mechanisms which influences the development of thymus and mTECs, still not in every single details. To date molecular details are still missing how an immature mTEC develops to a mature mTEC, especially molecular mechanisms, which influence the maturation and what controls each single step to its final maturation. Previous researches have found in the immature mTECs four new subpopulations present in fetal and embryonic mTECs, which have not been shown in adult mice. Until now only a handful of microRNAs (miRNAs) have been found to play a role in mTECs, but no miRNAs have been founds to play an active role in the maturation process of mTECs. The scope of this doctorate thesis was to characterise the four subpopulations in adult mice with the help of mRNA and miRNA microarray analyses. With the help of the mRNA gene arrays we could decipher a lineage sequence within the four subpopulations stating that Sca1 as the most progenitor population Sca-1 < DP < CD24 < DN. According to the analyses DN was the closest cell population to mature mTECs, probably one step before coming a mature mTEC. Thereupon we used bioinformatics to filter the most prominent miRNAs, which might play an important role in the development of the four subpopulations. Those miRNAs were verified by using a SCID mouse model, which emulates the dynamic development of the four subpopulations. Subsequently we inhibited or overexpressed those

miRNA candidates in freshly isolated mTECs to see their influence of the development of the four subpopulations. At the end we found 11 promising miRNAs that play a role in the maturation of the four subgroups. This finding resulted in a deeper search for possible mRNA interaction partners for those miRNA candidates. After investigating 35 mRNAs we found 4 miRNA-mRNA interactions, which have not been described in the mTEC development before. Following miRNA-mRNA networks have been found: miR-34a interacts with Foxp1, Mir-125b-5p with Bak1 and Mapk14, miR-125a-3p with Fam83e. This thesis completes the understanding of the four subpopulations in adult mice as well discovered 4 new miRNA-mRNA interactions, which are important for the maturation of the four subpopulations. With this newly gained knowledge we can apply it to the human thymus and their mTEC development with the hope to boost the thymus function after chemotherapy and organ transplantations.

1 Introduction

The immune system is crucial for the survival of an organism as it is responsible for the detection and elimination of pathogens. It can be divided into two mechanisms, the innate and adaptive immune responses. The innate or naïve immune response can be typed as a fast but not specific response while the adaptive response develops after birth and advances itself during encountering with pathogens and becomes very specific. The T lymphocytes, short T cells, are playing a central role in the adaptive immune response, which have the feature to recognise self and non-self antigens with the involvement of self-major histocompatibility complexes (MHC). A highly diverse repertoire of T cells, up to 10^8 different variations, is generated in the thymus. The thymus provides T cells a place to mature and ensures a strict control of T cell development and elimination in case of self-reactivity, thus providing self-tolerance in the periphery (Parkin and Cohen, 2001). The function of the thymus and its importance for the T cell immune response was discovered over 50 years ago (Miller 1961). Today we know that the thymus is the essential site of T cell lineage commitment, T cell repertoire generation and subsequent stringent selection of T cell receptor specificity.

1.1 The evolution and development of the thymus

The thymus is a primary lymphoid organ for the generation of a variety of T cell repertoire, which has been reported to be only present in jawed and non-jawed vertebrates. Their numbers and positions can vary differently among species. In mammals the location of the thymus is located in the upper chest area and contains of two main lobes, where each lobe consists of plentiful lobules packed into a capsule. The architecture of the thymus can be separated into four compartments, which includes the sub-capsular area, the cortical-medullary junction (CMJ), the cortex and the medulla. Within the thymus different cell types are harboured in it, which includes cells from the lymphocyte lineage (T cells, NK cells and NKT cells), hematopoietic cells (dendritic cells, macrophages and B cells) and thymic stroma cells (thymic epithelial cells, fibroblasts and endothelial cells) (Boyd et al. 1993). The

thymic epithelial cell (TEC) population can be divided into two components based on their function, morphology and antigen specific profiling, the cortical TECs (cTECs) and medullary TECs (mTECs). TECs have a very special morphology compared to typical epithelial cells, which are densely packed and mostly stratified on the basement membrane, while TECs are having a three-dimensional structure and different density packed areas (Brelinska and Warchol 1997, van Ewijik et al. 1999). Therefore the thymus with its unique feature provides an exceptional microenvironment for thymocyte migration, development and selection (Anderson and Jenkinson 2001, Prockop and Petrie 2000).

1.1.2 The evolution of the adaptive immune system

According to the history of evolution the adaptive immunity in vertebrates developed about 500 million years ago. It was the time frame where the occurrence of the thymus has been recorded, an organ that is responsible for the development of T cells. Both the anatomical position as well as the number of thymi differs from species to species (Rodewald 2008). Even though jawless and jawed vertebrates show similar features of an adaptive immunity such as the expression of a diversity of antigens, the mechanism of antigen composition vary in both families.

1.2.1 The maturation of thymocytes in the thymus

The differentiation and maturation processes of hematopoietic cell linages occur in two different sides, bone marrow and thymus. Whereas almost all linages differentiate and mature in the bone marrow, T cells are the only exception to develop in the thymus. T cells, which are still in the development and resided in the thymus, are called thymocytes. T cell progenitors are migrating from the bone marrow to the thymus and have to undergo a sequential of highly sophisticated and controlled selection processes. During those processes they encounter numerous cells of epithelial and mesenchymal origins, together these cells represent the thymic stroma and are resided in different compartments of the thymic microenvironment (Boyd 1993, Klein and Kyewski, 2000). The thymus consists of different compartments with diverse functions where thymocytes have to migrate

through them during the maturation process: subcapsular zone, the outer area cortex and the inner region medulla that are separated by the cortico-medullary junction. The developmental stage of thymocytes are categorized based on their expression levels of surface markers CD4 and CD8, which alters during the maturation starting from the immature stage $CD4^-$ $CD8^-$ double negative (DN) to $CD4^+$ $CD8^+$ double positive (DP) to be finally mature $CD4^+$ or $CD8^+$ single positive (SP) T cells (Rodewald 2007, Petrie and Zuniga-Pflucker 2007).

1.2.2 The selection of thymocytes and establishment of central tolerance

The selection process all starts when T cell progenitors migrate to the thymus via blood vessels and arrive at the CMJ, from there they follow a chemokine gradient (CCR7, CCR9 and CXCR4) to enter the subcapsular area by passing by the cortex (Fig.1). In the subcapsular area thymocytes are starting to proliferate and remain in that area for several days (Benz et al. 2004, Plotkin et al. 2003). In that stage thymocytes are at their earliest immature stage and are described as DN as they do not express any CD4 and CD8 antigens. During that stage those DNs start to reshuffle their T cell receptor (TCR)α- and TCRβ-loci to generate unique and functioning αβ-TCRs by recombining V-, D and J gene cassettes in a stochastic mode to create diversities up to 10^{15} unique receptors (von Boehmer et al. 1989, Ciofani and Zuniga-Pfluecker 2007). Those who fail to reorder their T cell gene cassette properly will undergo apoptosis also named as death by neglect. Thymocytes are becoming DP after two weeks of entering the thymus and makes up to the biggest population of thymocytes in the thymus. DP thymocytes are harboured in the cortex compartment and only those DP thymocytes, which carry a functional TCR and have the ability to interact with peptide-MHC complexes presented by cTECs, in a particular affinity and/ or avidity will transcend the so-called positive selection process (Anderson et al. 1999, McCaughtry et al. 2008). Before entering the medulla along another chemokine gradient (CCR7) those positively selected thymocytes with a TCR bound to a MHC class I molecule will become a $CD8^+$ SP thymocyte, whereas TCR bound to a MHC

class II molecule will become a $CD4^+$ SP thymocyte (Kurobe et al. 2006, Takahama 2006). In the medulla SP thymocytes come across with dendritic cells and mTECs and initiate the so-called negative selection process. This process is crucial to establish a central tolerance, as thymocytes with TCRs binding to self-peptides with a high affinity will undergo apoptosis. Those thymocytes with a medium binding capability will become regulatory T cells (Tregs). Non- to low binding of thymocytes to self-peptides will become naive T cells and will be released to the periphery. For a long time it was believed that the negative selection happens only in the medulla but recently a study has shown that the negative selection can also occur in parallel to the positive selection in the cortex initiated by cTECs and DCs (McCaughtry et al. 2008).

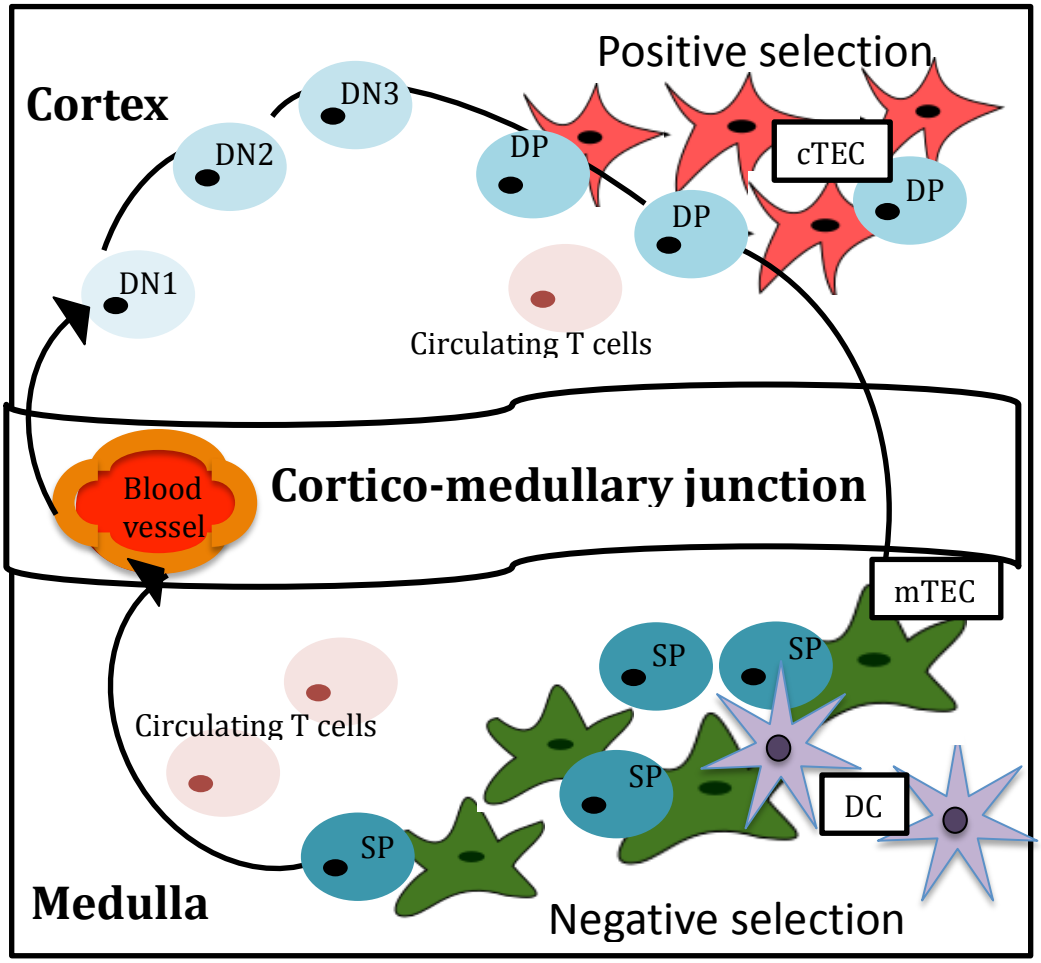

Figure 1 The thymus compartment: Thymus consists of two compartments, cortex and medulla. Immature thymocytes are undergoing two selection progresses. Firstly positive selection occurs where only T cells with T cell receptors reactive to self-MHC proteins will pass. Then they move forward to the medulla where the negative selection starts. Medullary thymic epithelial cells, short mTECs are harboring in the medulla. Their importance is to present self-antigens to maturing T cells. T cells with TCRs binding to self- antigens will undergo apoptosis. By eliminating auto-reactive T cells autoimmunity is prevented. And non-reactive T cells will be released to the periphery.

Addition to mTECs with the ability to present self-antigens to thymocytes, DCs in the thymus play also a central role for tolerance induction (Jenkinson et al. 1992) as they have the feature to take up and present extracellular antigens (Kyewski et al. 1986, Klein et al. 2001). It has been reported that DC have the ability to take up self-antigens such as TRAs from mTECs and present them to developing thymocytes (Gellgos and Bevan 2004, Koble and Kyewski 2009). As mTECs can represent only 1-3% of TRAs at a time, thus having mTECs as well as DCs to present self-antigens to maturing thymocytes, will increase the chances the encounter and capture of auto-reactive thymocytes, (Klein et al. 2009).

1.2.3 The thymocyte selection model

The selection process of TCRs is according to different models a process where certain levels of affinity and avidity between TCR and APC can determine the destiny of maturing thymocytes. Taking together the TCR affinity to self-peptide-MHC complex and the interaction with co-receptors on APCs, which determines the avidity, can resolve to the development of different cell types of T cells (Jameson and Bevan 1995). A thymocyte interaction with low avidity results favourably to a naïve T cell, while an interaction with higher avidity preferentially develops to a natural regulatory T cell. Developing thymocytes with a very high avidity leads to negative selection and will be induced to go to apoptosis (Hogquist 2001, Hogquist et al. 1994).

1.2.4 The discrepancy between cTECs and mTECs

A set of cells which play a major role in the T cell development and in the TCR repertoire selection process are called thymic epithelial cells (TECs). The development of TECs is regulated by two transcription factors Foxn1 and Aire, both are essential for their development and their functional maturation. TECs can be subdivided into two subgroups cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) and can be discriminated based on their expression of specific surface markers, their cell morphology and their location in the thymus. The progenitor of cTECs and mTECs derived from non-hematopoietic cells and are CD45 negative but epithelial cell

adhesion molecule 1 (EpCAM-1) positive. Furthermore cTECs are Ly51 positive while mTECs are Ly51 negative. Whereas cTECs are Ulex eurpopaeus agglutinin-1 (UEA-1) negative, mTECs do express UEA-1. Another way to differentiate cTECs and mTECs are the expression of cytokeratins. cTECs express cytokeratin 8 (K8) and mTECs express cytokeratin 5 (K5). Studies have shown that TEC progenitors express both K8 and K5 (Ucar et al. 2015, Bleul et al. 2006).

1.2.5 The maturation of mTECs

The developmental sequence of the MTEC development can be followed and determined by three markers such as CD80, MHC II and Aire. The immature mTECs show the following pattern $CD80^{low}MHCII^{low}Aire^{-}$, during the intermediate stage they are $CD80^{high}MHCII^{high}Aire^{-}$ and finally during the mature stage they are $CD80^{high}MHCII^{high}Aire^{+}$. After a certain period those mature mTECs become post mature mTECs and are $CD80^{low}MHCII^{low}Aire^{low}Ivl^{+}$ and can also be detected by adding an additional marker involucrin (Ivl) (Metzger et al. 2013). During the maturation of mTECs from immature to mature mTECs an increase of promiscuous gene expression (pGE) can be tracked and is been reported to have the highest degree of TRA expression and the most diversity of pGE (Fig.2). The unique feature of mTECs is the vast variety of TRAs presented to developing T cells to establish a central tolerance (Derbinski et al 2001, Klein et al. 2009).

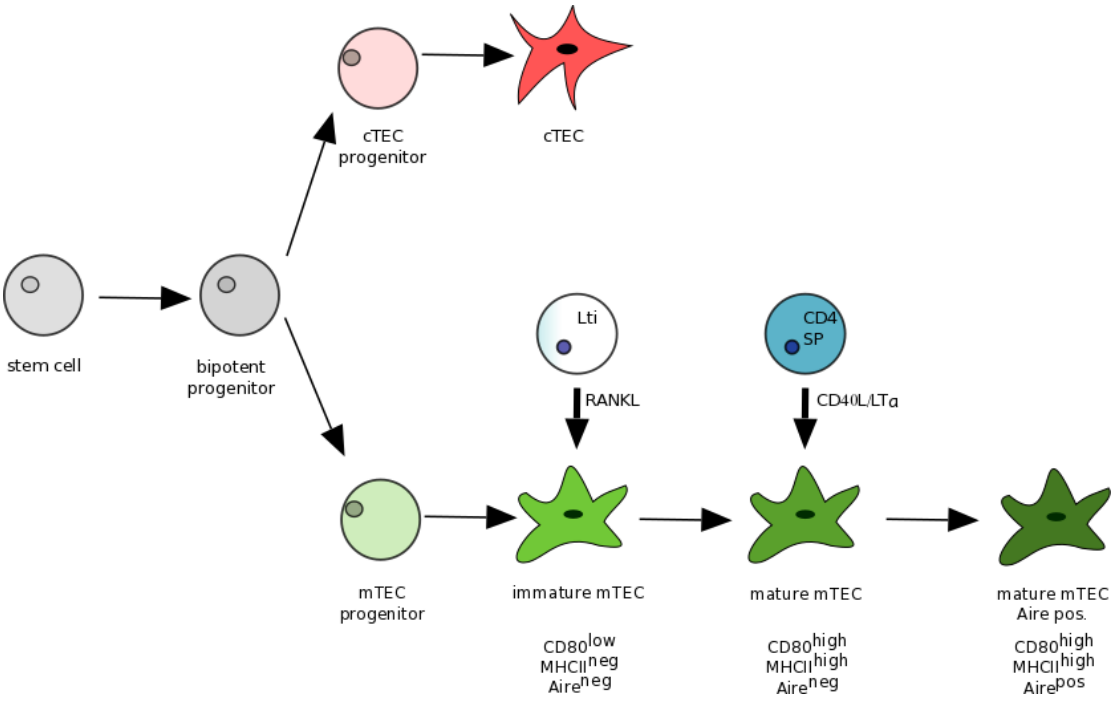

Figure 2 The maturation of TECs: The stem cell develops further to a bipotent progenitor, dependent on the signals it can derive to cTEC or mTEC. The immature mTEC undergoes maturation in different steps by expressing different expression markers such as Aire, CD80 and MHCII.

1.2.6 The crosstalk between developing thymocytes and thymus

The function of the thymus is to generate functional T cells, which have the capacity to distinguish self and non-self. On the other hand SP thymocytes play a role in the organization of the medulla hence having an impact on the mTEC development. This interaction between thymocytes and thymus is called “thymic crosstalk” (Ewijk et al. 1994). Herby mice with the lack of functional B and T lymphocytes have a deficiency in the thymus development such as severe combined immunodeficiency (SCID) mice. Not only the size of the thymus is up to ten times smaller compared to its wild type but also the thymus organ structure is impaired. The CMJ is lacking and the compartments are disorganised. The numbers of TECs are decreased; particularly the number of mTECs is affected. T lymphocytes, which express whole CD3/TCR ab complexes, have the possibility to influence the maturation and organization of the medulla compartment by sending certain signals to mTECs such as RANK and CD40 signals, which have been shown in experiments to play a crucial role in the mTEC differentiation in adult mice. RANKL is expressed in CD4+ as well as in CD8+ thymocytes while CD40 is preferably expressed in CD4+ T cells (Hikosaka et al. 2008). The absence of

CD28 on CD4+ thymocytes, its binding partner CD80/86 on mTECs, is not causing major perturbation, but if CD28-CD80/86 and CD40/CD40L are inactivated a decrease of mTECs could be observed as dramatically as the total absence of CD4+ thymocytes. This demonstrates that the interplay between CD28-CD80/86 and CD40/CD40L play as well a profound role in the development of a proper medulla. Without the activation of those two signalling pathways SP T thymocytes are not properly screened for their auto reactivity as mTECs are not able to develop properly to provide proper pGE and TRA expressions thus it is more likely that autoimmunity occurs (Williams et al. 2014). A subset of Natural killer T cells (NKT), called invariant NKT cells (iNKT), also express RANKL, have been reported to play a role in the mTECs by their interplay with CD4+ T cells (White et al. 2014), (Bosma and Carroll, 1991), (Lopes et al. 2015). Taking together the signalling mechanisms how mTECs are influenced by external signals to induce differentiation and medulla formation needs to be further elucidated. Experiments have shown that the thymus compartments especially the medulla and its mTECs can be restored by a bone marrow transplantation which gives the conclusion that hematopoietic cells are playing a crucial role in the proper medulla development and in turn a functional thymus is needed to provide a niche for developing T cells (Frenz et al. 1996).

1.3 The Signalling pathways in TEC development

The TEC development is stringently regulated by numerous pathways, which are known to play an essential role in the development of mTECs and thymocytes. One of the well-known pathways is the non-canonical nuclear factor κB (NF-κB) pathway that includes members of the tumour necrosis factor (TNF) receptor family involving RANK, lymphotoxin β receptor (LTβR) and CD40 (Akiyama et al. 2008). mTECs and thymocytes are partially communicating through (LTbR). Disruption occurs in the differentiation of mTECs, which will lead automatically to a reduced number of mTECs if LTβR is not present (Boehm et al. 2003). Disturbance of any of those above receptors or their downstream signalling molecules, such as the nuclear factor-κB-inducing kinase (Nik), which interacts with LTβR, can cause

autoimmunity as well as an impaired development of mTECs. Other components like IkB kinase α (IKKα), RelB and TNF receptor associated factor 6 (TRAF6) can also induce the production of autoantibodies due to the failure of developing functional mTECs (Burkly et al. 1995), (Akiyama et al. 2005), (Kinoshita et al. 2006) (Thomas, 2005).

Another important pathway, which influences the thymic architecture and mTEC development, is the signal transducer and activator of transcription (Stat) 3 signalling pathway. This molecule has been reported to play an essential role in cell proliferation, differentiation and cell survival by its anti-apoptotic effect (Hirano et al. 2000, Damell JE Jr., 1997). When deleting Stat3 in the thymus, a premature involution of the thymus occurs during adulthood, the cellularity got lost over time, no separation between cortex and medulla can be seen after 4 months, as well as its increased sensitivity to environmental stress; even though preadolescence did not shown any difference between control and $Stat3^{-/-}$ mice. Interestingly was to observe that thymocytes were not affected by the Stat3 deletion in spite of thymus hypoplasia but their numbers are decreased. Deletion of Stat3 also causes an expansion of the medulla, mostly $mTEc^{low}$ due to lower apoptotic effects in this subset, but a severe decline of the cortex. In overall it did not affect TRA expression or induce any autoimmune diseases compared to control mice. It is suggesting that Stat3 plays a role in the maintenance of thymus architecture especially for the formation of the medulla region as well as for the maintenance of mTECs but is unessential for the mTEC maturation. Stimulatory proteins, cytokines and growth factors mediate activation of Stat3. It has been reported that CD40 or epidermal growth factor (EGF) can mediate Stat3 activation and EGF-R has an influence in mTEC growth and medulla morphology in adult mice (Shigetoshi Sano et al. 2001), (Dakshayani Lomada et al. 2016), (Rumi Satoh et al. 2016).

1.4 microRNAs and their influence in post-translational regulation

MicroRNAs (miRNAs) are small RNAs, which have the capacity to regulate genes post-transcriptionally. They have been previously found in *Caenorhabditis elegans* (Pasquinelli and Ruvkun, 2002) and since their

discovery thousands of miRANs have been found in different species (Ambros, 2003), (Cai et al. 2009) and researchers have been trying to find their function in different cell types (Bartel, 2004), (He and Hannon, 2004). Their size range from 21-25 nucleotides, single stranded, mostly binds to the 3' untranslated region (3'UTR) of a mRNA that has a perfect or imperfect complementary sequence. So far only one miRNA and mRNA target has been reported to have a perfect complementarity. As the most of the cases is has multiple mismatches and several nucleotide bulges between the miRNA and its mRNA target (Pasquinelli, 2012). The function of miRNAs is diverse as it can degrade the mRNA target, can induce translational repression as well as deadenylation of the mRNA target strand. MiRNAs are fine-tuners and act accordingly to their cellular requirements. The canonical maturation of a functional miRNA starts with the transcription of the gene where the miRNA is located (Fig.3). In general the same cluster of miRNAs are co-transcribed and their functions can be similar or different even coming from the same miRNA cluster. Primary miRNA (pri-miRNA) is generated during the transcription by RNA polymerase (Pol II, occasionally Pol III) and cleaved by a complex called Drosha-DGCR8 to generate the precursor miRNA (pre-miRNA). Afterwards the pre-miRNA is transported with the help of Exportin-5 outside the nucleus to the cytoplasm. Another cleavage is followed by Dicer, which finally leads to a mature miRNA. The leading strand of the mature miRNA duplex is forming with Argonaute (Ago) proteins a RNA-induced silencing complex (RISC) that has the function to modulate the mRNA on a post-transcriptional level (Winter et al. 2009), (Ha and Kim, 2014). The other strand, which is the passenger strand, will be cleaved and degraded and not incorporated into RISC (Liu et al. 2013).

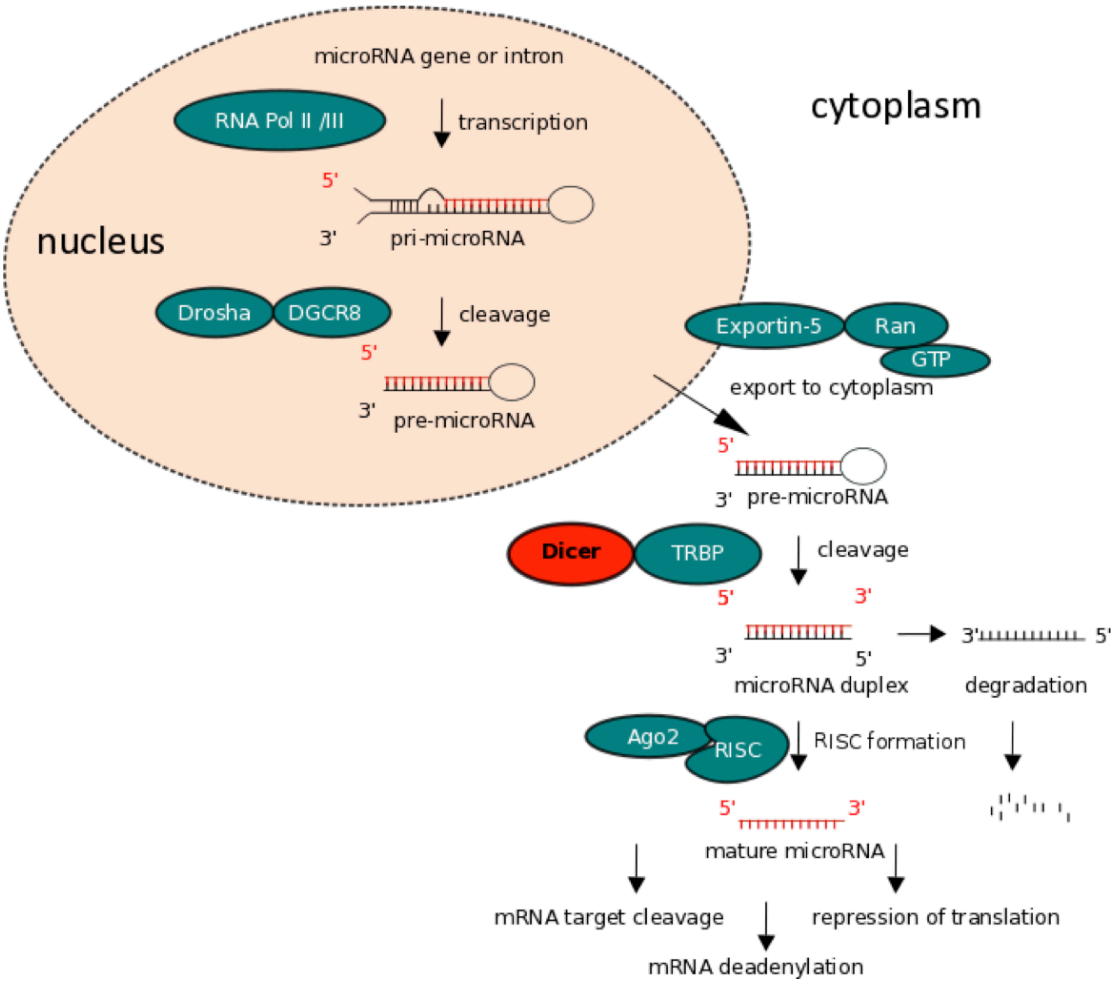

Figure 3 The maturation of miRNAs: The first step of the maturation starts in the nucleus and generates a pre-miRNA which then will be transported to the cytoplasm where the pre-miRNA undergoes several cleavage steps to become a mature miRNA.

There is the non-canonical pathway how miRNAs can be loaded to the RISC complex without the involvement of dicer. This pathway is called the dicer-independent miRNA biogenesis. It is believed that the pre-miRNA is loaded directly to Ago2, where the miRNA is sliced and unwound and later processed by so far unknown nucleases to become a functional mature miRNA (Yang and Lai, 2010), (Yang et al. 2012). Today there are many papers describing the involvement of miRNAs in cancer, cell regulation and differentiation (Mehta and Baltimore, 2016), (Baumjohann and Ansel, 2013) as well as in signalling pathways (Avraham and Yarden, 2012). The first alteration of miRNAs was found in 2002 in chronic lymphocytic leukemia (CLL). Since then miRNAs and their function and role in diseases have been elucidated and their use in the clinics to predict the outcome of a treatment or use miRNAs as biomarkers to diagnose a disease (Hayes et al. 2014).

The Notch signalling is also an important pathway in T cell lineage commitment and mTEC development (Feyerabend et al. 2009). The development of immature ab T cells to mature ab T cells is driven by Notch signalling, having its highest contribution in immature ab T cells, which includes early thymic progenitors, double-negative 2 (DN2) and DN3 thymocytes. After the b-selection, which is after the DN3 stage, Notch 1 is downregulated and only a low expression is remained in double positive (DP) thymocytes (Radtke et al. 2013). Recently it has been found that expression of Notch 1, Notch 2, Notch 3, Hey 1 and Hey 2 are present in immature mTECs. Data are suggesting that downregulation of Notch 1 in mTECs have to be reduced to ensure a proper mTEC development. As a remaining activation of Notch 1 in mTECs will lead to a decrease of mature mTECs and an increase of immature mTECs (Goldfarb et al. 2016).

1.4.1 The involvement of microRNAs in signalling pathways

The major role of miRNAs is to regulate mRNA targets and influence their expression levels but they can also influence the cascade of a signalling pathway and act as a feedback loop (Taganov et al. 2006). High-throughput screenings have been done to reveal miRNAs in different signalling pathways, like the well-conserved NF-κB pathway (Yang and Wang, 2016) or the Notch signalling pathway (Olarerin-George et al. 2013). MiRNAs discovered to play a role in NF-κB are for instance miR-125a and miR-125b which are constantly activating this pathway by repressing TNFα-induced protein 3 (TNFAIP3) which is known to be an important player in this signalling cascade (Kim et al. 2012). Falling in a proper regulation of NF-κB signalling leads to diseases like incontinentia pigmenti, autoimmunity or cancer (Courtois and Giilmore, 2006). The Notch signalling pathway is involved in many biological processes like cell differentiation, development, survival and apoptosis. That is why miRNAs have a significant function in this part by fine-tuning the signals to assure a proper communication between cells. Prominent miRNAs have been discovered to play a crucial role in Notch such as miR-326, which interacts with Notch and other Notch proteins and acts as a feedback loop to maintain a proper communication (Kefas et al. 2009).

1.5 microRNAs in thymus development

In the recent years researchers have focused on revealing microRNAs, which play a role in the T cell development and selection, thymus microenvironment and as well as differentiation and development of mTECs. As the thymus is proving T cell precursors a unique microenvironment to become mature T lymphocytes with the property to discriminate self and non-self to avoid autoimmune reactions, development of resided cells in the thymus as well as the microenvironment need to be regulated tightly to ensure a proper selection and maturation of T lymphocytes. Deletion of Dicer in Foxn1 Cre Dicer$^{fl/fl}$ mice results in the absence of mature miRNAs processed in the canonical pathway of Dicer-dependent miRNA biogenesis, which causes reduction of the thymus size as well as an impairment of thymus cellularity and development of T cells. Consequently the development of Foxp3 Treg is impaired and shows a lost of suppression function in vivo and mice are developing a lethal systemic autoimmune disease which resembles the FoxP3 knockout (KO) phenotype (Zhou et al. 2006). In aged Dicer deficient animals a complete absence of DP thymocytes can be observed that then lead to a disruptive development of SP T cells and naïve T cells. The declined number of T cells at the early age of Dicer deficient mice was already caused by the change of the microenvironment as numbers of cTECs increased which lead to a defective positive selection, mTEC numbers declined and the location of mTECs were predominantly located in the CMJ. In later stages the typical cortico-medullary organization got lost and TECs were not organized properly anymore. The property of the MHC II profile in mTEC has changed as the expression of immature mTECs has reduced and the proportion of mature mTECs has increased. Interestingly mature mTECs were not affected by the absence of mature microRNAs and still provided a range of self-antigens to ensure a proper negative selection process. Still T cells from Dicer-deficient mice are rather developing autoimmune diseases than control mice (Zuklys et al. 2012). In another approach where Dgcr8 is deleted by Cre recombinase in TEC showed a very similar result with an impaired thymic structure with a loss of keratin-8 (K8) and keratin-5 (K5), which results to a disrupted separation of

the cTEC and mTEC compartment. Aire$^+$ cells were reduced over time drastically and an increase of immature mTEC proliferation could be seen. Even though the numbers of cTECs and mTECs are aberrant and its thymic structure disorganised thymocyte development is still functioning and mice did not develop any spontaneous autoimmune diseases (Khan et al. 2014). Aire regulates the expression of microRNAs as the knockdown of Aire in mTEC2.10 cell line resulted in a changed miRNA expression level of 30 miRNAs indicating that Aire controls the expression of certain miRNAs (Macedo et al. 2012). MiR-155, miR-150, miR-93 and miR-181b were found to play an important role in thymus involution. Deletion of miRNAs in the thymus has been reported to lead to a progressive loss of pGE, both Aire-dependent and Aire-independent genes, thus miRNAs and Aire share interdependence in pGE (Ucar et al. 2013). Numerous microRNAs have also been found in thymic epithelial tumours (TETs), a disease, which occurs normally rare, but is very heterogeneously and mostly benign but with infiltrating and metastasizing behaviour. In those significantly downregulated miRNAs found in this disease a prediction analyses could shown that they are all linked to important pathways such as Wnt, Notch, Transforming growth factor-β (TGF-β) and other prominent pathways (Ganci et al. 2014). Thus a tight regulation of miRNA expression is important for proper T cell maturation, maintenance of the thymus microenvironment and the proper development of TECs to provide an efficient selection of functional T lymphocytes. Failing to do so can cause autoimmunity and cancer. On the other hand stress can influence the expression of thymic miRNAs thus resulting in an impairment to provide an effective selection and maturation of T lymphocytes (Belkaya et al. 2011).

1.5.1 Functional miRNAs in mTECs

In the past few years' three miRNAs have been discovered to play a crucial and functional role in mTEC development. MiR-29a was found to control thymic microenvironment and has a high expression in cTECs as well as in mTECs. Interestingly miR-29a deficient mice have a normal thymus architecture, normal development of TECs as well as proper T cell development but in the age of 4 weeks mice have a disruptive thymus

architecture which looks very similar to Dicer deficient mice with a profile indicating them undergoing thymic involution already in that early stage. It has been shown that miR-29 is essential for the maintenance of the thymic epithelial compartment and its hypersensitivity to pathogen-associated molecular pattern (PAMP) (Papadopoulou et al. 2012).

Another miRNA is miR-205, which is found in mTECs with a high expression level compared to $CD45^+$ cells and cTECs. The levels of miR-205 are comparable between $Aire^-$ and $Aire^+$ mTECs, eventually slightly higher in immature mTECs. Mice with a deficiency of miR-205 did not show any differences in Aire expression, thymus architecture or influence the expression and maturation of mTECs. Nor did it show any reduction of T cell development compared to wild type or a reduced thymic cellularity. Additional to that no significant differences could be observed in the numbers of spleenocytes, Tregs and memory T cells comparing to controls thus indicating that miR-205 is not influencing the peripheral T cell homeostasis. Spontaneous autoimmunity could not be found in miR-205 deficient mice. There is a possibility that the relevant function of miR-205 in mTECs was not revealed eventually due to other members of the miR-200 family, which might compensate the loss of miR-205 in mTECs (Khan et al. 2015).

Recently a new miRNA was found to play a role in mTEC by the interaction of TGF-β. MiR-181a-5p is lowering its expression levels in adult or aged mice compared to young mice. To investigate the role of miR-181a-5p in mTECs a cell line called murine thymic epithelial cell line 1 (MTEC1) was used and transfected with miR-181a-5p mimics or inhibitors. The cell viability was increased when MTEC1 was transfected with miR-181a-5p mimics, while inhibition of miR-181a-5p showed the opposite effect. MRNA levels of Cyclin1, Cyclin D3, Cyclin B1, Cyclin E1, Cdk4, Cdk6 and C-myc were all significantly increased when MTEC1 was upregulated and again the opposite expression levels of those genes were seen in miR-181a-5p inhibition. MiR-181a-5p was found to have a complementary sequence to Tgfbr1, a gene, which belongs to the TGF-β signalling pathway. When upregulating miR-181a-5p the mRNA expression level of Tgfbr1 went down as well as the protein level. Smad2 level

seem to be down as well on western blotting after the upregulation of miR-181a-5p. Tgfbr1 expression increases during aging thus indicating a relation between miR-181a-5p, Tgfbr1 and thymic involution (Dongguan et al. 2016).

1.1 Objective of this study

Previous studies have shown the involvement of particular microRNAs in terms of aging in mTECs, maintenance of thymic epithelial architecture and Aire expression on mTECs. However, the influence of microRNAs in the regulation of mTEC development from immature to mature mTECs has not been discovered yet. Thus, finding particular microRNAs playing a role to drive mTEC development will bring clarity how mTECs are regulated on the molecular level.

The prime focus of this thesis was to identify microRNAs in different developmental stages and their mRNA targets. Three different experimental approaches have been used:

1) Isolate murine mTEC subpopulations from different developmental stages by using surface markers such as MHC II, SCA-1 and CD24. Afterwards do microarray analysis to find specific microRNA and mRNA targets in those subpopulations.
2) Use a mouse model to reconstruct a thymus, as the generation and development of mTEC can be followed in a dynamic manner.
3) Generation of a miRNA-mRNA network by bioinformatics and prove the findings in a cell culture model to see their functional interaction with each other.

The aim of the thesis is to understand how mTEC development is regulated to eventually use this knowledge to reconstruct a complete thymus or reprogram a thymus in terms of autoimmune diseases or cancer.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Product	Company
2.4G2	DKFZ (Bruno Kyewski lab)
4',6-diamidino-2-phenylindole (DAPI)	Sigma
6x DNA loading Dye	Fermentas
100 bp DNA Ladder	Fermentas
Acetone	Riedel-de Haën
Acetic Acid	Sigma
Agarose	Invitrogen
B27	Invitrogen
bFGF	Sigma
ß-Mercaptoethanol	Invitrogen
Bovine serum albumin (BSA), Fraction V	Sigma
Cell proliferation dye eFluor 450	Affymetrix eBioscience
Chloroform	Merck
Collagen I, rat tail	Invitrogen
Collagenase IV	Worthington
Collagenase D	Roche
CutSmart Buffer	New England Biolabs
Dharmafect	Dharmacon
Dispase Grade I (Neutral Protease)	Worthington
DNase I	Roche
dNTP mix 2mM	Thermo Scientific
DMEM (+ Glucose, + L-Glutamine, + HEPES)	Gibco
Doxycyline Hyclate	Sigma
EGF	Sigma

Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethanol (absolute)	VWR
Ethidium bromide	Roth
Fetal calf serum (FCS)	Biochrom
FuGENE HD	Promega
Gold Taq polymerase	Life technologies
GoTaq G2 Flexi DNA Polymerase (2500U)	Promega
Heparin	Sigma
HEPES (solution)	Invitrogen
Hydrocortisone	Sigma
Insulin	Sigma
Isofluran	Fluka
Isopropanol	Sigma
JM109 competent cells	Promega
Linear polyacrylamide (LPA)	Thermofisher
Lipofectamine 2000	Life technologies
Magnesium Chloride	Sigma
MEBM medium, pH 7.2-7.	Lonza
NaCl, 0,9%	Braun
Non-essential amino acids (NEA)	Gibco
Novalgin, 50 mg/ml	Sanofi Aventis
Nuclease-free water	Ambion
Oligo(dT)	Life Technologies
Opti-MEM mediu	Thermofisher
Paracetamol	Ratiopharm
PFA (Paraformaldehyde)	Merck
PmeI	New England Biolabs
1x Phosphate Buffered Saline (PBS)	Life technologies
10x Phosphate Buffered Saline (PBS)	Biochrom
Penicillin/Streptomycin	PAA
Prolong Gold Antifade Reagent with DAPI	Life Technologies
Propidium Iodide	Sigma

Proteinase K recombinant PCR Grade	Roche
RNAlater	Life Technologies
RPMI 1640 with L-Glutamine	Gibco
SiGlo Green	Thermo Scientific
Sucrose	Sigma
SOC medium	Thermofisher
Sodium Acetate	Sigma
T4 DNA ligase	New England Biolabs
T4 DNA ligation reaction buffer	New England Biolabs
Tris/Acetate	Sigma
Triton-X-100	Merck
TRIzol	Invitrogen
Trypan Blue	Merck
Trypsin	Gibco
ViaFect	Promega
XbaI	New England Biolabs

2.1.2 Dispensable materials and commercial kits

Product	Company
AutoClip 9mm, wound clips	BD
Auto MACS columns	Milteny Biotech
Cell culture dishes (6 wells, 24 well)	TPP
Cell strainer cap tubes (5 ml)	BD
Cell strainer (40, 70 100 µm)	BD
Cell-Strainer-Cap Tubes (5ml)	BD
Cell Strainer (40, 70, 100 µm)	BD
Centrifuge Tubes (15 and 50 ml)	TPP
DNeasy Blood & Tissue Kit	Qiagen
Dual-luciferase reporter assay	Promega
Falcon Tubes (15 and 50 ml)	BD

Flat-bottom plates (6, 12, 24, 96 well)	TPP
Filter papers Ø 55mm	Macherey-Nagel
Filter tips (10, 20, 100, 200, 1000 µl)	Starlab
First-Strand cDNA Synthesis Super Script II RT	Invitrogen
Foliodrape	Hartmann
Glasses with ventilation holes	DKFZ
High Pure RNA isolation kit	Roche
High Pure miRNA isolation kit	Roche
Latex surgical gloves, powdered	Ansell medical
Microlance (different sizes)	BD
Miniprep	QIAGEN
mRNA microarray mouse	Agilent
miRNA microarray mouse	Agilent
Neubauer Improved C-Chip DHC-N01	Digital Bio
Nucleopore filter, 0.8 µm	Whatman
Ophthalmic ointment Bepanthen	Bayer
OptiPlate 96 white	Perkin Elmer
Pasteur pipette	Brand GmbH
PmirGLO Dual-luciferase miRNA target expression vector	Promega
Parafilm	Pechiney Plastic Packaging
PCR reaction tubes (200 µl)	Biozym
Pipet tips without Filter (10, 200, 1000 µl)	Starlab
Polypropylene round bottom tubes (5 ml)	BD
Power SYBR Green PCR Master Mix	Applied Biosystems
RNaseZap	Ambion
Round bottom plates (96 well)	TPP
Round bottom screw-cap tubes	Nunc
Safe-lock tubes (0,5, 1,5, 2 ml)	Eppendorf
Sterile filters	Millipore and Sartorius
Sponge, 2 x 2 cm	Baumarkt Obi
Suggi, mounted swabs	Kettenbach

Surgical suture material, PGA Resorba	Resorba
SuperScript II RT	Invitrogen
Syringes (1, 5, 10, 20, 50 ml)	Terumo
TaqMan microRNA Reverse Transcription kit	Applied Biosystems
Taqman Universal PCR Master Mix UNG	Applied Biosystems
TRIzol	Invitrogen
Ultra low attachment plates (6, 96 well)	Corning
Vacuum filtration – Filtermax	TPP
VIROMER Green	Lipocalyx

2.1.3 Media, buffers and commercial solutions

Trypan Blue

Trypan Blue	0.2%
NaCl	
pH 7.0	150 mM

RTOC and cell culture medium

FCS	10%
Pen/Strep	50 µg /ml
HEPES	10 mM
β-Mercaptoethanol	50 µM
Non-essential amino acids	1x
B27	1x
βFGF	20 ng/ml
EGF	20 ng/ml
Heparin	4 µg/ml
Hydrocortisone	0.5 µg/ml
Insulin	5 µg/ml
in DMEM with L-Glutamine	

Collagenase solution

Collagenenase IV	0.25 mg/ml
DNase I	25 µg/ml
HEPES	10 mM
FCS	3% (v/v)
in RPMI 1640 medium pH 7.2	

Collagenase/ Dispase solution

Collagenenase IV	0.2 mg/ml
Dispase Grade I	0.2 mg/ml
DNase I	25 µg/ml
HEPES	10 mM
FCS	3% (v/v)
in RPMI 1640 medium pH 7.2	

FACS buffer

FCS	3%
in PBS pH 7.2-7.4	

GM buffer

BSA	0.5% (v/v)
EDTA	5 mM
in PBS pH 7.2-7.4	

RBC lysis buffer

H_2O	900 ml
NH_4Cl	8.26 g
$KHCO_3$	1 g
EDTA (0.1 mM final)	0.037 g
Make up to 1l pH 7.4	

RPMI medium

FCS	3%
Pen/Strep	50 µg/ml
HEPES	10 mM
in RPMI 1640 pH 7.2	

Trypsin solution for embryonic thymus digest

Trypsin	0.25%
EDTA	0.2%
in PBS pH 7.2-7.4	

1.2% Agarose gel

Agarose	1.2 g
1x TAE buffer	100 ml
Ethidium bromide	5 ml

Tris-acetate-EDTA (TAE) buffer

Tris/Acetate	40 mM
EDTA	1 mM
in distilled water pH 7.5-8.0	

2.1.4 Magnetic beads and Microbeads used for MACS purification

Product	Company
Anti-CD45 microbeads	Miltenyi Biotech
Anti-CD90.2 microbeads	Miltenyi Biotech
OneComp eBeads	affymetrix eBioscience

2.1.5 Antibodies

Product	Species	Isotype	Conjugate	Clone	Company
CD11c	rat	IgG	Biotin	HL3	BD
CD11b	rat	IgG	Biotin	M1/70	BD
CD19	rat	IgG	Biotin	ID3	BD
CD24	rat	IgG	PE-Cy5, PB	M1/69	eBioscience
CD24	rat	IgG	FITC	M1/69	Biolegend
CD25	rat	IgG	Bril. Violet421	PC61	Biolegend
CD3	rat	IgG	Pe-Cy5	17A2	BD
CD4	rat	IgG	PE, FITC	H129.19	BD,BioLegend
CD44	rat	IgG	Alexa647	IM7	BioLegend
CD45	rat	IgG	PerCP	30-F11	BD
CD8	rat	IgG	PE, PE-Cy5	53-6.7	BD
CD8	rat	IgG	FITC	53-6.7	BD
CD80	ham	IgG	PE	16-10A1	BD
CD119	mouse	IgG	Biotin	2E2	BD
EpCAM	rat	IgG	Alexa 647	G8.8	DKFZ
I-A/I-E	rat	IgG	PE, FITC	M5	BD
Ly51	rat	IgG	FITC, PE	6C3	BD
Ly-6A/E	rat	IgG	PE-Cy7	D7	BD
MHCII	rat	IgG	FITC, Biotin	2G9	BD
Sca-1	rat	IgG	PE-Cy7	D7	BD
Streptavidin	-	-	APC-Cy7	-	BD
Ter119	rat	IgG	Biotin	Ter119	Invitrogen

2.1.6 MiRNA primers, siRNA, antagomiRs and mimics

Product	Target	Company
Hsa-miR-125b	miR-125b	Life technologies
Hsa-miR-203	miR-203	Life technologies
Hsa-miR-29a	miR-29a	Life technologies
Hsa-miR-34a	miR-34a	Life technologies
Hsa-miR-92	miR-92	Life technologies
Mmu-miR-1a-1*	miR-1a-1	Life technologies
Mmu-miR-125a-5p	miR-125a-5p	Life technologies
Mmu-miR-200c	miR-200c	Life technologies
Mmu-miR-203*	miR-203	Life technologies
Mmu-miR-205	miR-205	Life technologies
Mmu-miR-324-5p	miR-324-5p	Life technologies
Mmu-miR-34a-3p	miR-34a-3p	Life technologies
Mmu-miR-34b-3p	miR-34b-3p	Life technologies
Hsa-miR-34b-5p	miR-34b-5p	Life technologies
Mmu-miR-494	miR-494	Life technologies
Mmu-miR-709	miR-709	Life technologies
Mmu-miR-761	miR-761	Life technologies
Mmu-miR-877*	miR-877	Life technologies
Mmu-miR-let-7c-1*	let-7c-1	Life technologies
RNU48	RNU48	Life technologies
U6 snRNA	U6	Life technologies
mirVana inhibitor miR-125	miR-125a-3p	Life technologies
mirVana inhibitor miR-125	miR-125a-5p	Life technologies
mirVana inhibitor miR-125	miR-125b-1-3p	Life technologies
mirVana inhibitor miR-125	miR-125b-5p	Life technologies
mirVana inhibitor miR-200	miR-200c-3p	Life technologies
mirVana inhibitor miR-203	miR-203a	Life technologies
mirVana inhibitor miR-205	miR-205-5p	Life technologies

mirVana inhibitor miR-29	miR-29a-3p	Life technologies
mirVana inhibitor miR-34a	miR-34a-5p	Life technologies
mirVana inhibitor miR-34	miR-34b-3p	Life technologies
mirVana inhibitor miR-34c	miR-34c-5p	Life technologies
mirVana inhibitor miR-494	miR-494-3p	Life technologies
mirVana inhibitor miR-709	miR-709	Life technologies
mirVana inhibitor miR-761	miR-761	Life technologies
mirVana inhibitor miR-877	miR-877-3p	Life technologies
mirVana inhibitor miR-92	miR-92a-3p	Life technologies
mirVana inhibitor let-7c	let-7c	Life technologies
mirVana inhibitor neg. ctrl	scramble miRNA control	Life technologies
mirVana mimic miR-125	miR-125a-3p	Life technologies
mirVana mimic miR-125	miR-125a-5p	Life technologies
mirVana mimic miR-125	miR-125b-1-3p	Life technologies
mirVana mimic miR-125	miR-125b-5p	Life technologies
mirVana mimic miR-200c	miR-200c-3p	Life technologies
mirVana mimic miR-203	miR-203-3p	Life technologies
mirVana mimic miR-205	miR-205-5p	Life technologies
mirVana mimic miR-29	miR-29a-3p	Life technologies
mirVana mimic miR-34a	miR-34a-5p	Life technologies
mirVana mimic miR-34b	miR-34b-3p	Life technologies
mirVana mimic miR-494	miR-494-3p	Life technologies
mirVana mimic miR-709	miR-709	Life technologies
mirVana mimic miR-761	miR-761	Life technologies
mirVana mimic miR-877	miR-877-3p	Life technologies
mirVana mimic miR-92a	miR-92a-3p	Life technologies
mirVana mimic neg. ctrl	scramble miRNA control	Life technologies
silencer siRNA Abcb1b	Abcb1b	Ambion
silencer siRNA Acsbg2	Acsbg2	Ambion
silencer siRNA Apln	Apln	Ambion
silencer siRNA Arid3b	Arid3b	Ambion
silencer siRNA Bak1	Bak1	Ambion

silencer siRNA Bcl6	Bcl6	Ambion
silencer siRNA Btg2	Btg2	Ambion
silencer siRNA Col4a4	Col4a4	Ambion
silencer siRNA Dcx	Dcx	Ambion
silencer siRNA Dhcr24	Dhcr24	Ambion
silencer siRNA Dusp7	Dusp7	Ambion
silencer siRNA Egr2	Egr2	Ambion
silencer siRNA Fam83e	Fam83e	Ambion
silencer siRNA Fbxo45	Fbxo45	Ambion
silencer siRNA Fgf23	Fgf23	Ambion
silencer siRNA Fli1	Fli1	Ambion
silencer siRNA Foxp1	Foxp1	Ambion
silencer siRNA Gapdh	Gapdh	Ambion
silencer siRNA Grtp1	Grtp1	Ambion
silencer siRNA Jun	Jun	Ambion
silencer siRNA Mapk14	Mapk14	Ambion
silencer siRNA neg. ctrl	scramble mRNA control	Ambion
silencer siRNA Psmb11	Psmb11	Ambion
silencer siRNA Rab11b	Rab11b	Ambion
silencer siRNA Rps15a	Rps15a	Ambion
silencer siRNA Sall4	Sall4	Ambion
silencer siRNA Sgms2f	Sgms2f	Ambion
silencer siRNA Sirt1	Sirt1	Ambion
silencer siRNA Sirt3	Sirt3	Ambion
silencer siRNA Slc22a17	Slc22a17	Ambion
silencer siRNA Tbx5	Tbx5	Ambion
silencer siRNA Tirap	Tirap	Ambion
silencer siRNA Tnf	Tnf	Ambion
silencer siRNA Trp63	Trp63	Ambion
silencer siRNA Ubac1	Ubac1	Ambion
silencer siRNA Zeb1	Zeb1	Ambion
silencer siRNA Zfp113	Zfp113	Ambion

silencer siRNA Zfp300	Zfp300	Ambion

2.1.7 Oligonucleotides for vector cloning

Product	Sequence	Company
Bak1-miR-125b-5p		
5’AAACTAGCGGCCGCTAGTCGGCGGCTGGACTCTCAGGGATTCT3’		
3’TTTGATCGCCGGCGATCAGCCGCCGACCTGAGAGTCCCTAAGAGATC5’		
Bak1-miR-125b-5p mut		
5’AAACTAGCGGCCGCTAGTCGGCGGCTGGACTAGGAGGGATTCT3’		
3’TTTGATCGCCGGCGATCAGCCGCCGACCTGATCCTCCCTAAGAGATC5’		
Mapk14-miR-125b-5p		
5’AAACTAGCGGCCGCTAGTTAAAGATGAATCCTCAGGGGTGAAGT3’		
3’TTTGATCGCCGGCGATCAATTTCTACTTAGGAGTCCCCACTTCAGATC5’		
Mapk14-miR-125b-5p mut		
5’AAACTAGCGGCCGCTAGTTAAAGATGAATCCTAGCGGGTGAAGT3’		
3’TTTGATCGCCGGCGATCAATTTCTACTTAGGATCGCCCACTTCAGATC5’		
Fam83e-miR-125a-3p		
5’AAACTAGCGGCCGCTAGTTCAGCTGCCTCACCATGTACTATT3’		
3’TTTGATCGCCGGCGATCAAGTCGACGGAGTGGTACATGATAAGATC5’		
Fam83e-miR-125a-3p mut		
5’AAACTAGCGGCCGCTAGTTCAGCTGCCTCGAAATGTACTATT3’		
3’TTTGATCGCCGGCGATCAAGTCGACGGAGCTTTACATGATAAAGATC5’		
Foxp1-miR-34a-5p		
5’AAACTAGCGGCCGCTAGTGCATGTTGATACCACTGCCTT3’		
3’TTTGATCGCCGGCGATCACGTACAACTATGGTGACGGAAGATC5’		
Foxp1-miR-34a-5p mut		
5’AAACTAGCGGCCGCTAGTGCATGTTGATACCAGGACCTT3’		
3’TTTGATCGCCGGCGATCACGTACAACTATGGTCCTGGAAGATC5’		

2.1.8 PCR primers

Product	Sequence	Company
Aire fwd	ATAGCATCCTGGACGGCTTC	Eurofins
Aire rev	GCTCCTCCAGTGCTTTTCTC	Eurofins
Bak1 fwd	TACATCGCCAGATGGATCGC	Eurofins
Bak1 rev	TCCCCTGTCATAGTGGCTGA	Eurofins
Casein b fwd	TCTGTCTAAGAGGATTTCCAGGTT	Eurofins
Casein b rev	CATTTCCAGTTTCAGTCAGTTCA	Eurofins
CD80 fwd	TCAGAAGAAATGAGGCAAGCAG	Eurofins
CD80 rev	AAGGAAGACGGTCTGTTCAGCT	Eurofins
Dicer fwd	CCATTTGCTGGAGTCACTCTG	Eurofins
Dicer rev	TAAATCTGGCAAGcGAGACG	Eurofins
EpCAM fwd	CTTGCAGACTGCGCTTCA	Eurofins
EpCAM rev	CCCCCTTCACA TCTTTTTCA	Eurofins
Fam83e fwd	AGAGCGGCAATTCCACTTCG	Eurofins
Fam83e rev	TGCCAGAATGAAGGCCAAGG	Eurofins
FoxN1 fwd	CTCTTCCCAAAGCCCATCTA	Eurofins
FoxN1 rev	AGGCTTCCGGTCTTACTGTTC	Eurofins
FoxN1 Cre fwd	CATTGTAGCTGGCTTTCTTCGAG	Eurofins
FoxN1 Cre rev	GAAGCATGTTTAGCTGGCCC	Eurofins
Foxp1 fwd	CAGCCACCCTCTCTATGGAC	Eurofins
Foxp1 rev	AGCGCATGCTCACTGTTG	Eurofins
Gad1 fwd	GGTTCGCACAGGTCACCC	Eurofins
Gad1 rev	GCCATTCACCAGCTAAACCAA	Eurofins
Gapdh fwd	GGGTTCCTATAAATACGGACTGC	Eurofins
Gapdh rev	CCATTTTGTCTACGGGACGA	Eurofins
Mapk14 fwd	CCCAGCAACCTAGCTGTG	Eurofins
Mapk14 rev	GCTCGGTACCACCTGGTAG	Eurofins
Meis1 fwd	GACGCTTTAAAGAGAGATAAAGATGC	Eurofins
Meis rev	CATTTCTCAAAATCAGTGCTAAGA	Eurofins

MHC II fwd	TGCCTTAGAGATGGCTCTGC	Eurofins
MHC II rev	CCATGAACTGGTACACGAAATG	Eurofins
Pdpn fwd	GTTTGGTTCTGGGACTCTGC	Eurofins
Pdpn rev	TCCAGTAGCACCTGTGGTTG	Eurofins
Sirt1 fwd	TGGAAGGAAAGCAATTTTGGT	Eurofins
Sirt1 rev	CTGAGTTACCTTAGCTTGGC	Eurofins
Trp63 fwd	TTCCGTGAGCCAGCTTATCA	Eurofins
Trp63 rev	GTCTCCAGCCATTGGCATGT	Eurofin

2.1.9 Equipment

Product	Company
Aluminium Plate	DKFZ
AutoMACS Pro Separator	Miltenyi Biotech
Axio Imager.Z1 Microscope	Carl Zeiss
BD FACSAria Cell Sorter	BD
BD FACSCantoTM Flow Cytometer	BD
Bone cutters	Fine Science Tools
Bone Scraper	Fine Science Tools
Bulldog Type Serrefine straight, 35mm	Fine Science Tools
Cell Observer	Zeiss
Centrifuge Rotanta 460 R	Hettich
Confocal microscope	Olympus
Cryostat (CM3050 S)	Leica
Curved forceps, 7-Inox-H	Fine Science Tools
Fine forceps, 5-Inox-H	Fine Science Tools
Forceps	Fine Science Tools
Gel electrophoresis Camera	AGS
Heating Block	Eppendorf
Hemostat, straight, 14 cm	Fine Science Tools
Ice machine AF80	Scotsman

Incubator HeraCall 240i	Thermo Scientific
Incubator Memmert	Memmert
Isoflurane evaporator	DKFZ
Magnetic Bar (MR200)	Heidolph
Micro pipets (2-1000µl)	Gilson
Micro scale CP224S	Sartorius
Microwave	Siemens
Millipore Milli-Q Plus	Millipore
Multichannel pipet (8,12 channels)	Eppendorf
Nano-Drop® 100 photometer	Thermo Scientific
Neubauer-Counting chamber	Brand
PCR-Machine (PTC100TM)	MC Research
Reflex 9 Wound Clip Applicator	Fine Science Tools
Reflex Wound Clip Remover	Fine Science Tools
Scissors	Fine Science Tools
Sterile Hood Hera Sate	Heraeus
Stereo dissecting microscope Stemi DV4	Zeiss
Table Centrifuge (Biofuge)	Heraeus
Tabletop microscope Axiostar Plus	Zeiss
Thermo mats	Dehner
Vortexer	Scientific industries
Water Bath	Braun

2.1.10 Cell lines

HEK293T cells were kindly provided by Olga Ucar (DKFZ).

2.1.11 Softwares and homepages

Product	Provider / Company
Adobe Acrobat X Pro	San Jose
AxioVision LE (4.4)	Zeiss
EndNote (X4)	Adept Scientific
FACS Diva software (6.0)	BD
FlowJo (8.8.7)	Tree Star
GraphPad Prism (6.0)	GraphPad
Inkscape	Inkscape
IOS	Apple
microRNA sequence	www.microRNA.org
microRNA-mRNA interactions	TargetScan
Microsoft Office 2010	Microsoft
SoftMax Pro	Molecular Devices
Spectra Max M5 plate reader	Molecular Devices

2.1.12 Mouse strains

C57BL/6 mice were purchased and/or time mated from the animal facility at German Cancer Research Center (DKFZ) as well as from Charles River (Germany). The time point where a female mouse was declared as pregnant “day 0” was based on the vaginal plug detection.

SCID mice (CB17/Icr-Prkdc^{scid}/IcrIcoCrl), nude mice, NMRI mice, balb/c mice were purchased at Charles River (Germany and USA). ROSA26-tmTomato-tmGFP mice, which express constantly tomato fluorescent protein, were kindly provided by Roger Sandhoff (DKFZ).

Dicer KO mice are mice originally Foxn1^{cre} mice which were inter-crossed with Dicer$^{flox/flox}$ mice to produce mice with thymic epithelial cell-specific excision of Dicer on the C57BL/6 background. Those mice were provided from Olga Ucar (DKFZ).

All mice were kept under specific pathogen-free conditions in the animal facility at the DKFZ.

2.2 Methods

2.2.1 Isolation of murine tail DNA

The isolation of genomic DNA from murine tails was performed with the DNeasy Blood & Tissue Kit. A short piece of tail in the length between 2-5mm is needed for the DNA isolation. DNeasy Blood & Tissue Kit from Qiagen was used and the instructions were followed according to the manufacturer's manual. The final sample was eluted with 50 ml of buffer and the concentration was determined by nano drop. Afterwards the DNA is ready for PCR or it can be stored in the freezer (-80 degrees) for long-term storage.

2.2.2 Isolation of RNA from sorted cells or tissue

For the isolation of total cellular RNA two methods were used either with the High Pure RNA isolation kit from Roche and the instruction was followed by the manufacturer's protocol and the other method was an isolation of RNA with TRIzol. If using tissues, the tissue has to be homogenized in 1 ml of TRIzol per 50-100 mg of tissue. Let sample rest for 5 minutes at room temperature and spin down the tube and transfer the supernatant to a fresh eppendorf tube. If using cells in suspension, spin down cells for 5 minutes at 300 x g and remove the media and re-suspend the cells in ice-cold PBS and repeat that step but instead of re-suspending cells in PBS use 1ml TRIzol. Use a P100 and pipet up and down for at least 30 times to ensure the disruption of the cell walls. Now add 200 ml of chloroform per 1 ml of TRIzol reagent and vortex the sample vigorously for 15 seconds and let the sample incubate for 2-3 minutes at room temperature. Then centrifuge the sample at 12,000 x g for 15 minutes at 4 degrees. After the centrifugation the mixture has separated into a lower red phenol-chloroform phase, an intermediate phase and an upper colourless aqueous phase. The RNA is only in the upper phase and can be transferred carefully with a plastic Pasteur pipette to a fresh tube, be aware not disturbing the other phases. Measure the volume, which has been transferred to the new tube. To precipitate the RNA, the protocol for RNA precipitation has been used.

2.2.3 Isolation of miRNA

The isolation of miRNA was done with the High Pure miRNA isolation kit from Roche. The instruction was followed by the two-column protocol to isolate purified small RNAs. The miRNA concentration was measured by nana drop.

2.2.4 RNA precipitation

RNA precipitation can to reduce the volume size of the RNA sample. Place 50 ml of RNA sample in an eppendorf tube and add 5 ml of 3M Sodium Acetate and 1 ml linear polyacrylamide (LPA) to the sample. Add additional 125 ml ice-cold 100% ethanol and invert the sample twice. Precipitate for 30 min at -80 degrees or at -20 degrees overnight. Then spin the eppendorf tube at 13,000 x g for 30 minutes at 4 degrees. Afterwards decant the supernatant and pipet the pellet dry. Next add 500 ml of 70% ice-cold ethanol, vortex, spin down at 13,000 x g for 5 minutes at 4 degrees and discard the supernatant. Repeat this step. Pulse-spin the tube at maximum speed to collect all residual ethanol and let the pellet air dry for 5-10 minutes. Finally re-suspend the pellet with 10 ml RNase-free water.

2.2.5 MiRNA Reverse Transcription

The conversion of RNA to cDNA for miRNA RT-qPCR has been done with the TaqMan microRNA reverse transcription kit from Applied Biosystems. The protocol from the manufacturer has been used. The cDNA has been stored in -80 degrees or has been processed immediately for RT-qPCR.

2.2.6 cDNA Synthesis

The conversion of RNA to cDNA for RT-qPCR has been performed based on the SuperScript II reverse transcription protocol from Invitrogen.

2.2.7 Genotyping PCR

Prepare the master mix on ice by adding the following 2 ml of 10x buffer, 2 ml of $MgCl_2$ 25 mM, 4 ml of dNTP's 2 mM, 0.1 ml of forward primer 100 pM, 0.1

ml of reverse primer 100 pM, 0.2 ml of Gold Taq Polymerase, 14 ml of water and 2 ml of genomic DNA.

The following PCR settings has been used:

- Activation of polymerase for 9 minutes at 94 degrees
- Denaturation for 1 minute at 94 degrees
- Annealing for 1 minute at 60 degrees
- Elongation for 1.5 minutes at 72 degrees

} 30 cycles

- Elongation for 9 minutes at 72 degrees

2.2.8 Thymus dissection and medullary epithelial cell preparation

Sacrifice mice with CO2 and remove their thymi by ripping up the rib cage upwards. Place thymi into the petri dish that is filled with RPMI medium and place the dish on ice. Clean the thymi by removing blood vessels and connective tissues and transfer those clean thymi into a fresh petri dish filled with fresh RPMI medium Then chop those thymi in small pieces and transfer them with a Pasteur pipette into a round-bottom tube (in each tube place up to 6 adult thymi) and add a small stir bar into the tube. Use for each thymus 1 ml pre-warmed collagenase solution. Place the round-bottom tube into a water bath at 30 degrees for 15 minutes and turn the magnetic stirrer on. After 7 minutes of incubation re-suspend the collagenase thymi solution with a broken pipette. After 15 minutes transfer the supernatant to a falcon tube (placed on ice) and add 5 ml of RPMI medium to stop the enzymatic reaction. Add new collagenase solution to the round-bottom tube and stir for another 15 minutes, while re-suspending the solution with a broken pipette after 7 minutes to avoid clotting of the tissues. After 15 minutes transfer the supernatant to the same falcon tube. That tube consists mostly of T cells and no medullary thymic epithelial cells and can be used for T-cell analysis or discarded. Now add 1 ml collagenase/dispase solution per thymus to the round-bottom tube and increase the water bath temperature to 37 degrees and stir now for 25 minutes, while after 15 minutes of incubation re-suspend the solution with a pipette. After the 25 minutes transfer the supernatant to a fresh falcon tube filled with fresh RMPI medium (5 ml). Repeat the digestion

with collagenase/dispase solution for another 4 times, where each time the supernatant is collected after 25 minutes of incubation. Now spin down all falcon tubes for 1,400 rpm for 5 minutes at 4 degrees. Now re-suspend with 5 ml RPMI medium all falcon tubes, which have been used to collect all supernatant of the collagenase/dispase fraction. Then let the solution to go through a 70 mm pre-wet cell strainer and wash the cell strainer with another 5 ml RPMI medium and mix it well before counting the cells. After counting add 10 ml of GM buffer to the solution and centrifuge at 1,200 rpm for 5 minutes at 4 degrees. Discard the supernatant and add 1 ml GM buffer for each $1x10^8$ cells. Then add 40 ml of microbeads per 1 ml GM solution (1/25 volume of MACS beads) and incubate the solution for 30 minutes at 8 degrees in the dark, while stirring the tube after 15 minutes. Meanwhile prepare the MACS stand on ice and pre-wet the MACS separation columns with 3 ml GM buffer. Wait until the column is dry again before starting with the depletion; when the column is dry place a fresh falcon tube filled with 1ml RPMI medium under the column to collect the depleted cells. After 30 minutes of incubation wash the falcon tube with the same volume with GM buffer, vortex shortly, and centrifuge with 1,400 rpm for another 5 minutes at 4 degrees. Discard the supernatant and re-suspend the pellet with 1 ml GM buffer for $1x10^8$ cells. Apply for each column up to $2x10^8$ cells, wait until the solution went through the column and then wash the column twice with 3 ml GM buffer. Centrifuge the flow-through at 1,200 rpm for 5 minutes at 4 degrees, re-suspend the pellet with RPMI medium, filter through a pre-wet cell strainer and count the cells again. Place the cell solution in ice for further analysis.

2.2.9 Thymus dissection and isolation of embryonic medullary thymic epithelial cells

The removal of fetal thymi in the age of E14.5 was under microscope. Blood vessels were removed and transferred to an eppendorf tube filled with cold PBS. The thymi were washed four times and then transferred to a tube with 1.5 ml pre-warmed collagenase/dispase solution, up to 10 thymi per tube.

Tube was placed in a water bath with 37 degrees for 5 minutes. After 5 minutes a P200 pipette was used to enforce the digest mechanically by pipetting up and down for at least 30 times. This step was repeated twice. Alternatively this digestion can be done with Trypsin-EDTA but instead of 15 minutes, the time will be reduced to 8 minutes. Only 600 ml of Trypsin-EDTA is used and after 5 minutes, pipette with a P200 at least 30 times up and down to enforce the digest. Then place the tube back to the water bath for another 1 minute and repeat the digest enforcement with the P100 pipette. If the digest is still not complete, repeat the final step again. Trypsin digest is harsher compared to the collagenase/dispase solution. After all thymi were digested, RPMI medium was added to the tube to stop the enzymatic reaction. The cell suspension was then transferred to a FACS tube with a pre-wet filter on top of it. The eppendorf tube was washed twice, each time going through the same filter. FACS tubs were then centrifuged at 1,400 rpm for 5 minutes at 4 degrees. Remove the supernatant and re-suspend the pellet with RTOC/cell culture medium and placed in ice.

2.2.10 Re-aggregate Thymus Organ Culture (RTOC)

The re-aggregate thymus organ culture is a method to culture mTECs in a 3D structure. Put a 6 well plate under the sterile hood, place in each well a sterile sponge (in the thickness of around 5 mm) and then use a sterile syringe plunger and press on the sponge so it can soak up the medium, avoid any bubbles during that procedure. Place on top of the sponge a Whatman membrane. Place the well plate to the incubator. Meanwhile count the cells, which has been harvest from the E14.5 embryos and distribute $0.5x10^6$ cells in each eppendorf tube. Centrifuge all eppendorf tubes and remove the supernatant. Take out the well plate to a sterile place. Do a quick spin to remove the residual supernatant in the eppendorf tubes. Transfer with a P10 pipette the cell pellet onto the membrane. Place the well plate back to the incubator (37 degrees, 10% Co^2). The re-aggregation will take place in the next hours. A RTOC can be kept up to 7 days by replacing the medium in each well every 3 days.

2.2.11 FACS staining of mTECs

After the enrichment of TECs cells were re-suspended in RPMI medium. Centrifuge the cell suspension at 1,400rpm for 5 minutes at 4 degrees. Remove the supernatant and block for 15 minutes with anti-Fc-receptor solution (2.4G2), 1 ml of 2.4G2/ $1x10^8$ cells. Place the tube in the dark at 4 degrees. After the incubation time, wash the cells and stain the cells with the following antibody cocktails for 15 minutes for the primary antibody, wash with FACS buffer and incubate for another 15 minutes for the secondary antibody. Then wash again, centrifuge and then re-suspend in FACS buffer and place the tube on ice in the dark.

For mTEC detection following antibodies were used:

Antibody	Marker
CD24	T-cell and B-cell differentiation
CD45	Non-hematopoietic cells
CD80	mTECs
DAPI	dead cell exclusion
EpCAM	mTECs and cTECs express EpCAM
Ly51	cTECs
MHC II	mTECs
Sca-1	hematopoietic cells
Ter119	Erythroid cells

2.2.12 Bone marrow transplantation

The bone marrow was isolated from 6 weeks old balb/c mice. Balb/c were the background from the SCID mice, which were transplanted with the isolated bone marrow. The bone marrow was removed; erythrocytes and CD90.2 cells were depleted. SCID mice were 5 weeks old when they arrived the animal facility and were left for 1 week before transplantation. $2x10^7$ cells were injected intravenously (i.v.) per mouse when SCID mice were in the age of 6 weeks. All SCID mice were injected at the same time. For each time point 24

SCID mice have been injected with bone marrow and another 24 injected with PBS to serve as a control.

2.2.13 Transient transfections with freshly isolated cells

After freshly isolated mTECs have been sorted for CD80/MHC II low, cells were transferred to a falcon tube filled with RTOC medium and on ice. Then cells have been centrifuged and cells were counted. We used for each transfection 150.000 cells. Cells were transferred to a 24 well plate and placed to the incubator. Meanwhile miRNA mimics, antagomiRs or siRNAs have been diluted down to 11 mM using Buffer Green with a final volume of 15 ml. Place 3 ml of Viromer Green on the wall of an eppendorf tube and add immediately 270 ml of Buffer Green by flushing down the 3 ml off the wall and vortex shortly for 3-5 seconds. Then add 125 ml of the mixed solution to the 15 ml of miRNA mimic, antagomiR or siRNA and mix it well and incubate for 15 minutes in the dark at room temperature. After the incubation time add each transfection solution to the cells, by dropping slowly to the cell suspension and shake well. Cells were harvest and washed after 14-16 hours of incubation and were put into a RTOC system. After 4 days cells were analysed.

2.2.14 Vector generation, Transformation, Co-transfection

The vector used for the luciferase assay was the pmirGLO vector. The oligonucleotides were designed to have two restriction enzyme sites, one Pmel and the other one Xbal, thus to ensure the oligonucleotide to be placed into the right 5’ to 3’ direction. First vector is digested with Pmel and Xbal to generate the overhangs. Add Plasmid (in 1 mg/ ml concentration), CutSmart Buffer, Pmel, Xbal, BSA and sterile water to a clean tube and make it up to 20 ml. Incubate digestion for 1 hour at 37 degrees. Stop digestion at 65 degrees for 15 minutes. Check plasmid length with gel electrophoresis. Oligonucleotides were diluted down to 1 mg/ ml, oligonucleotides and oligo annealing buffer were mixed together and heated it up to 90 degrees for 3 minutes and then transferred to a 37 degrees water bath for 15 minutes. Then

mix 4 ng of annealed oligonucleotides with 50 ng of linearized vector, as well as ligation reaction buffer and T4 DNA ligase and mix it thoroughly. Incubate for 30 minutes at room temperature. Afterwards place it on ice. Preheat the SOC medium to 42 degrees and meanwhile chill sterile polypropylene culture tubes on ice. Thaw the JM109 competent cells on ice and transfer 100 ml of competent cells to the culture tubes and add 25ng of DNA to the 100 ml competent cells and quickly flick the tubes for several times. Place the tube immediately on ice for 10 minutes. Then heat-shock the cell mixture for 50 seconds in a water bath at 42 degrees. Afterwards place it back to ice for another 2 minutes. Add 900 ml of cold SIC medium to the cell suspension and incubate for 60 minutes at 37 degrees in a shaker. Finally cells were diluted to 1:10 and 1:100 and plated on LB agar ampicillin containing plates. Plates were placed to an incubator at 37 degrees for overnight. Then pick two clones for overnight growing by placing it back to the shaker at 37 degrees. Plasmid DNA was purified by using a Miniprep from QIAGEN. The size of the plasmid is checked via agarose gel. HEK293T cells were cultured a day prior to transfection. Dilute plasmid to 250 mg/ ml and all miRNA mimics and antagomiRs accordingly. Lipofectamine 2000 were incubated with Opti-MEM medium at room temperature for 5 minutes. Then mix this solution with the plasmid and mimic or antagomiRs. Incubate for 20 minutes at room temperature. Remove the medium from HEK293T cells and wash it twice with PBS. Then remove the PBS and add the transfection solution on top of it. Incubate for 24 hours and replace the transfection medium with a DMEM medium. After 48 hours luciferase assay can be performed.

2.2.15 Luciferase assay

The preparation for the measurements of the luciferase and renilla signals was done with the Dual-luciferase reporter assay kit from Promega. The protocol was followed according to the manufacturer's instructions. Measurements were done at the Spectra Max M5 plate reader.

3 Results

3.1 Four subpopulations within the immature mTEC pool

In the past years researchers have been intensively trying to solve the conundrum how the development of mTECs is regulated and maintained in adulthood and what driving forces are present in mTECs to drive immature cells to maturation. The mTEC population can be collected by the following scheme, namely: $DAPI^{-}CD45^{-}EpCAM^{+}MHCII$ (Fig.4). Recently a study demonstrated that immature mTECs ($MHCII^{low}$) can be subdivided into four subpopulations based on their surface expression of Sca-1 and CD24, calling them: double negative (DN), Sca1-CD24+ (CD24), double positive (DP) and Sca1+CD24- (Sca-1). These four subpopulations are different in terms of their ontogeny, long-term reconstitution ability, label retention and the capability to give rise to mature mTECs (Klug, 2013).

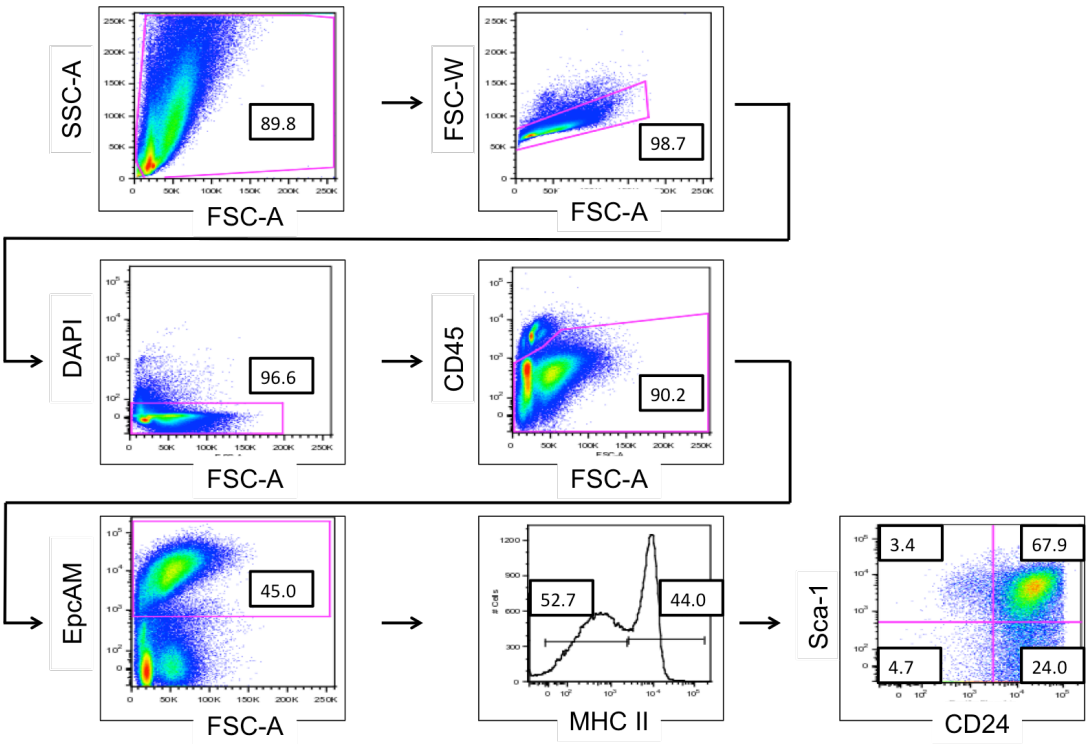

Figure 4 The four subpopulations: The gating strategy to find the four subpopulations. Starting by the exclusion of duplets (FSC-W/ FSC-1A) and dead cells (DAPI). Followed by the inclusion of non-hematopoietic cells by excluding CD45 positive cells. Then gate for EpCAM positive cells because mTECs are EpCAM positive. Low MHC II expression indicates immature mTECs, therefore we gate for left bump. By using Sca-1 and CD24 we can segregate the four subpopulations.

To understand the regulation of how immature mTECs are developing into mTECs it is necessary to understand those cells first and see what kind of

molecular driving forces are present to drive immature mTECs to become mature mTECs. To do so we started to look for the four subpopulations in different mouse strains such as severe combined immunodeficiency (SCID) mice, nude mice and Dicer$^{flox/flox}$ x FoxN1^{Cre} mice (henceforth referred to as Dicer knockout (KO) mice). In all three strains we could identify a deficiency in the development of the four subpopulations as well as their potential to drive mTECs to maturation. SCID and Dicer knockout mice were further analysed to characterize their involvement in the mTEC development. As is hasn't been shown how those four subpopulations relate to each other we compared their mRNA and miRNA expression levels to decipher a potential lineage progression towards mature mTECs. Potential functional miRNAs were selected and analysed, selected miRNA were proven for their relevance by using a SCID reconstitution model, potential to manipulate the development of the four subpopulations were investigated by overexpression and downregulation of miRNAs and finally finding their negative correlated mRNA targets and the relevance of those targets in the role of mTEC maturation.

3.2 The four subpopulations in distinct mouse strains

We have recently seen that Dicer KO mice show a decline in Sca-1 and DP subpopulations. This phenomenon could also be seen in other strains with a block in mTEC development, such as in nude and SCID mice. The distribution of the four subpopulations is always similar, no matter from which background they come from. Black6 and Dicer KO are sharing the same background, as well as SCID with balb/c or nude with NMRI (Fig.5). Also the percentages of mature mTECs are declined in those three mouse strains (Fig.6). In all wild types DP is the population, which has the highest percentage of cells (65 - 85%), second biggest population is CD24 (14 - 24%), DN ranges between 0.4 – 4% and Sca-1 is around 0.4 – 7%. In Dicer KO, SCID and nude mice the biggest population is gone, while the distribution in CD24 and DN increased dramatically. The percentage of DP (12 -18%) went down, while CD24 (76 – 82%) and DN went up (0.6 – 12%), however the population of Sca-1 remained small (0.1 – 0.8%). Importantly to mention is that nude mice have a mTEC-intrinsic genetic defect (a mutation in the transcription factor FoxN1), the

similar phenotype in the SCID model results from the absence of T cells, which influence the thymic epithelium development through the thymic crosstalk. Notably, this block ultimately leads to the absence or great reduction of terminally differentiated mature mTECs and impaired T cell selection in all three different mouse strains.

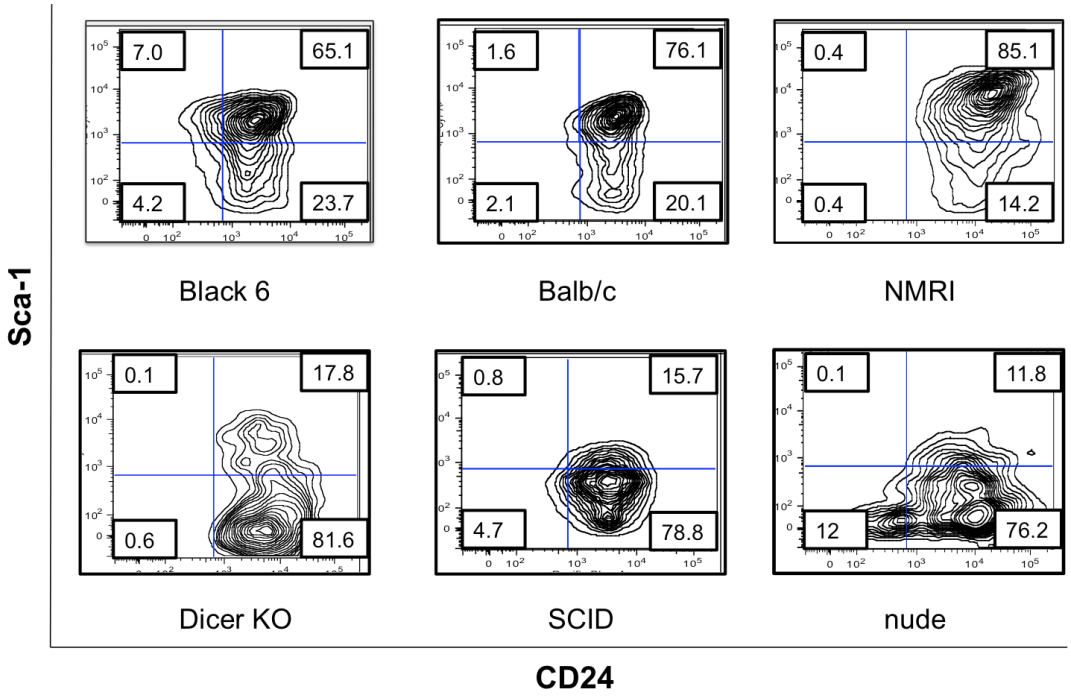

Figure 5 Different mouse strains shows different patterns of the four subpopulations: Black6, balb/c and NMRI mice express a similar pattern of the four subpopulations where you find the biggest population residues in Sca-1^{+}/CD24^{+}. Dicer KO, SCID and nude mice show a totally different distribution, where the biggest population is inhabits in the Sca-1^{-}/ CD24^{+} population. Even though the modifications made in Dicer KO, SCID and nude differ, the results of their distribution of the four subpopulations remain similar.

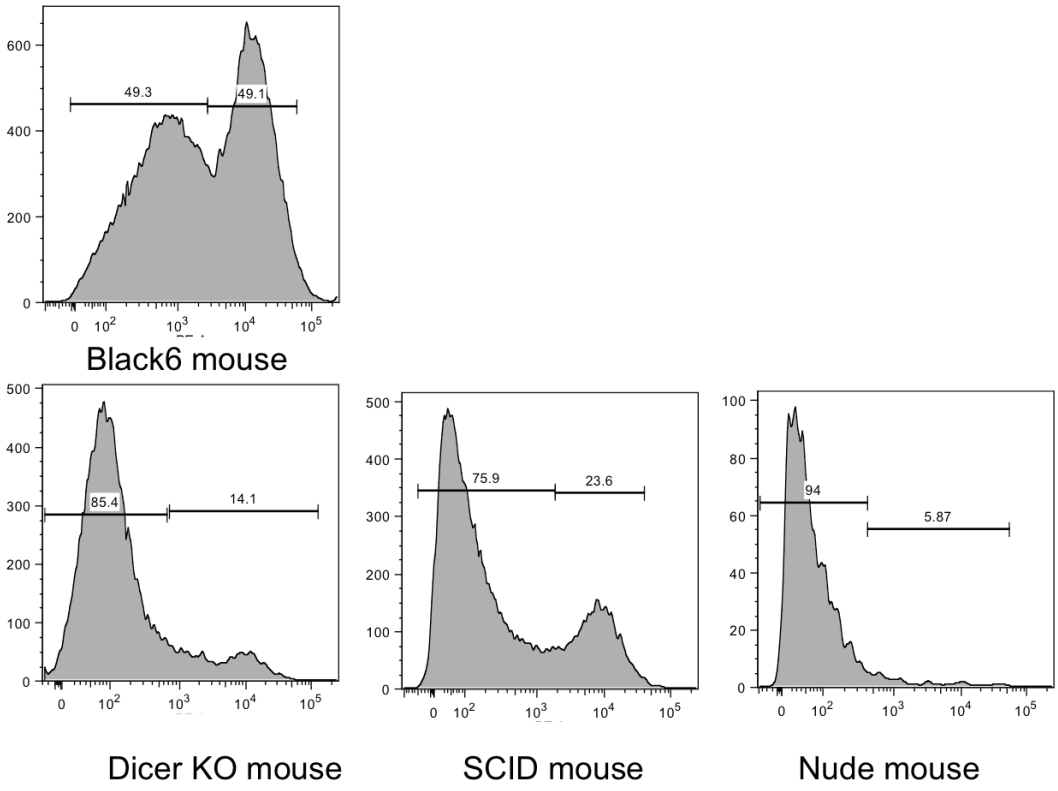

Figure 6 Development of mTECs: Normally mTECs can be divided in immature and mature mTECs (Black6 mice). Immature mTECs replenish the mature mTEC populations. In mouse strains such as Dicer KO, SCID and nude mice we saw an impairment of the four subpopulations thus effecting as well the development of mature mTECs.

The remarkable similarity of the distribution of the four subpopulations between nude, SCID and Dicer KO mice hinting the fact that these subpopulations might represent sequential lineage progression stages of mTEC and that this lineage progression is dependent on two type of signals: cell-intrinsic and cell- extrinsic factors. It has been already published (Desanti et al. 2015) that mTEC maturation and development are depending on lymphotoxin and RANKL signals provided by several hematopoietic cells. Activation and inactivation of certain signalling pathways are also needed to ensure a proper mTEC development such as Notch and NF kappa b signalling. It is generally accepted that fine-tuning of intracellular signalling usually relies on posttranslational modulators such as miRNAs or long-non-coding RNAs (lncRNAs) to act as switches.

3.3 The mRNA expression profiling of the four subpopulations

To be able to determine the molecular mechanisms of mTEC lineage progression we performed mRNA and miRNA expression profiling of the four subpopulations of immature mTECs (Sca-1, CD24, DP and DN) and compared them to those of mature mTECs (high). This confirmed our hypothesis that those subpopulations are related to each other and eventually develop from each other. When we compare the mRNA expression levels and displayed the data in a dendrogram (Fig.7) we should see how the subpopulations are related to each other. One replicate from DP has been removed from all analyses as the sample turned out to be an outlier. DP and CD24 are close to each other as they have been paired, as well as DN with high. Sca-1 was not paired with any subpopulations thus leading to the conclusion that it may differ from the other subpopulations. Still we can assume that Sca-1 is closer to the two subpopulations DP and CD24 than to DN or high. Another demonstration of the relationship of all four subpopulations is shown in the unrooted tree (Fig.8). It endorses our hypothesis what we have seen already in the dendrogram, but also segregated the relationship between Sca-1, DP and CD24; as Sca-1 is closer to DP than to CD24. Taken together the data give us the hint that the subpopulation Sca-1 is different and might be a progenitor subpopulation, while DP acts as an intermediate stage between Sca-1 and CD24. The subpopulation DN is the closest to mature mTECs.

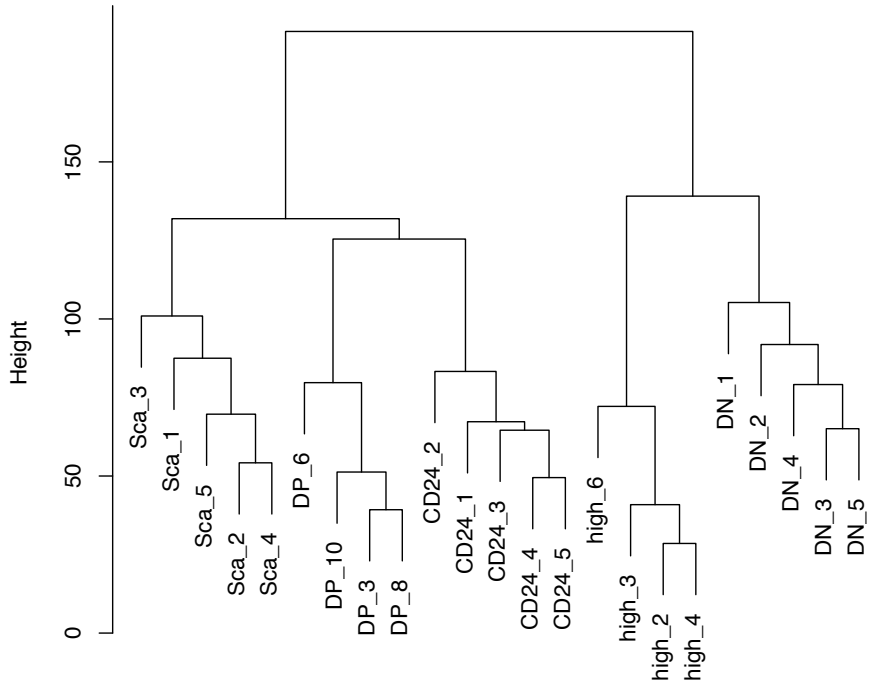

Figure 7 Dendrogram of mRNA profiling: The data have been generated with R. mRNA expression data, which were generated with mRNA microarray analysis, have been used for this analysis. For each subpopulation 5 replicates have been used, except high and DP, where only 4 replicates have been used. Each replicate consists of 24 mice, which have been used to pool the samples together.

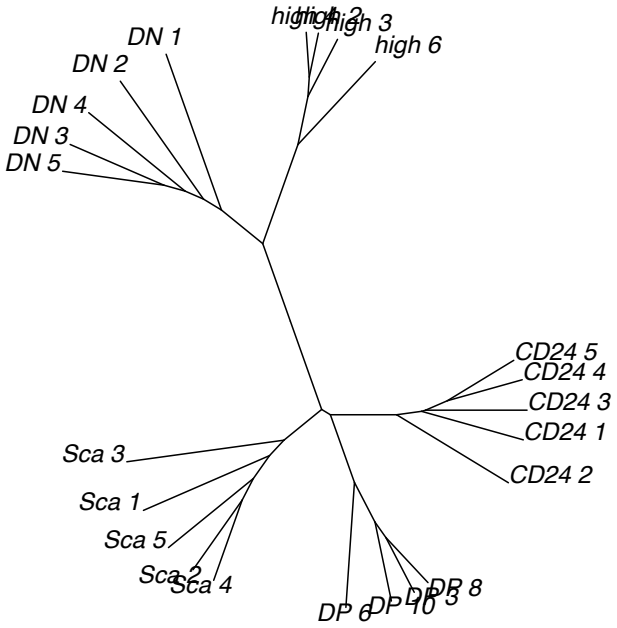

Figure 8 Unrooted tree of mRNA profiling: The unrooted tree is another illustration method to show the relationship between all five populations. Sca-1, DP and CD24 have similar mRNA expressions than DN and high. DN and high populations are more alike.

We did a principal component analysis (PCA) to see our findings from another perspective. The first component gives the highest degree of variability (48%) the mRNA expression data can give, while the next following component provides 15% of variability (Fig.9). Therefore the first two components were used to show the variability between the five populations. Different symbols have been used to represent the different populations, Sca-1 as a triangle, DP as a full coloured square, CD24 as an empty square, DN as a star and high as a circle (Fig.10). First it can be interpreted that all five populations are different from each other, as there is no overlap of two or more populations. It can be understood that the populations high and Sca-1 share the least similarity of mRNA expressions. As the population high have a high value of PC1 but a low value of PC2, whereas Sca-1 a high value of PC2 but an extremely low level of PC1. The interpretation of a potential developmental sequence or which populations are more alike is harder to read compared to a unrooted tree or a dendrogram.

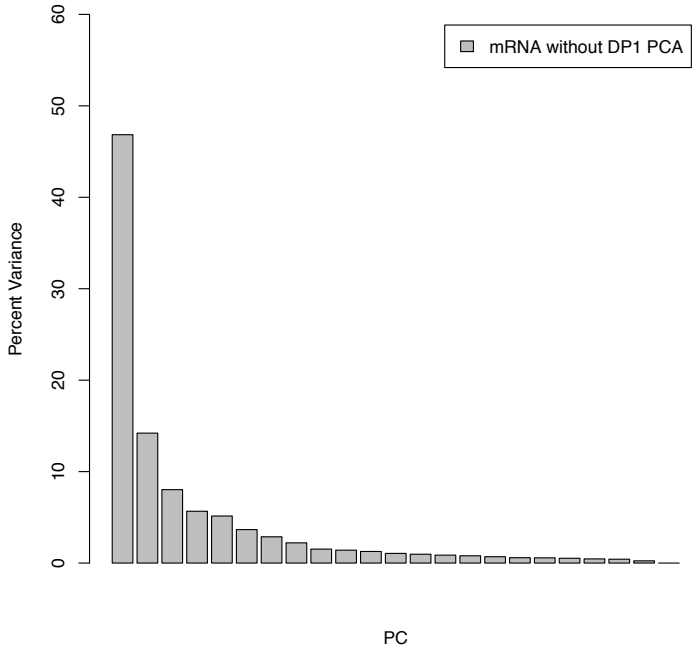

Figure 9: Principal Component Analysis: The percentage of variance in each principal component. PC1 contributes around 48%. Summing up all 24 PCs it will make up to 100%.

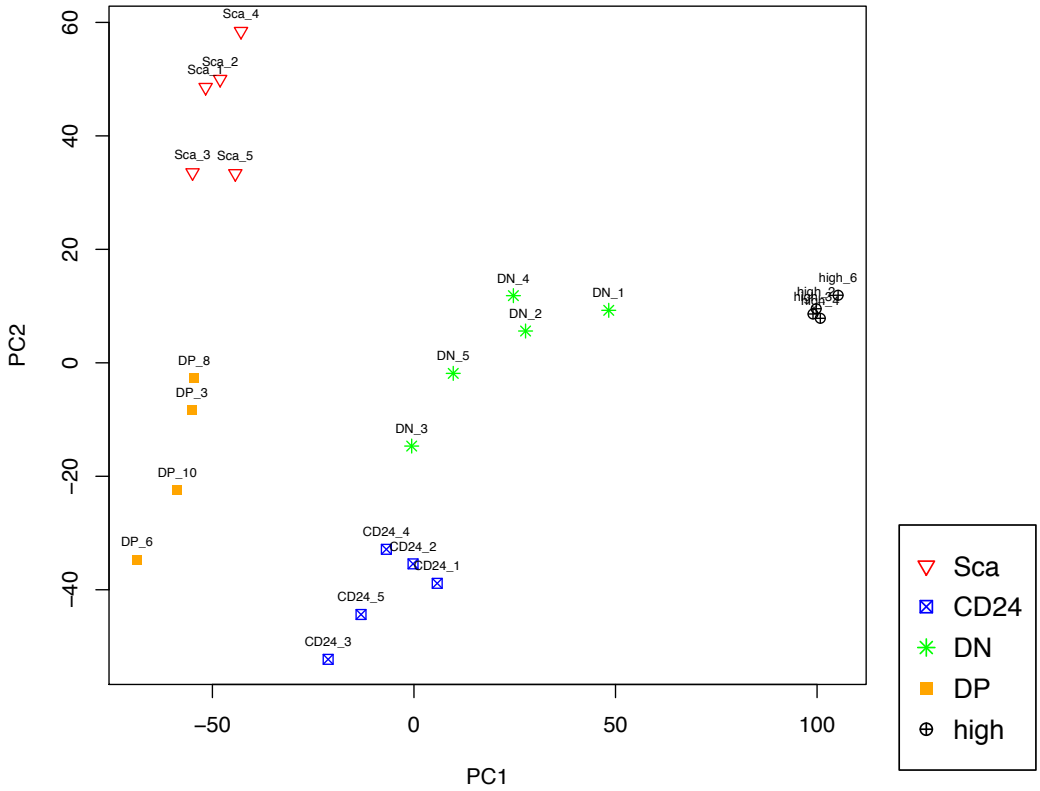

Figure 10 PCA of the four subpopulations: The principle component plot with PC1 and PC2 is displayed here. There are no overlaps of the populations thus we conclude that all five populations are different from each other. Because PC2 covers only 15% variance, the difference between DP and Sca-1 is just minor.

The Venn-diagram shows the number of genes that are unique for each subpopulation (Fig.11). Each subpopulation is compared to mature mTECs. The rationale behind this analysis is to reveal the numbers of unique genes between the populations. While Sca1-high represents 1381 unique genes, DP-high 1788 genes, CD24 only 486 and DN merely 324 unique genes, meaning that the difference between the mRNA expressions of each subpopulation to mature mTEC is getting less during the development. Thus we propose the following potential developmental sequence: Sca-1 → DP → CD24 → DN → mature mTECs (high).

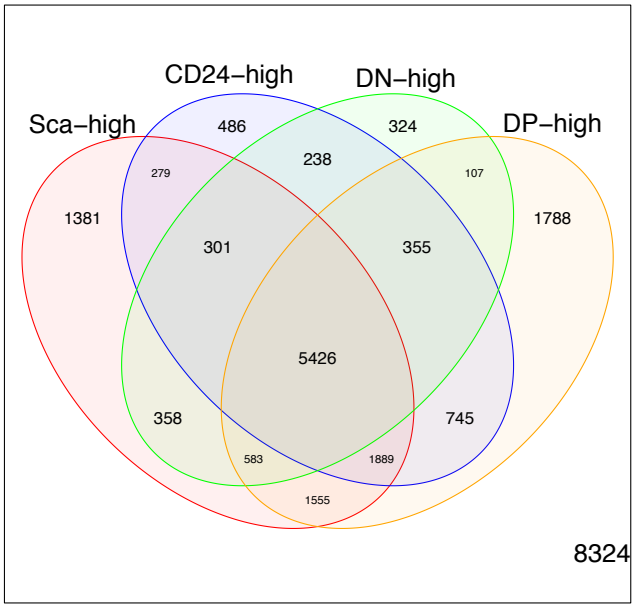

Figure 11 Venn diagram of the mRNA profiling: When each subpopulation is compared to mature mTEC (high) we can see that Sca-1 has 1381 unique genes compared to high, whereas DN only 324 unique genes. Therefore a Venn diagram can provide hints how much different each subpopulation is compared to high.

3.4 The miRNA expression profiling of the four subpopulations

Besides the mRNA profiling we did also with the same biological samples a miRNA profiling, as we are interested to see the different miRNA expressions during the development. Interesting was to observe that the four subpopulations showed a very different clustering in the miRNA expression data compared to the mRNA expression data. When we looked at the miRNA expression levels between the subpopulations we noticed that always two subpopulations are sharing the same sets of miRNA, while the set of miRNAs in mature mTECs is clearly very different from the two clustered subsets (Fig.12). The dendrogram shows that Sca-1 and DN are sharing the same miRNAs, while DP and CD24 are sharing the same miRNA sets. These results are indicating that miRNA expressions vary from the gene expressions during the development of mTECs. These variations follow the common knowledge how miRNA interacts to regulate gene expressions (Neilson et al. 2006). A developmental arrest can be seen as the four subpopulations are hampered and mature mTECs are not being developed or not being developed properly in the absence of miRNAs. Thus we are hypothesizing that miRNAs might play a role in the development of mTECs.

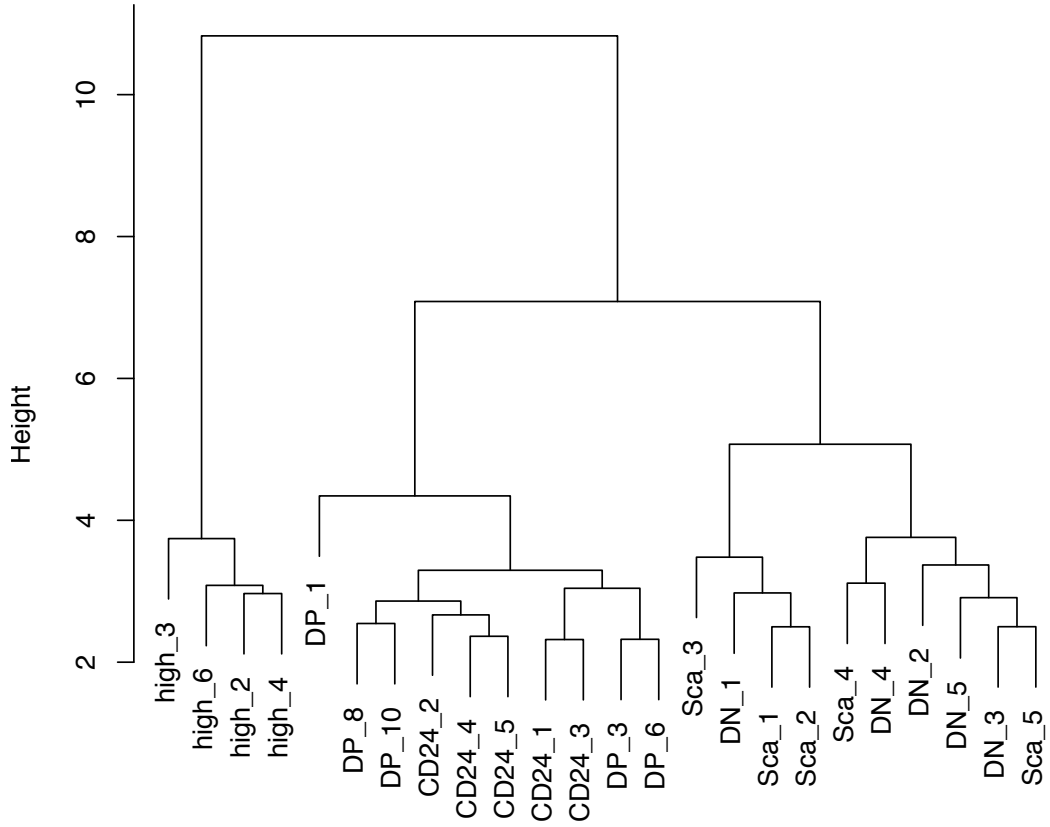

Figure 12 MiRNA profiling of the four subpopulations: The dendrogramm displays the five populations as a family tree. Where we can see that miRNAs from high are unique. DP is clustered with CD24, while Sca-1 is clustered with DN.

When we looked at the PCA on the miRNA level the difference between the two clustered subpopulations look similar to the dendrogram (Fig.13). MiRNAs in mature mTECs are very different from those of the four subpopulations. Again we can see a clustering of always two subpopulations, Sca-1 with DN and DP with CD24. Mature mTECs have a high value of PC1. While Sca-1 and DN have a high value of PC2, but a low value of PC1. And DP and CD24 have both low levels of PC1 and PC2. The results provide an indication that high and the two clusters are different from each other.

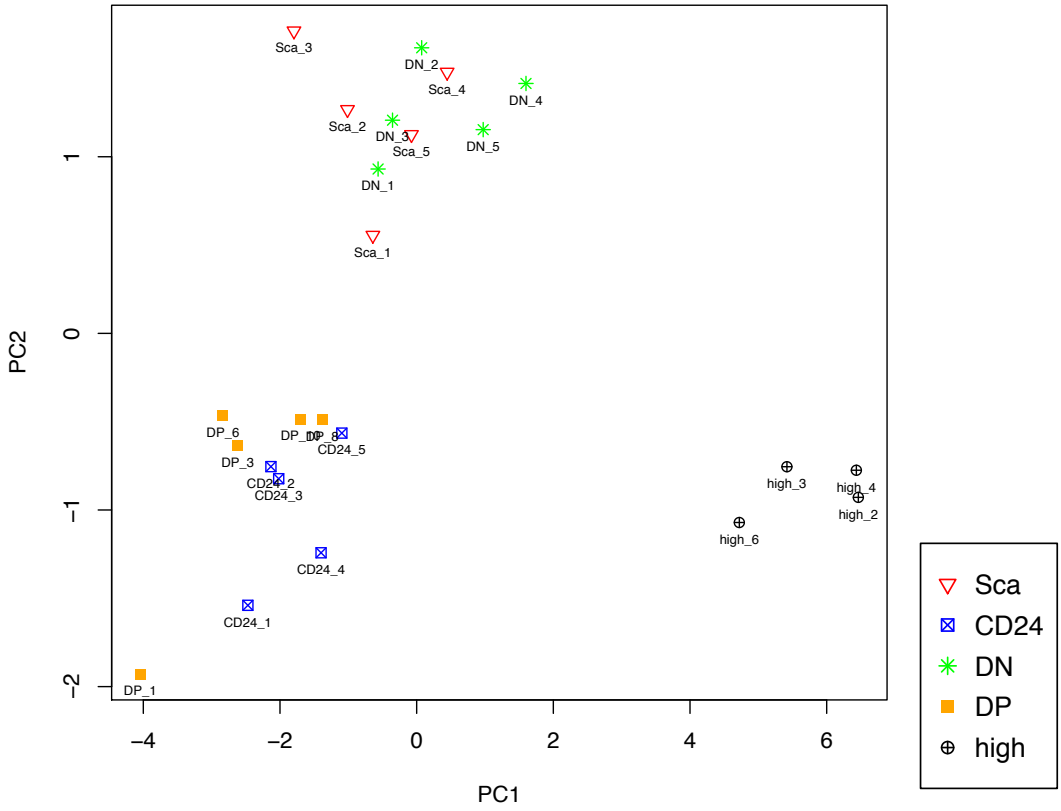

Figure 13 PCA of miRNA profiling: The PCA plot shows a clustering of Sca-1 with DN, while DP groups with CD24. The plot hints that high and the two clusters differ from each other. The per cent of variance in PC1 is 63% and in PC2 only 10%. Therefore the difference between DP/CD24 are minor compared to Sca-1/DN. But the variance difference between DP/CD24 to high, as well as Sca-1/DN to high, are huge.

The Venn-diagram for miRNAs (Fig.14) demonstrates the number of miRNAs from each subpopulation compared to high. Sca-1-high shows 13 exclusive miRNAs, as well as 13 unique miRNAs for DN-high. When comparing Sca-1-high with DN-high there are 8 miRNAs Sca-1 and DP share with each other. CD24-high displays 17 unique miRNAs and DP-high counts 42 distinctive miRNAs. CD24 shares according to the Venn-diagram 63 exclusive miRNAs with each other.

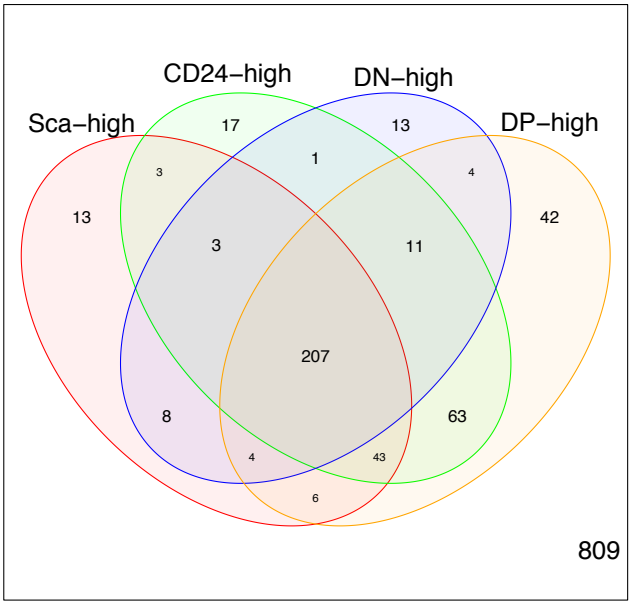

Figure 14 Venn-diagram of the miRNA profiling results: Sca-1-high has 13 unique miRNAs, CD24-high 17 miRNAs, DN-high 13 miRNAs and DP-high 42 miRNAs. When we take two subpopulations together, Sca-1 and DN, we can see an overlap of 8 miRNAs while the two populations CD24 and DP have 63 common miRNAs with each other.

Taking it all together miRNAs are present in the four subpopulations and show in miRNA clusters. We can see a clustering of miRNAs in Sca-1 with DN and DP with CD24. The miRNA set from mature mTECs seem to be different from the two clusters. The relevance of miRNAs in the development of mTECs still needs to be discovered.

3.5 A developmental progression among the four subpopulations

To look closer into the potential lineage progression sequence we looked into known markers, which have been published to play a role in mTEC development or TRAs (Fig.15). Known markers are such as CD80 (Akiyama et al. 2014), FoxN1 (O'Neill et al. 2016), Aire (Shi and Zhu, 2013), Pdpn, Gad1 (Rattay et al. 2016), Casein beta (Csnb), Trp63 (Wu et al. 2012), Pcp4 (Goldfarb et al. 2016), Sirt1 (Chuprin et al. 2015), CCR4 (Laan et al. 2009), Meis1 (Hirayama et al. 2014) and Pax1 (Romano et al. 2013). By plotting the expression values of those genes into our sequence we could actually see a lineage progression, indicating that the population Sca-1 might come first, then develops further into DP. From DP it differentiates to CD24, while CD24 becomes DN and very likely that DN becomes a mature mTEC. Just like the sequence, which we have observed.

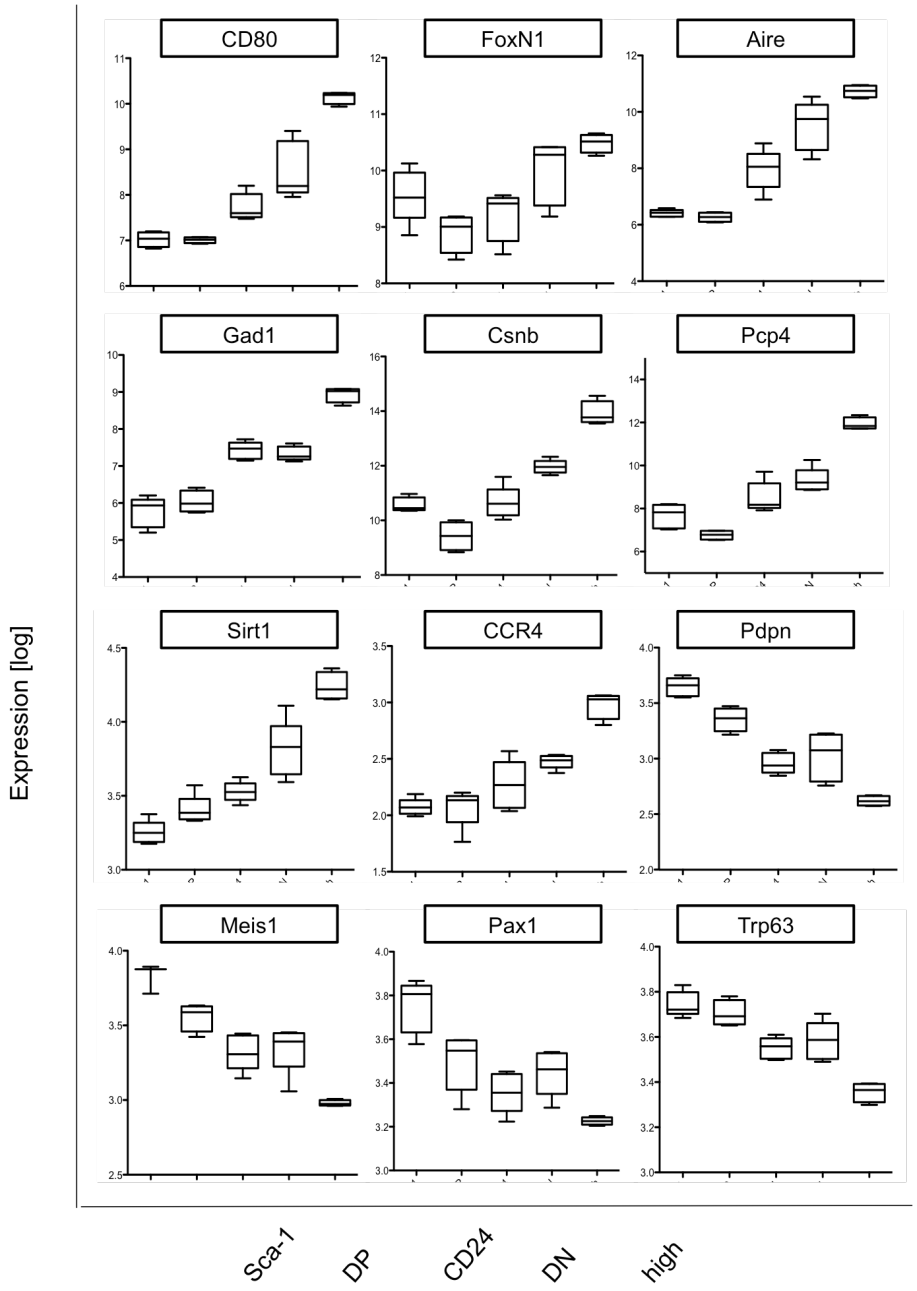

Figure 15 Potential lineage progression sequence: By plotting known mTEC markers, which have been published in the literature to play a role in the mTEC development, we can actually see that our suggested lineage sequence among the four subpopulations supports our claim. We used 5 replicates for each subpopulations, except DP and high, were we used only 4 replicates.

By comparing the expression of thousands of genes in the four subpopulations we could reveal a potential sequence how immature mTECs develop over different steps to become mature mTECs. To endorse our hypothesis we used another method to propose the progression sequence, by using a FoxN1 eGFP mouse line (Fig.16). There we look specifically for the FoxN1 expression levels in the four subpopulations as well as in mature

mTECs (MHCII high) and compared the expression values of FoxN1 among those cell populations. It is known in the literature that FoxN1 is upregulated during mTEC maturation and that immature mTECs express very low levels of FoxN1. So we looked at the expression values of each population and we can reveal that Sca-1 (black) has a very minor FoxN1 expression, while DP (red) has an average expression level. CD24 (blue) is almost comparable with mature mTECs (violet), whereas DN (green) is not distinguishable from mature mTECs. Therefore we propose the following lineage sequence: Sca-1 → DP → CD24 → DN → mature mTECs

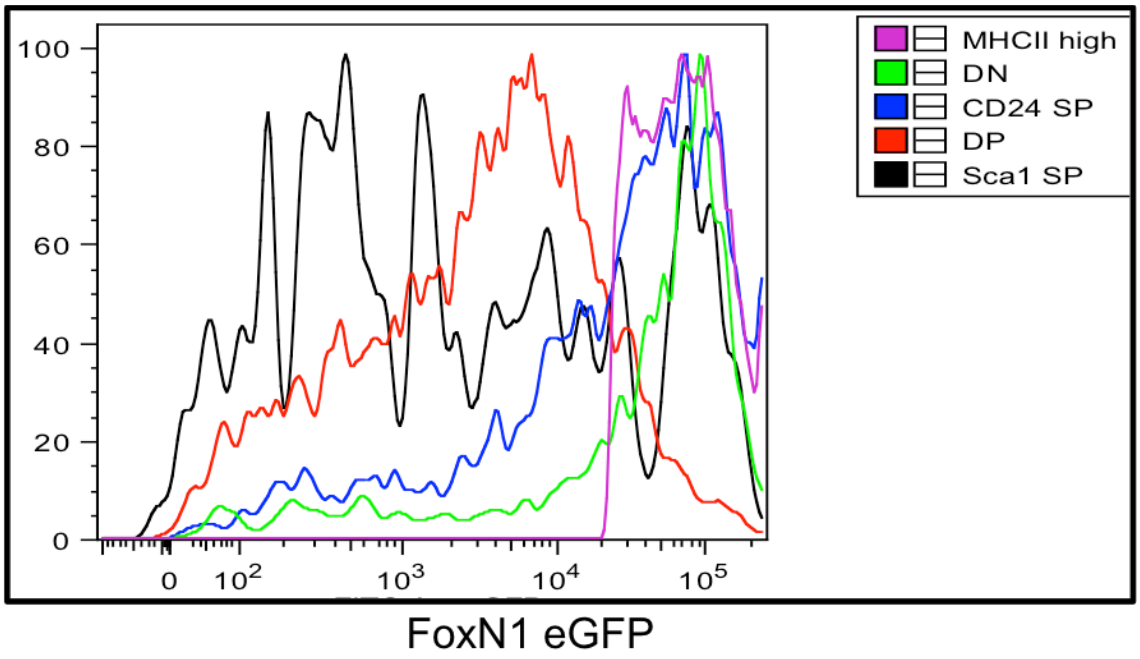

Figure 16 FoxN1 eGFP expression in the four subpopulations: FoxN1 is a known mTEC development marker. We used a FoxN1 eGFP mouse and checked the correlation between the FoxN1 eGFP signal and each subpopulation. This experiment has been done three times; each time one mouse was used.

3.6 Finding the most prominent miRNAs in the development of immature mTECs

To find the functional miRNAs, which are playing a role in the four subpopulations, data that have been collected by miRNA microarray analysis were used to find the most relevant ones. Afterwards those data were further processed in a PCA. Looking at the PCA results we saw a striking separation of miRNAs in the four subpopulations as well as in mature mTECs. In the PCA

we listed all miRNAs in different principle components (PCs) based on their percentage of variances (Fig.17). In the principal component 1 around 63% of miRNAs are showing exactly the phenotype that we have observed, clustering of CD24 with DP and Sca-1 with DN. PC 2 covers 10% of miRNAs and all following PCs are showing a less and less per cent of variances. All 24 PCs are summed up to 100%.

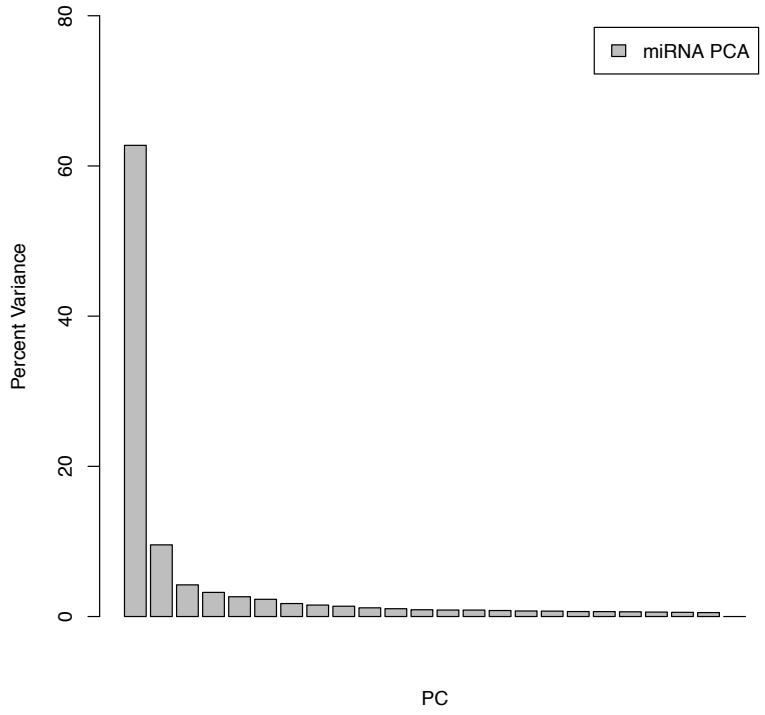

Figure 17 microRNA sets with their per cent of variations: The percentage of variance in each principal component. PC1 contributes around 63%. Summing up all 24 PCs it will make up to 100%.

3.7 Verification of the potential miRNAs in different mouse lines

From the PC1 be picked from the top of the list 25 miRNAs, as they seem to be the most prominent ones. Afterwards we ran a literature search to figure out whether those miRNAs have been mentioned to play a role in development, differentiation or are involved in a signalling pathway. Subsequently search for functional taqman primers for those miRNA candidates. Then we verify their existence in quantitative PCR (qPCR) in 3 biological independent sorted cells. The results in qPCR revealed that only 16 miRNAs are promising candidates. The other 20 miRNAs we have tested

failed to show a substantial signal. Thus we only proceed with those 16 miRNAs, which are listed in the table (Tab.1).

miRNA
mmu-let-7c-5p
mmu-miR-125a-3p
mmu-miR-125a-5p
mmu-miR-125b-1-3p
mmu-miR-125b-5p
mmu-miR-200c-3p
mmu-miR-203-3p
mmu-miR-205-5p
mmu-miR-29a-3p
mmu-miR-34a-5p
mmu-miR-34b-3p
mmu-miR-494-3p
mmu-miR-709
mmu-miR-761
mmu-miR-877-3p
mmu-miR-92a-5p

Table 1 Potential miRNAs from principal component 1: The most promising miRNAs after intensive literature search and qPCR.

Interestingly, our miRNA candidates have different expression dynamics (Fig.18). Some of them (miR-200c-3p, miR-92a-5p, miR-34a-5p, miR-494-3p) are going up during lineage progression, some are (miR-877-3p, miR-125b-5p, 125a-3p, miR-125a-5p, miR-205-5p) declining during maturation, while others (miR-709, miR-29a-3p, miR-203-3p, miR-34b-3p, miR-761, let-7c-5p, miR-125b-1-3p) are rising and falling during development. This kind of fluctuation of miRNA expressions might be important during the lineage progression thus worth to investigate further. To confirm our findings we went back to our miRNA microarray data and checked for the miRNA expressions of our candidate miRNAs. Indeed we saw the same dynamics in the microarray data as well.

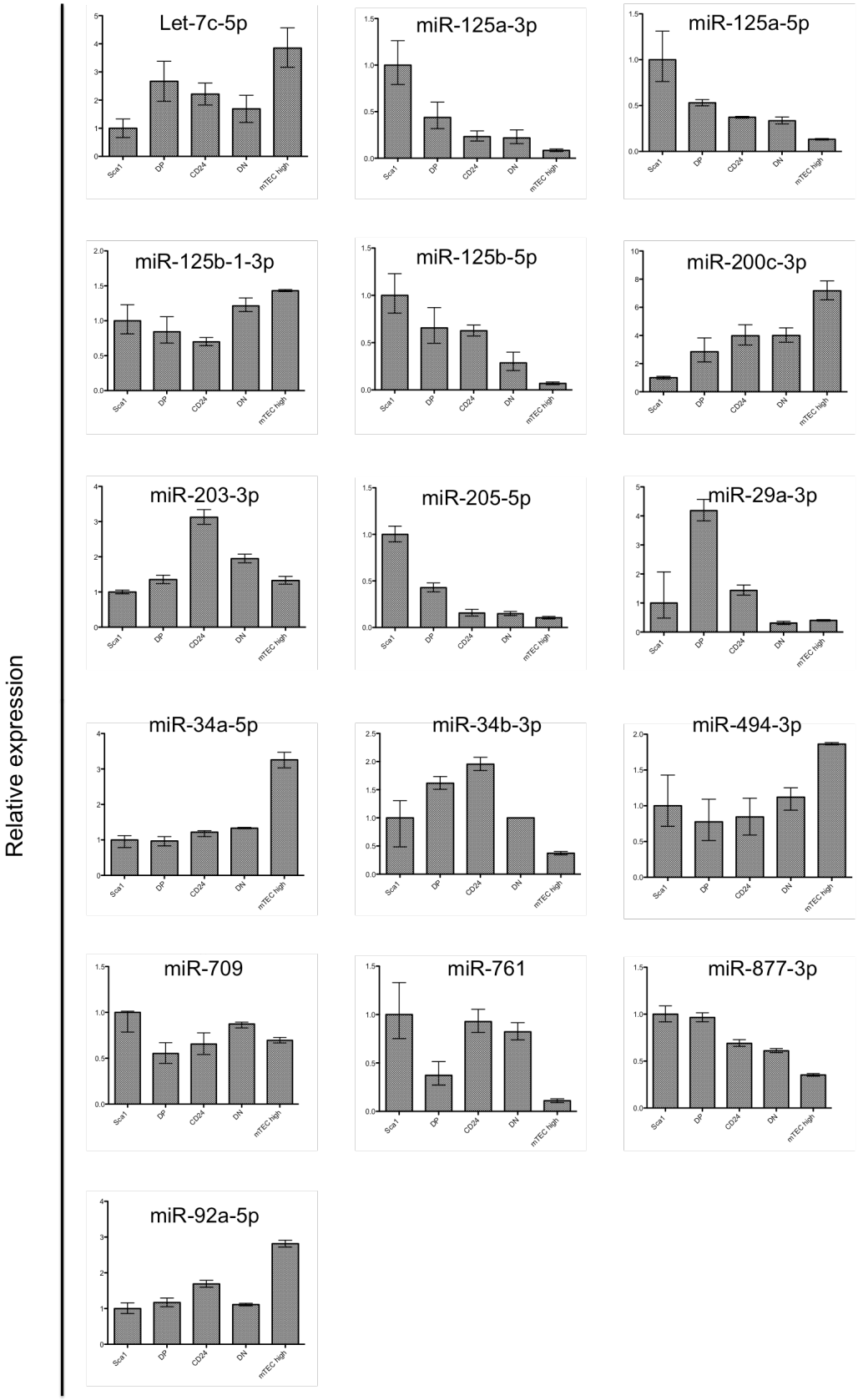

Figure 18 Different miRNAs plotted in our proposed lineage sequence: The qPCR was done with freshly isolated RNA from three independent sorts, each time with 6 female mice pooled together.

After we have confirmed the existence of our miRNA candidates in Black6 mice, we wanted to investigate their expressions in Dicer KO mice to see if their expressions are disturbed in those animals. In Dicer KO mice a shifted phenotype can be observed, where the biggest cell population in DP is extremely reduced while the cell population in CD24 is increased (Fig.19). Dicer KO mice have a loss of Dicer, an enzyme that cleaves pre-miRNA to become mature miRNA. If this fails all Dicer-dependent miRNAs are lost.

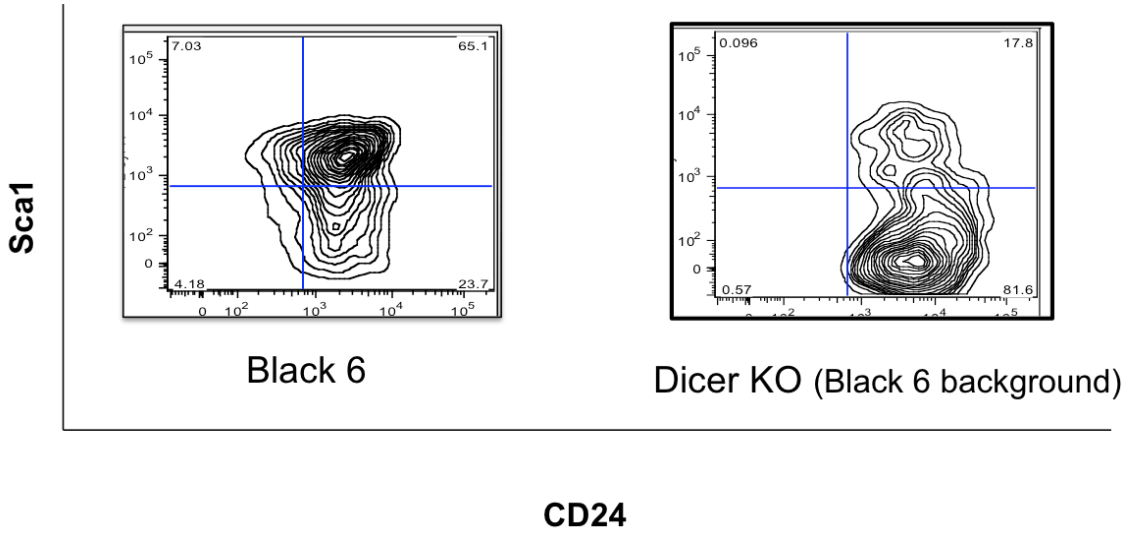

Figure 19 Distribution of the four subpopulations in Dicer KO mice: Sca-1 positive cells are on the upper two quadrants. CD24 positive cells are on the lower right quadrant. Cells with Sca-1$^+$ and CD24$^+$ are called double positive (DP) and is displayed on the upper right quadrant. Sca-1$^-$ and CD24$^-$ cells are on the lower left quadrant. A normal Black6 mouse has the biggest cell population in DP, followed by CD24+. While in Dicer KO mice the cell population CD24 becomes the biggest population and the population in DP drops dramatically.

As Sca-1 and DN are minor subpopulations, we needed to pool two populations together to have enough material to run our qPCR analysis. We pooled the populations Sca-1 with DP and CD24 with DN. Indeed all miRNAs were missing or expressed in a low expression level compared to the wild type. The threshold cycle (C_T) is shown between Sca-1 and DP versus CD24 and DN in Dicer KO (left bar) and Black6 mice (right bar), respectively (Fig.20). As some miRNAs were not detectable their C_T value is set as 40.

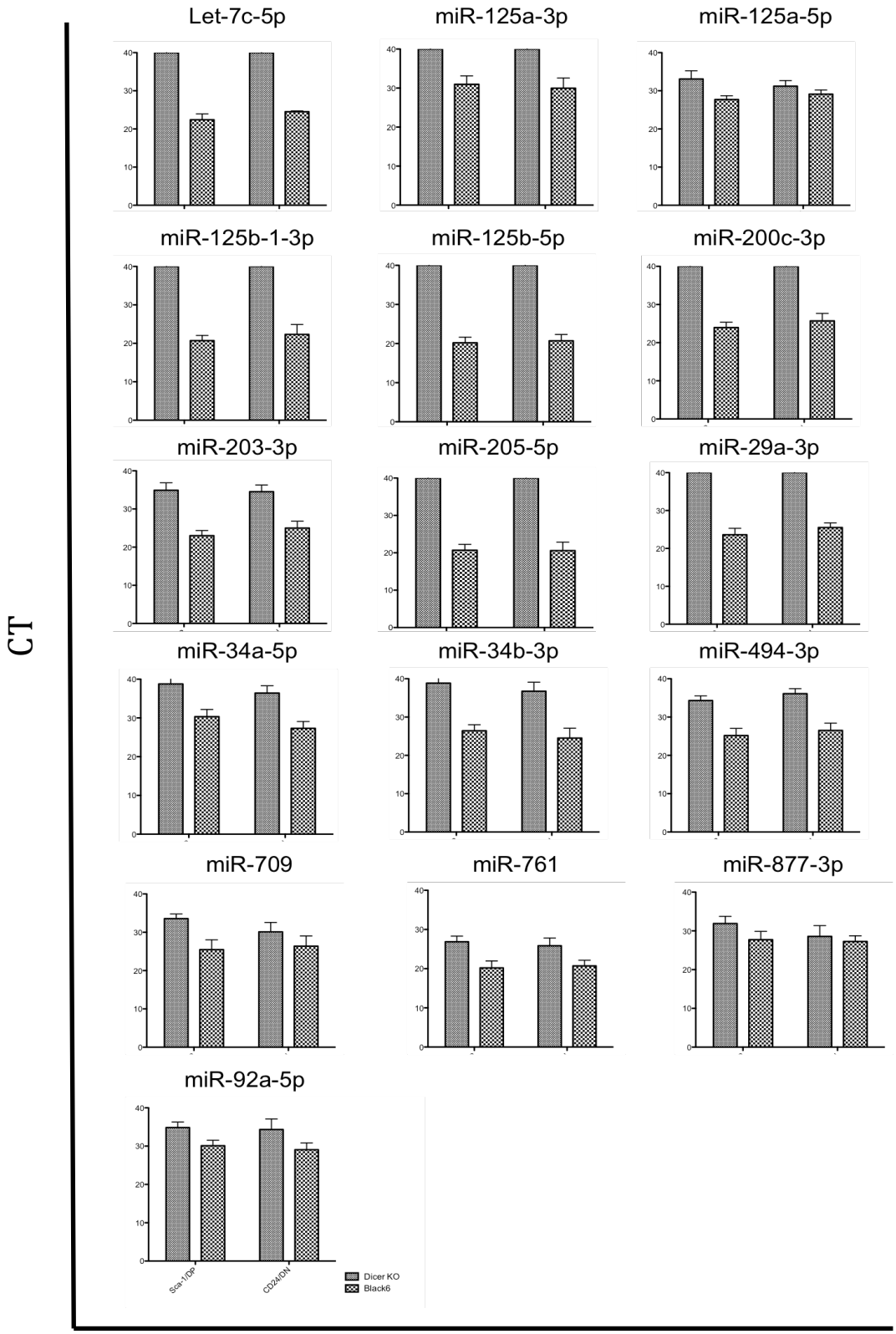

Figure 20 miRNA expressions in Dicer KO mice versus Black6 mice: For this experiment RNA were isolated from Black6 mice and Dicer KO mice. The experiment has been performed three times; each time 12 female mice were pooled. In all samples the difference between Black6 mice and Dicer KO mice were significant with a p-value <0.05 (t-test). Except for miR-200c-3p in CD24/DN sample and miR-877-3p in CD24/DN sample.

The results are indicating that our miRNA candidates in Dicer KO mice are expressed in very low up to non-detectable levels when compared to the wild type Black6 mice. Indicating that those miRNA expressions are hampered in Dicer KO mice, probably inducing the phenotype changes in the four subpopulations. For this reason we continue with our 16 miRNA candidates.

3.8 A dynamic model to follow the mTEC development

The amount of $2x10^7$ wildtype bone marrow cells, depleted for CD90.2, are injected intravenously into SCID mice (Fig.21) and the data indicated that bone marrow reconstitution in the adult SCID results in a restoration of the four subpopulations and reach their normal distribution after 8 weeks (Fig.22). The size of the reconstituted thymus is comparable to a young mouse thymus. The four subpopulations are back as well as a proper T cell development could be seen. Therefore, this experimental model is suitable to track adult mTEC lineage progression and thymus restoration.

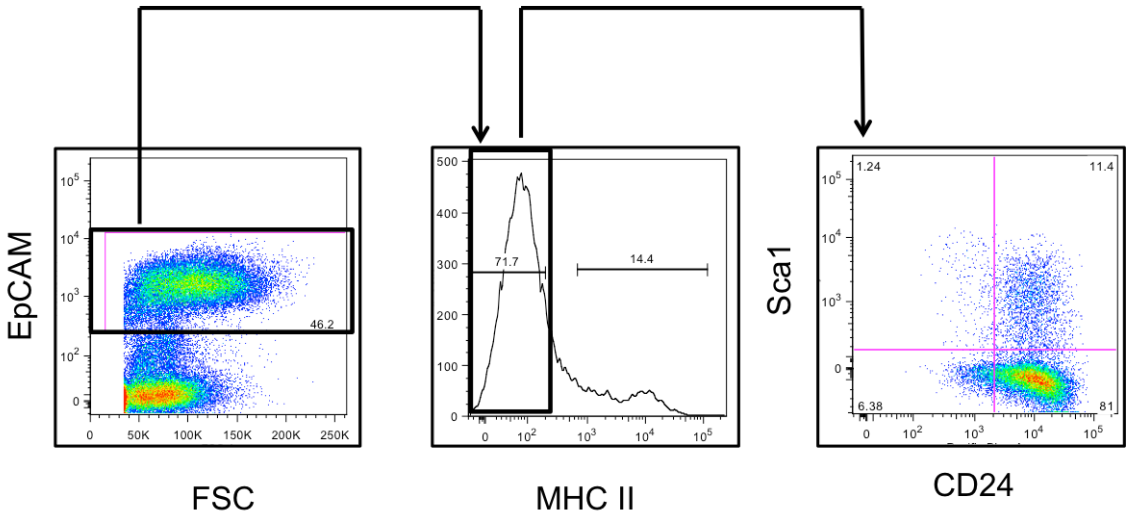

Figure 21 The four subpopulations in SCID mouse before bone marrow transplantation: MTECs are EpCAM positive cells therefore they have been gated. The four subpopulations can be found in MHCII lower part. Especially SCID mice do express a very low numbers of mature mTECs, MHC II high, therefore the cut between immature and mature mTECs can be segregated easily. In SCID mice the biggest subpopulation is CD24$^+$ with a 81% distribution of all immature mTECs.

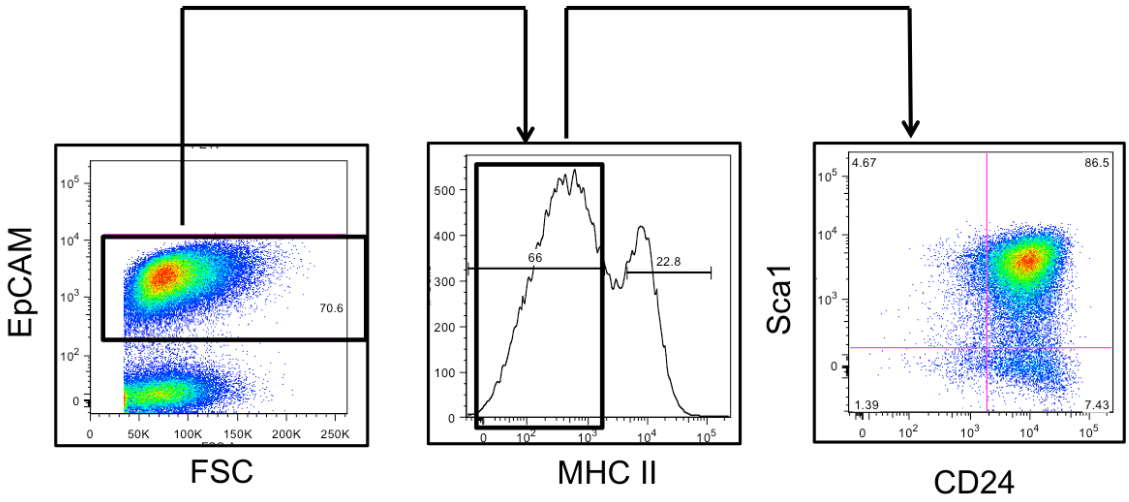

Figure 22 The four subpopulations after 8 weeks bone marrow transplantation: With the same gating strategy and a 8 weeks follow-up we can see an enrichment of EpCAM positive cells, but also two peaks in MHC II positive cells, indicating immature and mature mTECs. The bone marrow restored the function of mTEC development as well as the distribution of the four subpopulations. DP contains the biggest population of 87% like in Black6, balb/c or NMRI mice.

3.9 A dynamic model to reveal miRNA changes during mTEC development

The four subpopulations of mTECs, which represent distinct stages of mTEC lineage progression were isolated from the SCID thymi at different time points (3, 4, 5, 7 and 8 weeks) after bone marrow reconstitution, hence generating a time series of mTEC development during thymus restoration (Fig.23). Pooled wild type SCID mice show a 0.8%, 15.7%, 4.7% and 78.8% distribution, Sca-1, DP, DN and CD24 respectively. A small change of distribution 3 weeks post bone marrow transplantation can be observed 0.1%, 22.5%, 0.24% and 77.2%, Sca-1, DP, DN and CD24 respectively. After week 4 post bone marrow transplantation all three subpopulations increased their cell population (8.6%, 63.3%, 7.5%, Sca-1, DP and DN, respectively) except CD24 (20.6%) were we observe a decline. In week 5 the distributions of the four subpopulations look very similar to week 7 and week 8, indicating that during that time all four subpopulations have been recovered and the distribution of the four subpopulations are fixed. Sca-1 is around 0.4 -0.5 %, DP 73.2 – 77.6%, CD24 21.1 – 25.6% and DN 0.63- 1.1%. The variation between week 5 and week 8 are based on a t-test not statistically significant, p-values >0.05. The subpopulations Sca-1 and CD24 were statistically significant different

compared to SCID and/or previous time point until week 5 were only Sca-1 significantly changed compared to week 4, based on a t-test with a p-value <0.05. The thymus of SCID mice following bone marrow reconstitution has not previously been analysed with respect to the regeneration of the four subpopulations of mTECs. With our SCID model we can identify the time-frame of regeneration, which can be seen here as week 5 post bone marrow transplantation and therefore week 5 can be used as an end point of mTEC development.

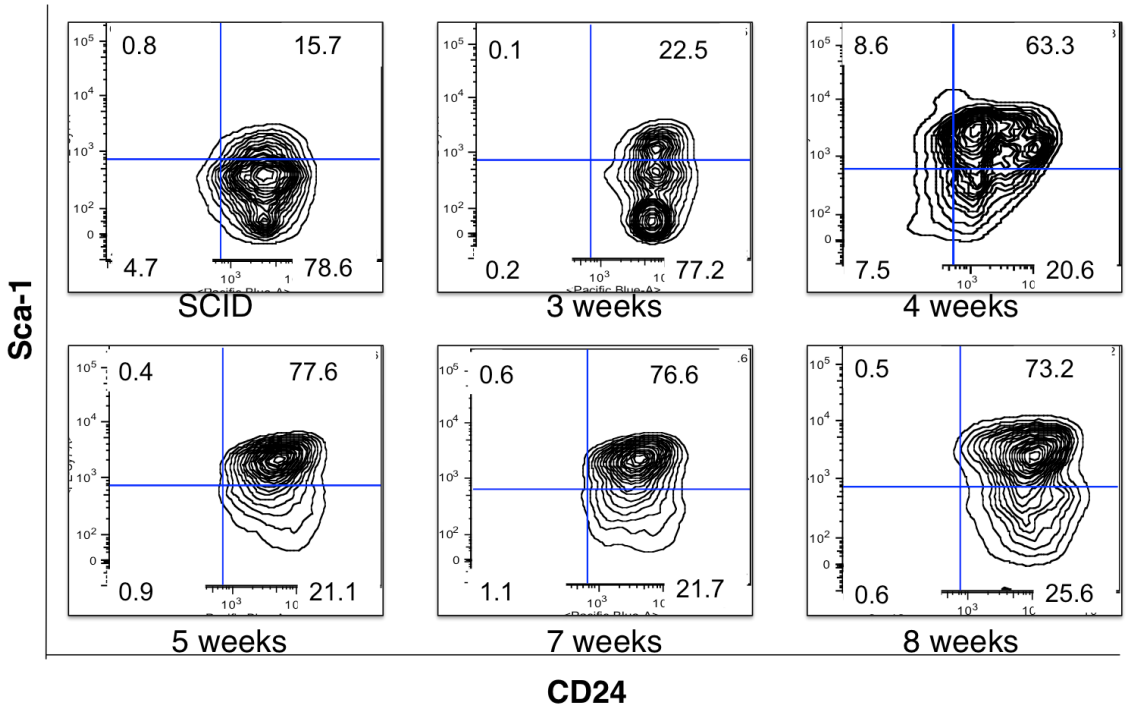

Figure 23 Time course after bone marrow transplantation: The development of the four subpopulations were observed over a time course of 8 weeks post bone marrow transplantation. In SCID mice the major subpopulation is CD24$^+$. During the bone marrow reconstitution the four subpopulations are changing their distributions, especially Sca-1$^+$ and CD24$^+$ cells. This dynamic remained until week 5. Afterwards the percentage of distributions remains the same.

The time frame until all four subpopulations was recovered could be also influenced by the regeneration of T cells. Therefore we checked for the generation of CD4 and CD8 T cells (Fig.24) as well as the expression of CD44 and CD25 in DN T cells stage (Fig.25). It is clearly to see that the generation of CD4 and CD8 thymocytes are normal after 4 weeks of post bone marrow transplantations. While the expression of CD44 and CD25 of week 3 looks already similar to week 8, indicating that CD44 and CD25 expression of DN T cells are already back after 3 weeks.

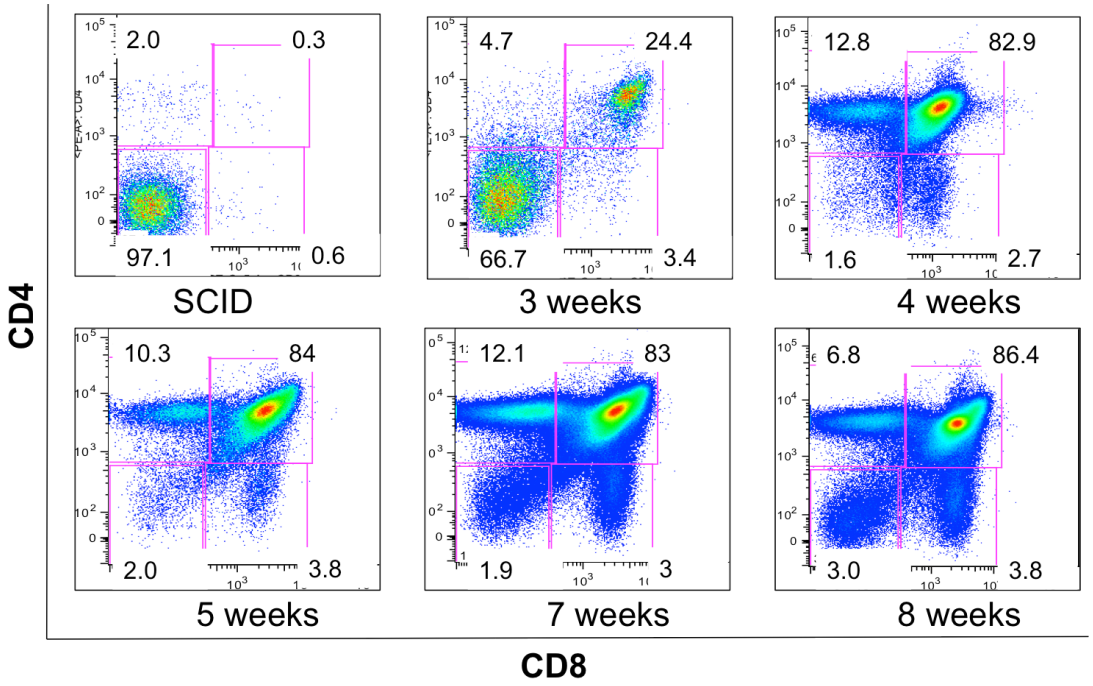

Figure 24 T cell regeneration after bone marrow transplantation in SCID mice: The development of $CD4^+$ and $CD8^+$ T-cells during the bone marrow reconstitution over a time course of 8 weeks. After3 weeks $CD4^+/CD8^+$ cells can be found. After week 4 post-transplantation the T-cell repertoire is recovered.

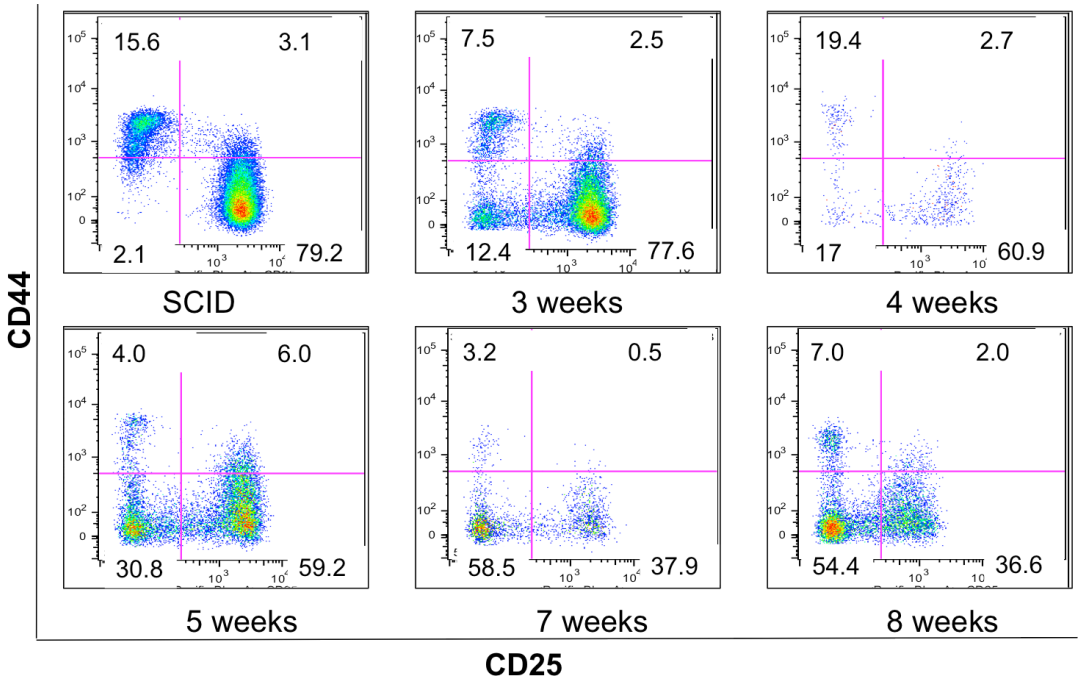

Figure 25 Development of CD44 and CD25 expression in T cells during bone marrow transplantation: The T cells, which are $CD4^-/CD8^-$ are called double negative (DN) and can further sub-divided into their CD44 and CD25 expressions. $CD44^-/CD25+$ lymphocytes undergo β-selection and after the selection for a proper re-arrangement of their TCR-b chain locus further differentiation steps occur to develop to $CD4^+$ and $CD8^+$ lymphocytes (Divya, 2007).

Then we generated a graph (Fig.26) with the kinetics of the generation of the four subpopulations as well as the generation of T cells during a time period of 8 weeks post bone marrow transplantation. Those distributions were compared with SCID and balb/c mice, where SCID acts as a starting point and balb/c as an end point to show how the distribution normally should be like.

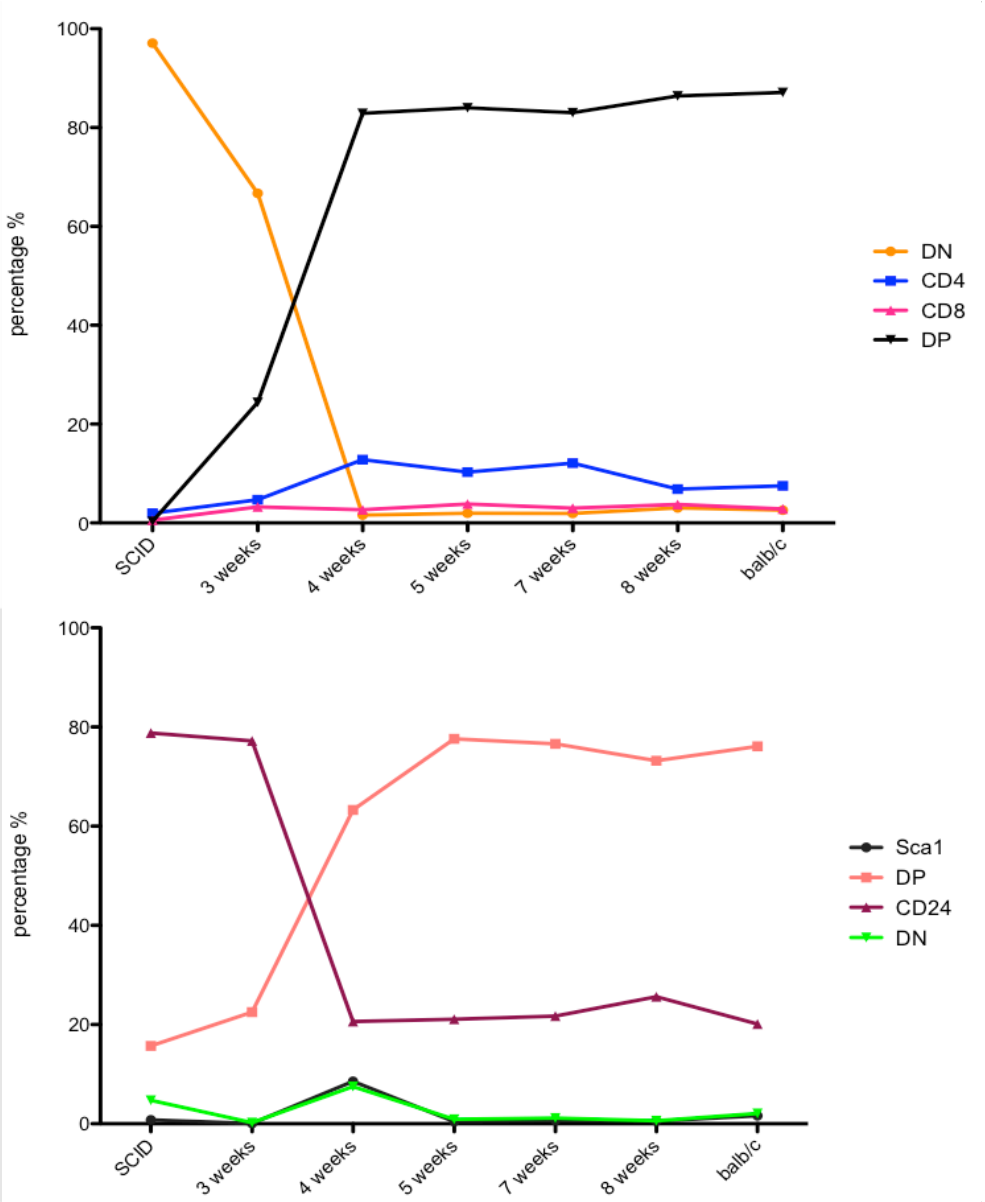

Figure 26 Time course of the generation of the four subpopulations and T cells: On the upper figure the development of $CD4^+$ and $CD8^+$ can be observed over a time course of 8 weeks. SCID is the starting before transplantation and balb/c is the mouse background of SCID but with an intact immune system. Therefore balb/c is used as a reference for an end point. We can note a dramatically change between $CD4^-/CD8^-$, called double negative (DN), and $CD4^+/CD8^+$, called double positive (DP) between week 3 and 4 post-transplantation. After week 4 all distributions remain stable. On the lower figure the development of the four subpopulations can be seen. The distribution among the four subpopulations change drastically between week 3 and 4 as well but the distribution itself start to become stable after week 5.

It is interesting to observe that the fully recovery of CD4 and CD8 T cells was reached after 4 weeks of post bone marrow transplantation, while the generation of the four subpopulations was reached after 5 weeks. We can see a shifted time point of one week. As it has never been observed how long it will take until the four subpopulations are recovered. With our findings we can claim that it takes around 5 weeks until their normal distribution of the four subpopulations is reached.

3.10 Discovery of dynamic miRNAs during mTEC development

The expression of our candidate miRNAs, which we picked in the previous experiments and selected based on several criteria, were analysed using a mouse model by inducing bone marrow transplantations in SCID mice and observe the development of our candidate miRNAs at different time points. The sorting needed to be adjusted, as the populations Sca-1 and DP are very small and are not adequate to run enough qPCRs. Thus Sca-1 and DP were pooled together (red rectangle), as well as CD24 with DN (blue rectangle) (Fig.27). In week 8 we isolated each subpopulation separately as Sca-1 (violet), DP (orange), CD24 (green) and DN (pink), respectively. The scrutiny of changes of miRNA expression during thymus regeneration will provide important hints to categorize “early” and “late” miRNAs, which are involved in regeneration of the mTEC compartment.

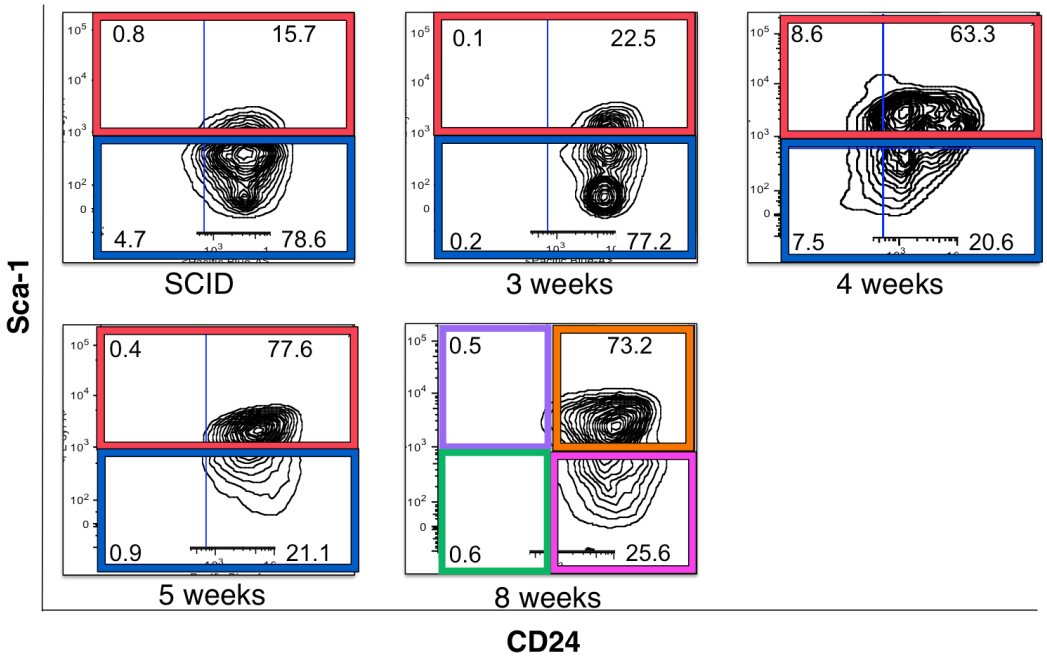

Figure 27 Sorting strategy of SCID mice to isolate the four subpopulations: The sorting strategy to obtain RNA samples to run for qPCR of our miRNA candidates. Due to a small number of RNAs in Sca-1 and DN, Sca-1 has been pooled with DP, while DN with CD24. In week 8 enough RNA can be obtained from each subpopulation.

Cells were isolated according to the isolation scheme above. RNA was isolated to verify our miRNA candidates during the SCID reconstitution on qPCR. Due to the low numbers of RNA samples we could only select few miRNA candidates to give an impression how dynamic miRNA expressions during development are. The selected miRNA candidates are: miR-125a-3p, miR-125b-5p, miR-200c-3p, miR-203-3p, miR-205-5p, miR-29a-3p and miR-709. The expression levels of each miRNA during the reconstitution are displayed from week 0 until week 5 post bone marrow transplantation (Fig.28).

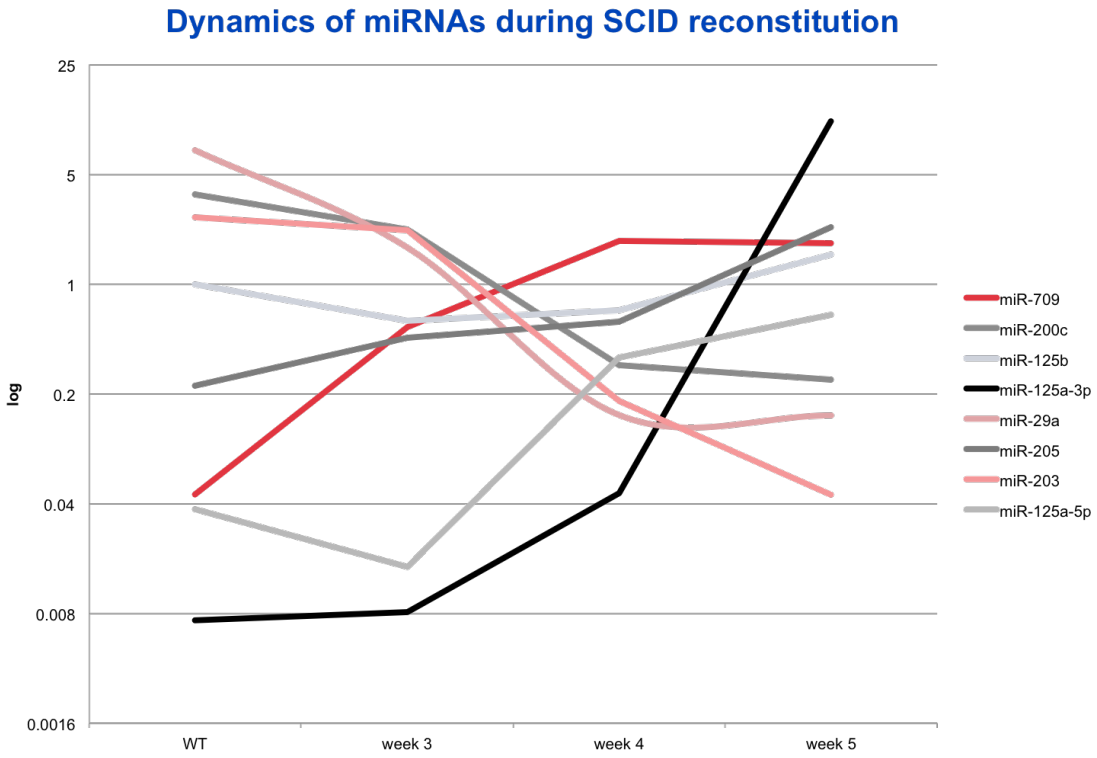

Figure 28 Dynamics of miRNAs during SCID reconstitution: The data shown here is part of the SCID reconstitution experiment where for each time point 24 SCID mice were injected with bone marrow. As a control PBS has been injected in another 24 SCID mice to serve as a control. Due to the low numbers of cells all four subpopulations were used and pooled as one sample. Then qPCR has been performed in three replicates. The results are shown in log base 5 scale.

3.11 Investigation of the functional role of our miRNA candidates

Interesting was to detect how dynamically the miRNA expression levels were during the SCID reconstitution, where mTECs have been gradually developed over the time course of 5 weeks. During that time period our candidate miRNAs have also undergone different expression levels. Thus we hypothesize that they might play a role during the development of the four subpopulations as well as having a role in maintaining the four subsets during adulthood. Therefore we wanted to investigate into those miRNAs by seeing if those miRNAs have a functional role. This can be investigated by manipulating the expression levels of mature miRNAs. To do so we are using a transient transfection method and transfect the tomato-red-labelled mTECs^{low} with either mimics or antagomiRs for 12 to 14 hours. Afterwards cells were mixed with embryonic thymic stroma from E14.5 old embryos and put into a culture system called re-aggregation thymic organ culture (RTOC) to harbour our cells for culture and give them a 3D culture microenvironment,

which those cells need to survive (Fig.29). After 4 days of cell culture cells were analysed with flow cytometry.

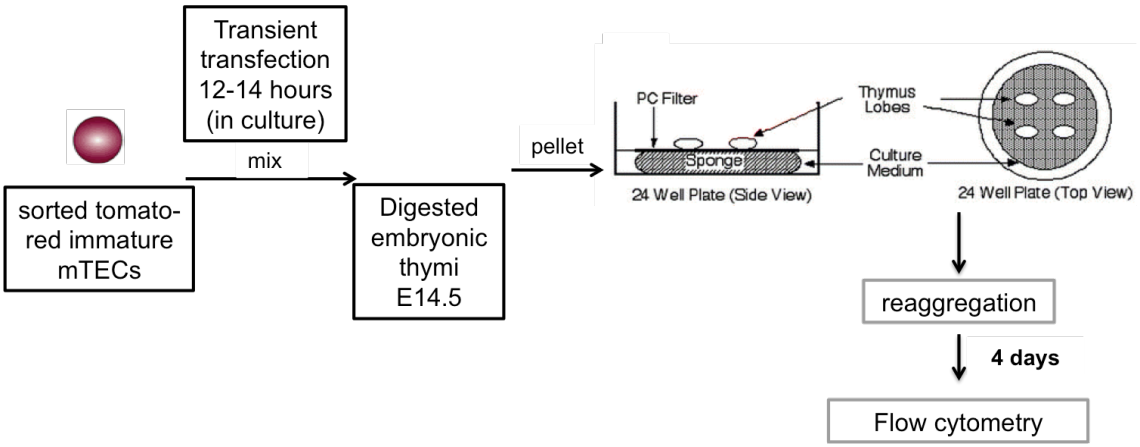

Figure 29 RTOC cultivation system: MTECs are primary isolated suspension cells and do not have ability to grow or to be maintained in the standard tissue culture. Therefore we use the RTOC system to cultivate our cells for a long period to be able to detect the miRNA changes we have induced. Our sorted cells are tomato-red labelled cells and transfected either with miRNA mimics, miRNA antagomiRs or siRNAs using a transient transfection method and then left for 12-14 hours in the well plates. After 12-14 hours cells were washed and mixed with digested embryonic thymi and placed on the top of a membrane. After 24 hours the cells form a 3D capsule where mTECs can grow and differentiate. After 4 days cells will be analysed with flow cytometry.

3.12 Transfection methods on primary cells

Primary cells are cells, which are hard to transfect and we tried different transient transfection reagents (Fugene, lipofectamine, Dharmafect and viromer Blue) to test the transfection efficiency and the viability (live and dead dye) of the cells after transfection (Fig. 30).

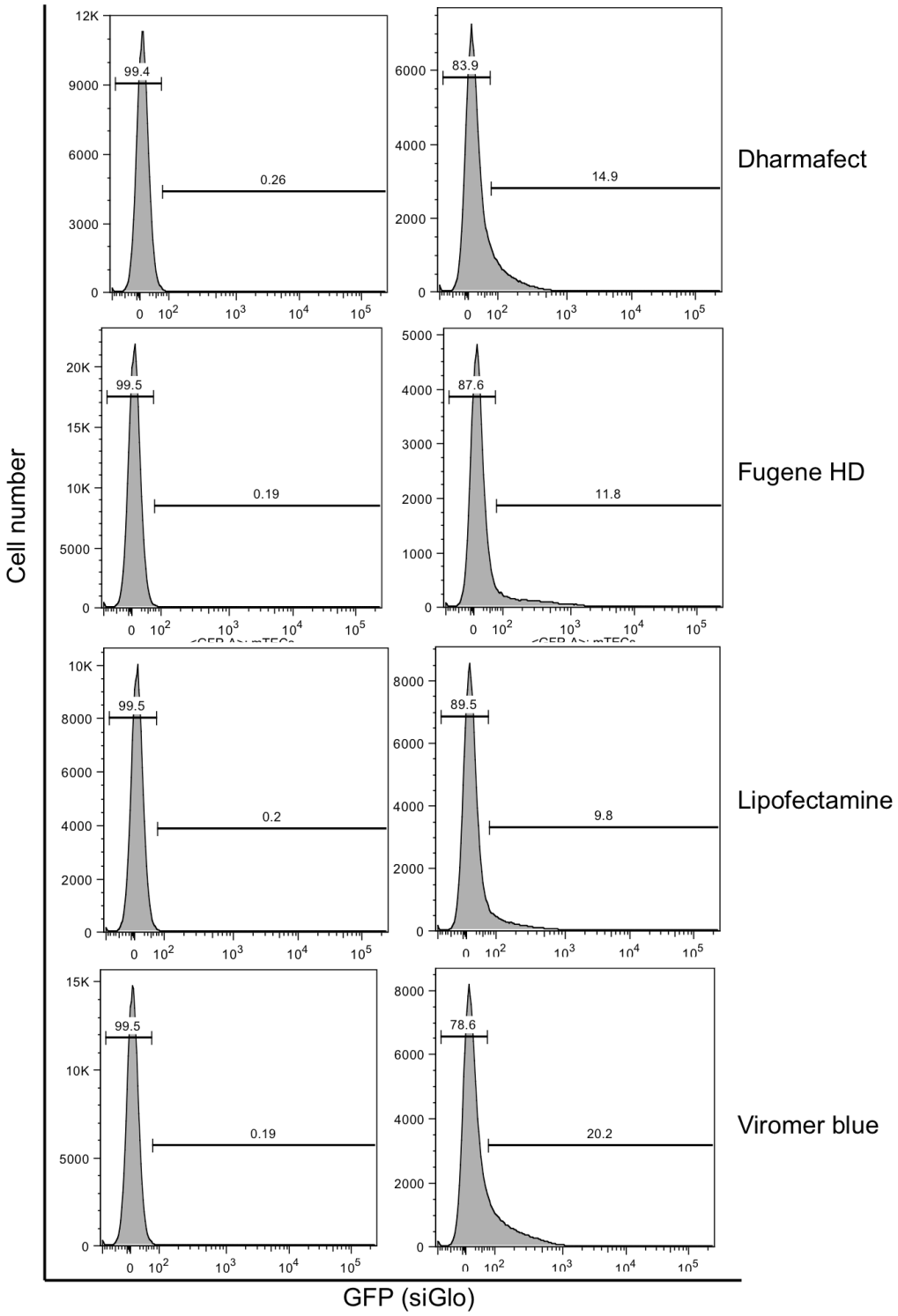

Figure 30 Transfection method: Different transient transfection reagents have been used and checked for their GFP expression after 12 hours of incubation. Left side control, right side transfected cells, 2 replicates.

We also approached a lentiviral transfection with GFP, incubated the cells for 24 hours and measured their viability and GFP expression levels. Even though the transfection efficiency was 60% the viability of the cells were very poor (Fig.31 and Fig. 32).

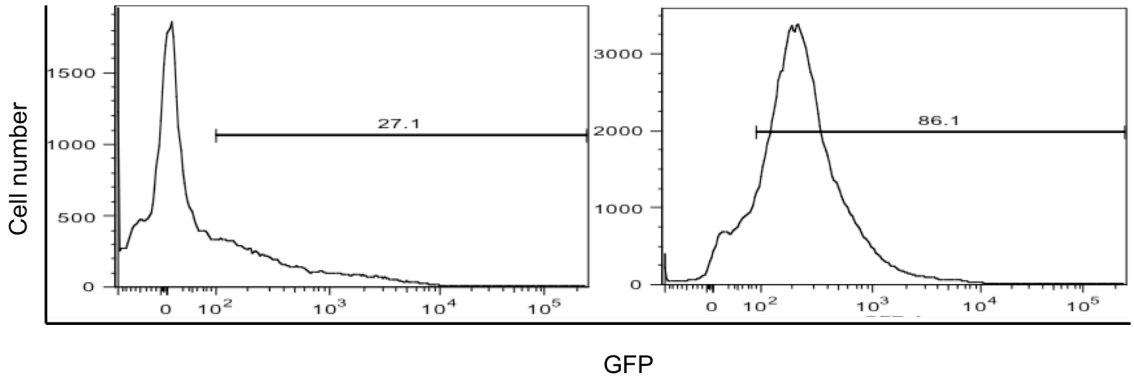

Figure 31 Transfection method lenti viral: A lentiviral transfection has been tested. The GFP expression level is high, unfortunately an incubation of 24 hours reduces the cell viability extremely, 2 replicates

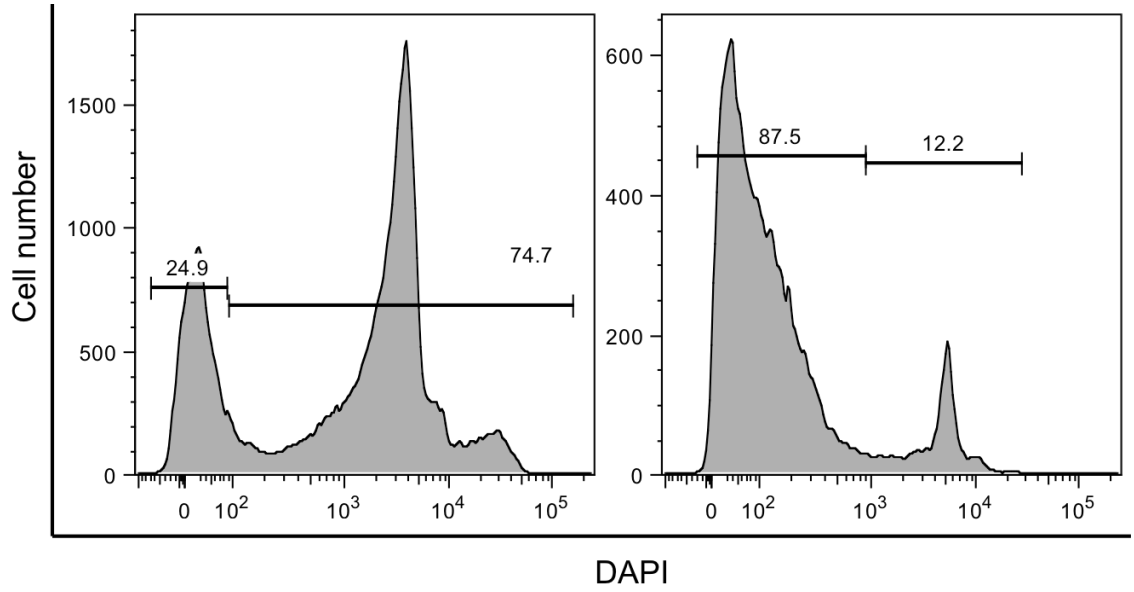

Figure 32 Viability of cells after transfection: The viability was meassured with DAPI. Left side transfected cells with lentivirus with a viability of 25% after 24 hours and on the right side with viromer Green with 88% living cells after 24 hours transfection, 2 replicates.

After several trials we decided to use a polymer-based reagent, called viromer Green that mimics a viral infection, which can promote an active endosome escape that will lead to a cytosolic delivery of our reagents. The transfection efficiency was measured by siGlo GFP, a fluorescence oligonucleotide duplex that localizes to the nucleus). SiGlo has the same length of a miRNA tagged with a GFP, thus provide us the ability to measure the efficiency of the transfection by measuring the GFP signal by flow cytometry. MTECs were isolated according to the mTEC isolation protocol and after the isolation cells were transfected with Viromer Green and siGlo for 12 hours. After 12 hours of

incubation cells were analysed by flow cytometry. We have reached a transfection efficiency of over 75% and 95% of our cells were viable. Therefore this method is efficient as well as suitable for our primary isolated cells (Fig. 33).

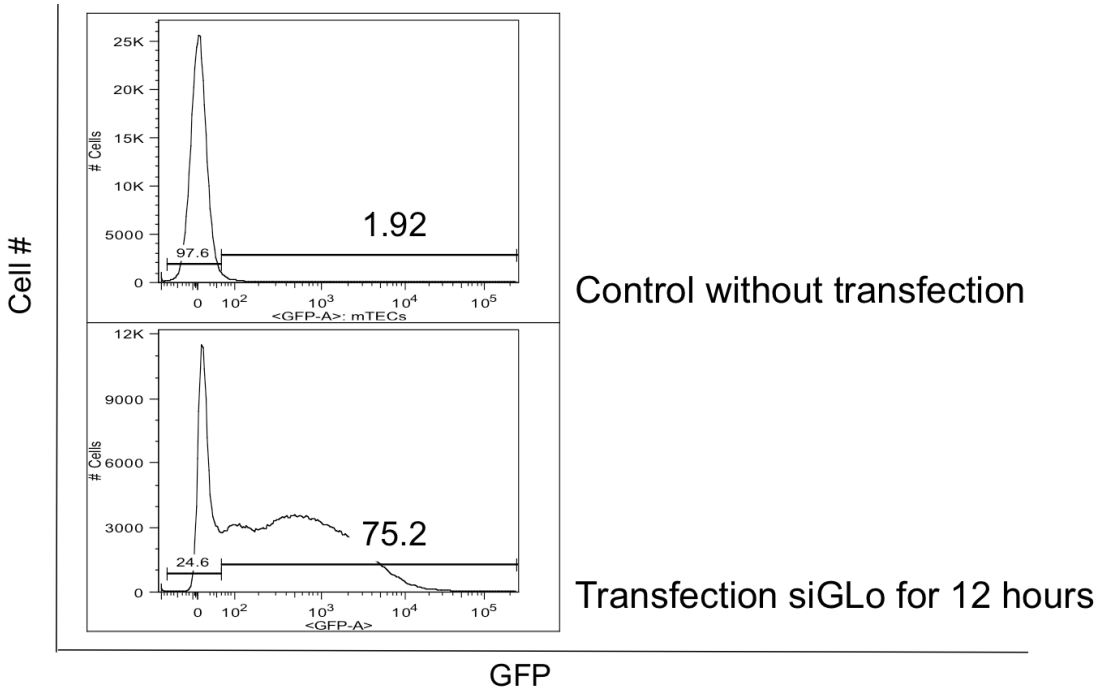

Figure 33 Transfection efficiency with viromer Green: The transfection efficiency was measured after 12 hours incubation of viromer Green with either PCS (control) or with siGlo (green fluorescence dye). The efficiency with viromer Green and siGlo was 75%.

3.13 Transfection efficacy of mimics and antagomiRs in mTECs

The next step was to figure out how well miRNA mimics or antagomiRs perform after the transfection to guarantee an up- and downregulation of a specific miRNA. To show the efficacy we have cells that are only transfected with the transfection reagent itself, with a scramble miRNA used here as a negative control, a mimic or an antagomiR. The list of miRNA mimics (Tab.2) and antagomiRs (Tab.3) are listed below.

miR-125a-3p	miR-125a-5p	miR-125b-1-3p	miR-125b-5p
miR-200c-3p	miR-203-3p	miR-205-5p	miR-29a-3p
miR-34a-5p	miR-34b-3p	miR-494-3p	miR-709
miR-761	miR-877-3p	miR-92-5p	

Table 2 List of mimics: The candidate miRNAs we have chosen are listed above and cells have been transfected with those mimics.

let-7c-5p	miR-125a-3p	miR-125a-5p	miR-125b-1-3p
miR-125b-5p	miR-200c-3p	miR-203-3p	miR-205-5p
miR-29a-3p	miR-34a-5p	miR-34b-3p	miR-494-3p
miR-709	miR-761	miR-877-3p	miR-92-5p

Table 3 List of antagomiRs: The candidate miRNAs we have chosen are listed above and cells have been transfected with those antagomiRs. Let-7c-5p has been chosen to be a positive control.

Transfected cells with miRNA mimics and antagomiRs were incubated for 14 hours, washed and then kept in culture for four days until their RNA were isolated. Afterwards qPCRs were performed to track the miRNA level changes between different samples. Overall we could show that the efficiency of the antagomiRs display a fully inhibition of the endogenous miRNA. While mimics raise the miRNA level up to thousand fold. Even though the scramble miRNA should not be affecting the cells, as the sequence is scrambled, still we can see a small interference in affecting the miRNA levels, but importantly not as effective as a mimic or an antagomiR (Fig. 34, 35).

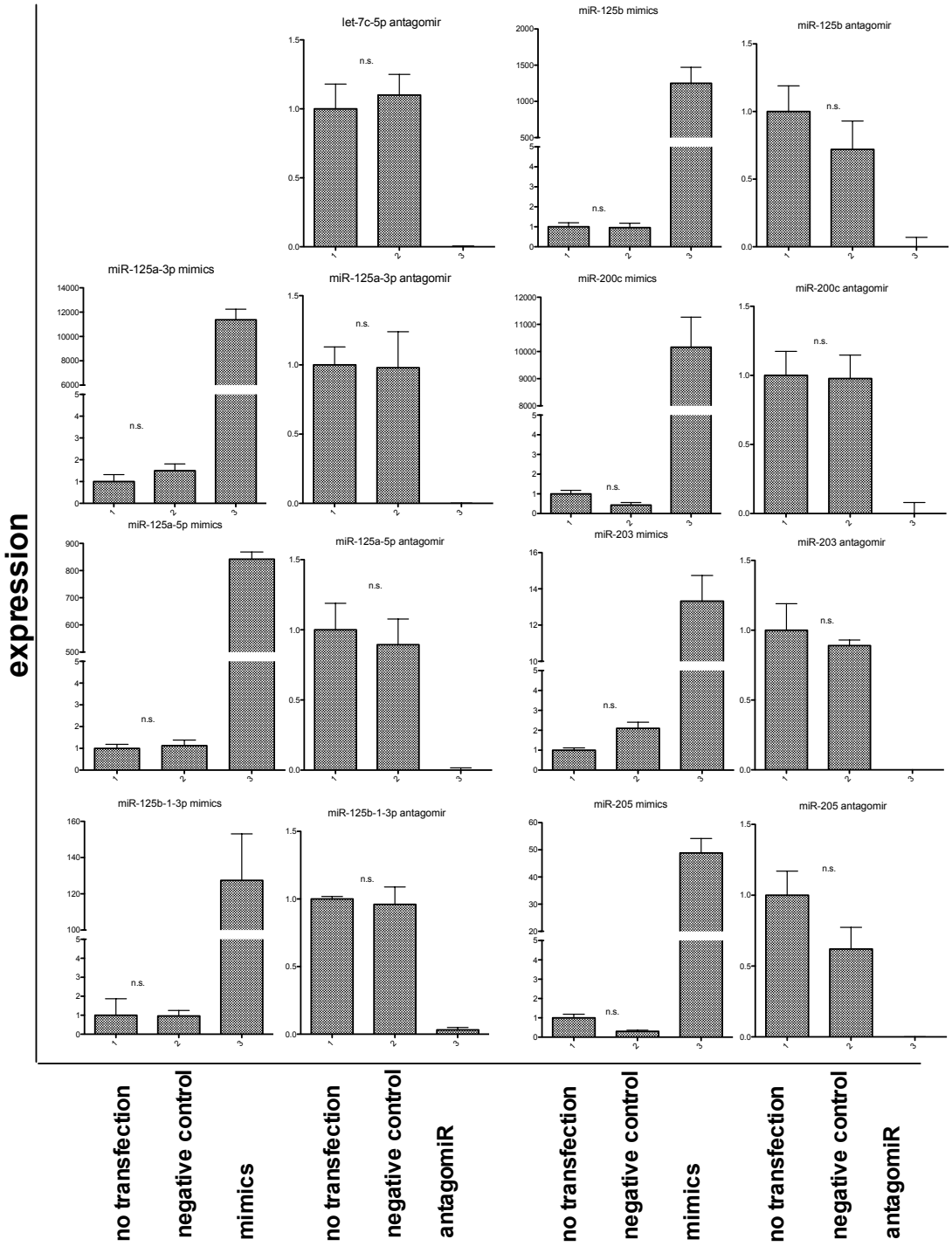

Figure 34: Efficiencies of transfection: In this experiment freshly isolated immature mTECs were transfected with either PBS (no transfection), scramble miRNA (negative control), let-7-c (positive control), miRNA mimic or miRNA antagomiR. This experiment has been done with two replicates. Then samples were collected and RNA was isolated from the cells. QPCR was performed with two replicates, each replicates consists of 3 biological replicates. Not only the transfection efficiency can be seen, but also the overexpression and downregulation of our miRNA candidates. The expressions between the samples no transfection and negative controls were statistically not significant, p-values were > 0.05.

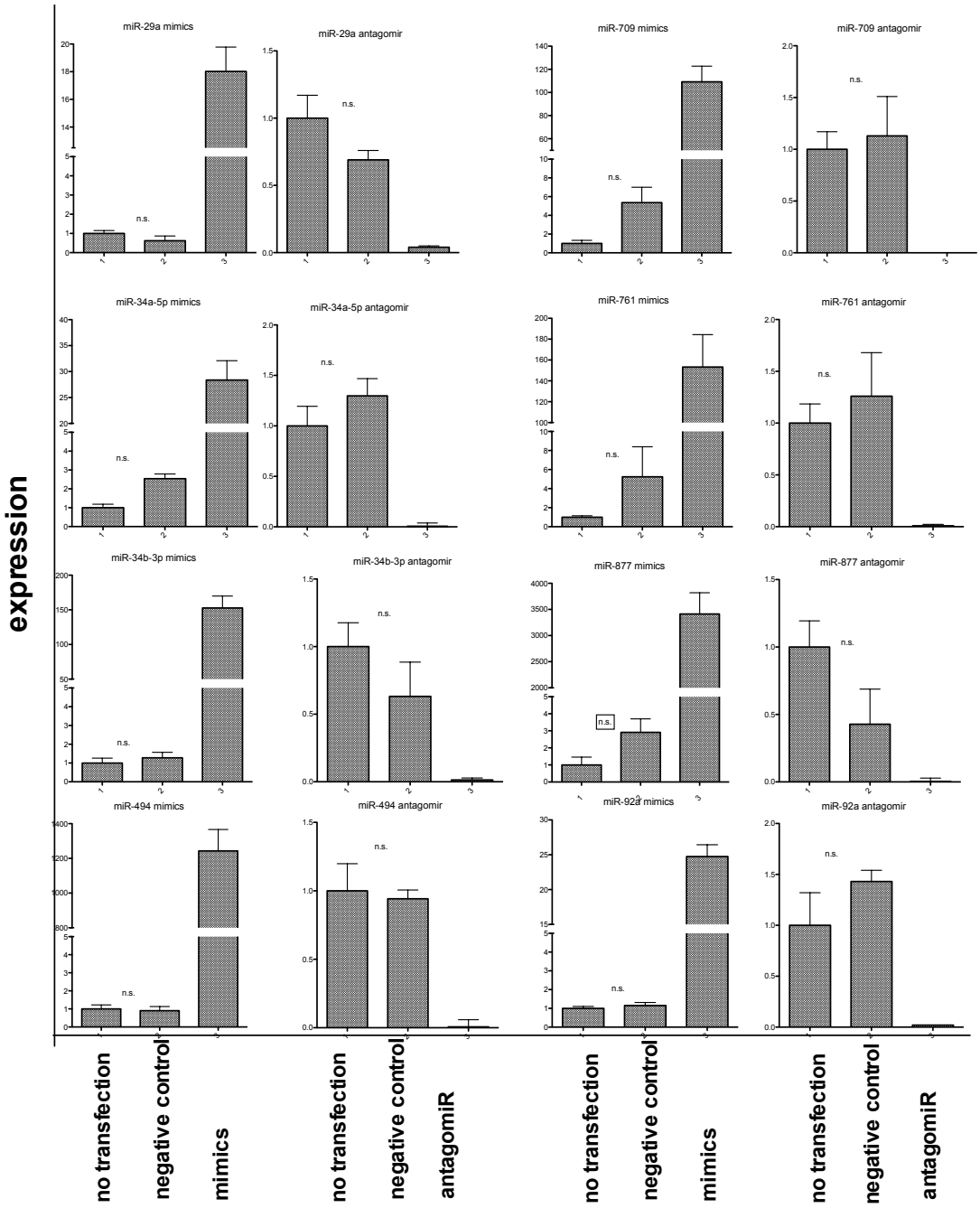

Figure 35: Efficiencies of transfection second part: The same experimental approach has been done as shown above.

3.14 Further adjustments and optimization of cell numbers and controls

Originally we have planned to transfect from each subpopulation a minimum number of 150 000 cells. It was a suggested cell number from the company's manual protocol. Due to the low amount of Sca-1 and DN cells extracted from 24 mice this cell number was not realistic. It would not be ethically correct to

increase the size of mice to gain such high number from each subpopulation. We would need to increase three times the size of mice, as for 24 mice the yield of cell numbers in Sca-1 and DN would be only 50.000. Thus we sorted only for immature MHCII low mTECs tomato red labelled (red rectangle), transfected those cells without sorting all four subpopulations separately to be able to screen all miRNA candidates (Fig.36). This give us unfortunately the disadvantage for not being able to see the effect of each miRNA affecting each subpopulation during the maturation, especially in regards of their lineage progression from one subpopulation to the next one. Nevertheless we will see if the miRNA can functionally change the phenotype of four subpopulations from a wild type to a more Dicer KO or SCID phenotype.

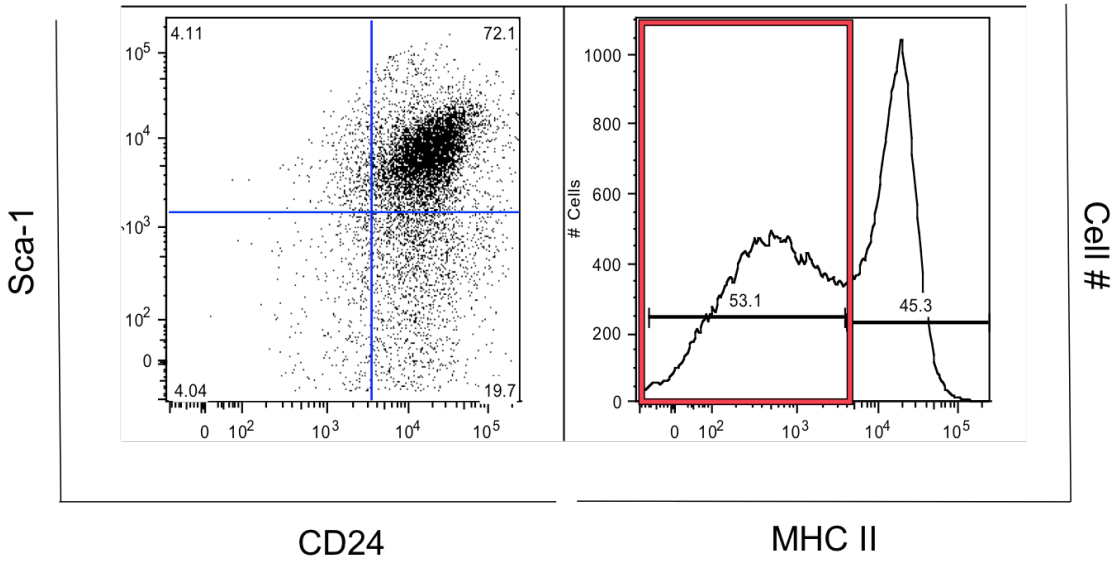

Figure 36 Sorting strategy of tomato red labelled mTECs: Gating strategy from sorting each individual subpopulation was changed to sort the entire immature mTECs. This increases the cell numbers in total.

For the judgment of a successful transfection with either a mimic or an antagomiR we used for the negative control a scramble miRNA, as a positive control we looked for a certain miRNA, which can generate a resemblance of the four subpopulations in Dicer KO or SCID. It has been reported in the context of mTECs that Hmga2 is regulated by let-7. Hmga2 is one of the many genes, under the control of AIRE, which influences the expression of certain peripheral tissue antigens (PTA) that is normally expressed on mTECs during their development (Oliveira et al. 2016). And this miRNA has been

recommended by the website of thermofisher to be suitable as a positive control as endogenous let-7c miRNA is known to negatively regulate High Mobility Group AT Hook 2 (HMGA2) (Shell et al. 2007). HMGA2 is ubiquitously expressed, a non-histone and is known to act as a transcriptional activator that can change the translation of genes through changing the chromatin structure. Our group has observed that the chromatin structure of mTECs is more opened during the development (not published yet, Rattay) thus changing the chromatin structure will cause disruption in the mTEC development. Consequently we thought that using let-7c might disturb the development of the four subpopulations. Surprisingly a downregulation of let-7c did show a disturbance in the distribution of the four subpopulations (Fig.35).

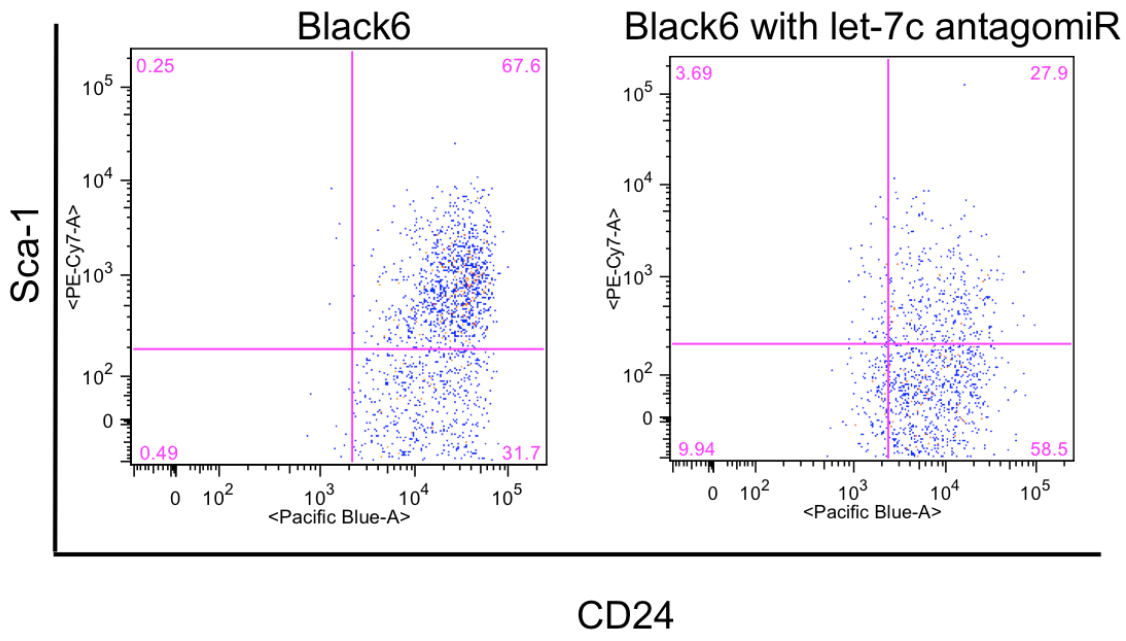

Figure 35 The distribution of the four subpopulations in Black6 mouse vs Black6 with let-7c: In this experiment we compared the distribution of the four subpopulations between immature mTECs from a Black6 mouse and mTECs from a Black6 mouse transfected with let-7c antagomiRs for 12 hours. Analysis with flow cytometry has been done post 4 days transfection (right picture) and 4 days cultivation without transfection (left picture). The experiment has been performed only once.

To ensure the same distribution of the subpopulations before and after transfection as well as four days tissue culture conditions we checked for their distributions in flow cytometry. Those cells were only transfected with the

transfection reagent viromer green, from this point will be called, wild type control.

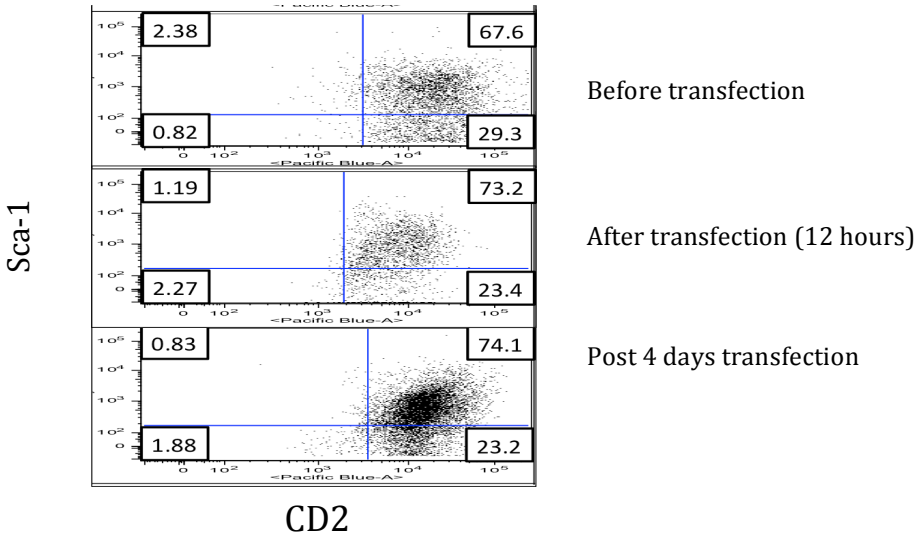

Figure 36 Distribution of the four subpopulations at different conditions: Here we are showing the distribution of the four subpopulations before the transfection, post-transfection after 12 hours and post-transfection after 4 days. The distribution of all three different time points is similar. While the before transfection has a lower DP population and a higher CD24 distribution compared to the transfected cells, but there are no significant differences between post 12 hours and post 4 days.

When we compare the distribution of each subpopulation between the wild type control and the negative control we do not see a difference between those samples (Fig.36). Thus our negative control is safe and will not cause a shift in our subpopulations. We also used the same isolated cell pool and transfected them with either a negative (Fig.37) or a positive control (Fig.38) and analysed them after 4 days.

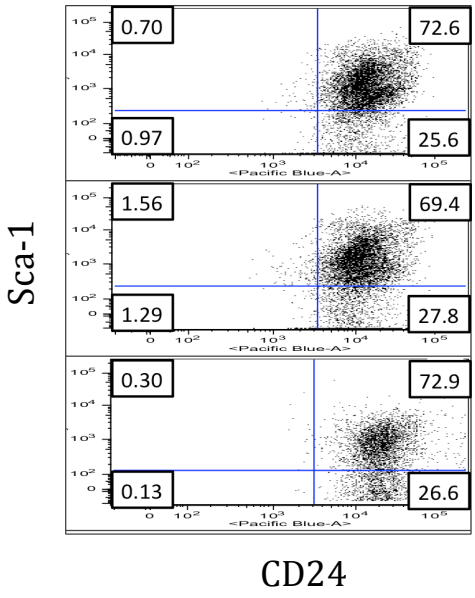

Figure 37 mTECs transfected with a negative control in 3 replicates: The distribution of the four subpopulations after the transfection of a negative control, a scramble miRNA. Here three independent replicates are displayed. The analysis with the flow cytometry has been performed after post 4 days transfection.

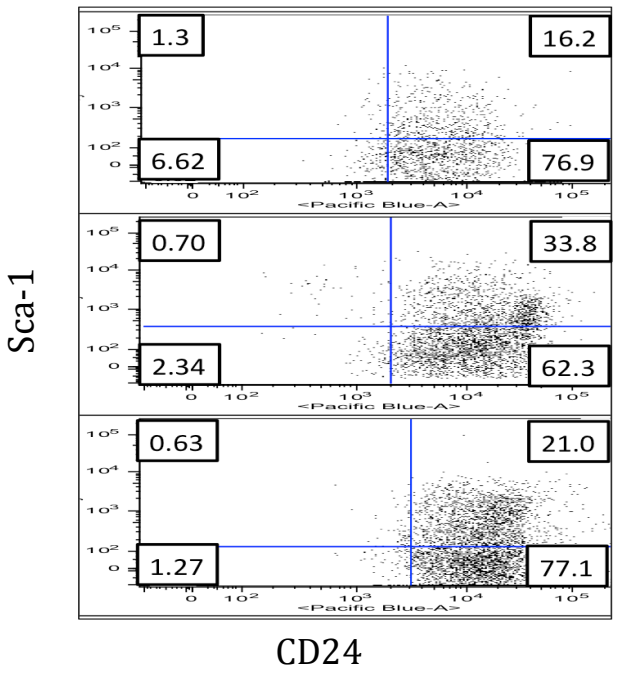

Figure 38 mTECs transfected with a positive control: The distribution of the four subpopulations after the transfection of a positive control, antagomiRs of miRNA let-7c. Here three independent replicates are displayed. The analysis with the flow cytometry has been performed after post 4 days transfection.

When we analysed the positive control where cells were transfected with an antagomir of let-7c, we could see a significant difference between two of the four subpopulations when comparing wild type with the positive control (Fig.39). The shift of the four subpopulations resembles the change of

population distributions in both SCID and Dicer KO mice. Therefore we found a positive control, which we can trust that can induce a shift in the four subpopulations to use as a positive control for the transfection efficiency as well as found a miRNA that could be relevant in terms of regulating the immature cells within the four subpopulations.

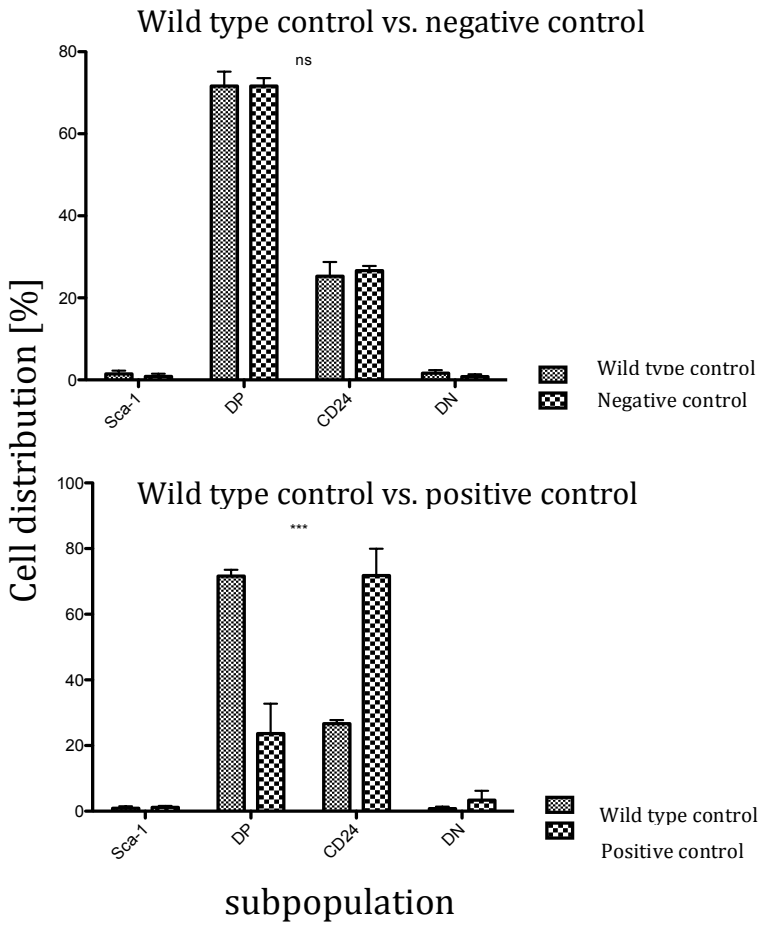

Figure 39 Wild type control versus negative or positive control: The experiment has been performed three times with three independent replicates. Analysis with flow cytometry has been done post 4 days transfection. The picture above shows the wild type control with transfection reagent without mimics or antagomiRs versus transfection with a negative control (scramble miRNA). The picture below shows the wild type control with transfection reagent without mimics or antagomiRs versus transfection with a positive control (antagomiRs let-7c). The distribution changes of the four subpopulations has been analysed with a t-test. Wild type control versus negative control shows no statistical significance. While the wild type control versus positive control shows a very high significance of p< 0.001.

3.15 A change in the miRNA level has an impact in the four subpopulations

The next step of our approach was to transfect our tomato red labelled immature mTECs with either a mimic or antagomiR. Then we compared the cell distribution of each subpopulation to the wild type control. The

experiments have been performed with three independent replicates. The results of the transfections with mimics and antagomiRs were very different. One miRNA, miR-203 (Fig.41), did not show any statistical changes in the four subpopulations when transfected either with mimics or antagomiRs. Some miRNAs such as miR-125a-3p (Fig.40), miR-200c (Fig.41), miR-205 (Fig.42), miR-494 (Fig.44) and miR-877 (Fig.45) did not show any distribution changes in the four subpopulations while transfected with a mimic, but with their respective antagomiRs we saw statistical significances (t-test based) p> 0.05. The statistical significances were from * = p>0.05, ** = p>0.01 and *** = p>0.0001. Other miRNAs such as miR-125b-5p (Fig.40) and miR-34a-5p (Fig.43) did not show any distribution changes when transfected with an antagomiRs, but when transfected with a respective mimic it did show a statistical significance. Interestingly we saw in five miRNAs miR-29a (Fig.42), miR-34b-3p (Fig.43), miR-709 (Fig.44), miR-761 (Fig.45) and miR-92a-5p (Fig.46) in both mimics and antagomiRs significant changes in the distributions of the four subpopulations. Some miRNAs need a tight regulation of their endogenous miRNA levels such as miR-29a, miR-34b-3p, miR-709, miR-761 and miR-92a-5p because an imbalanced level of these miRNAs can induce either an increase or a decrease of DP and CD24, while the other remaining 7 miRNAs can only influence the four subpopulation with either an overexpression or a downregulation of the miRNA. Taken together 12 miRNAs seem to be relevant to play a role in the development of the four subpopulations.

miR-125a-3p and miR-125b-5p

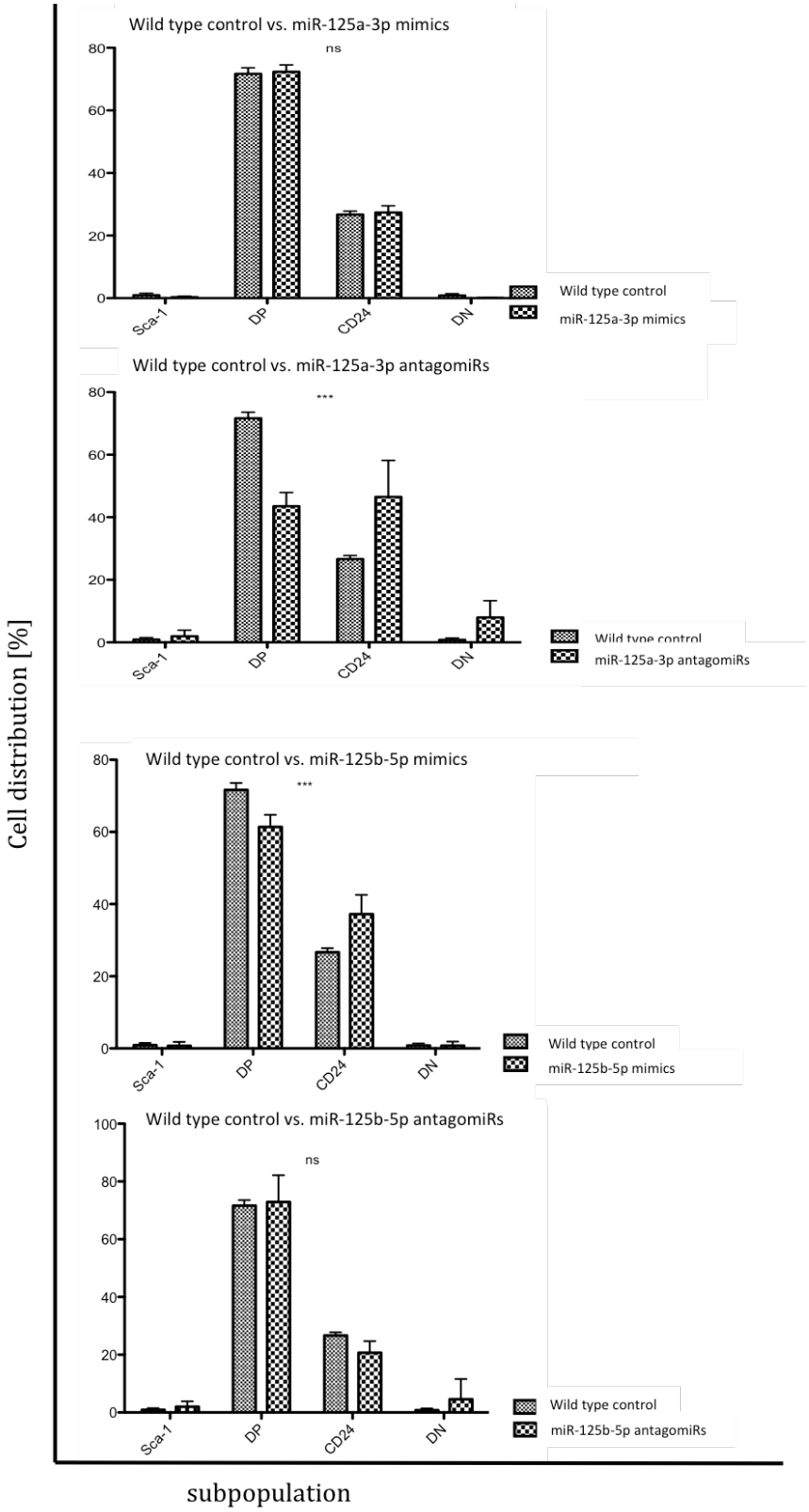

Figure 40 The effect of overexpression and downregulation of miR-125a and miR-125b: In this experiment we used three independent replicates either transfected only with the transfection reagent but without any mimics or antagomiRs, called wild type control, transfected with a miRNA mimic or transfected with an antagomiR. Based on the t-test we saw either no statistical significance (ns) p>0.05 and *** = p<0.001.

miR-200c and miR-203

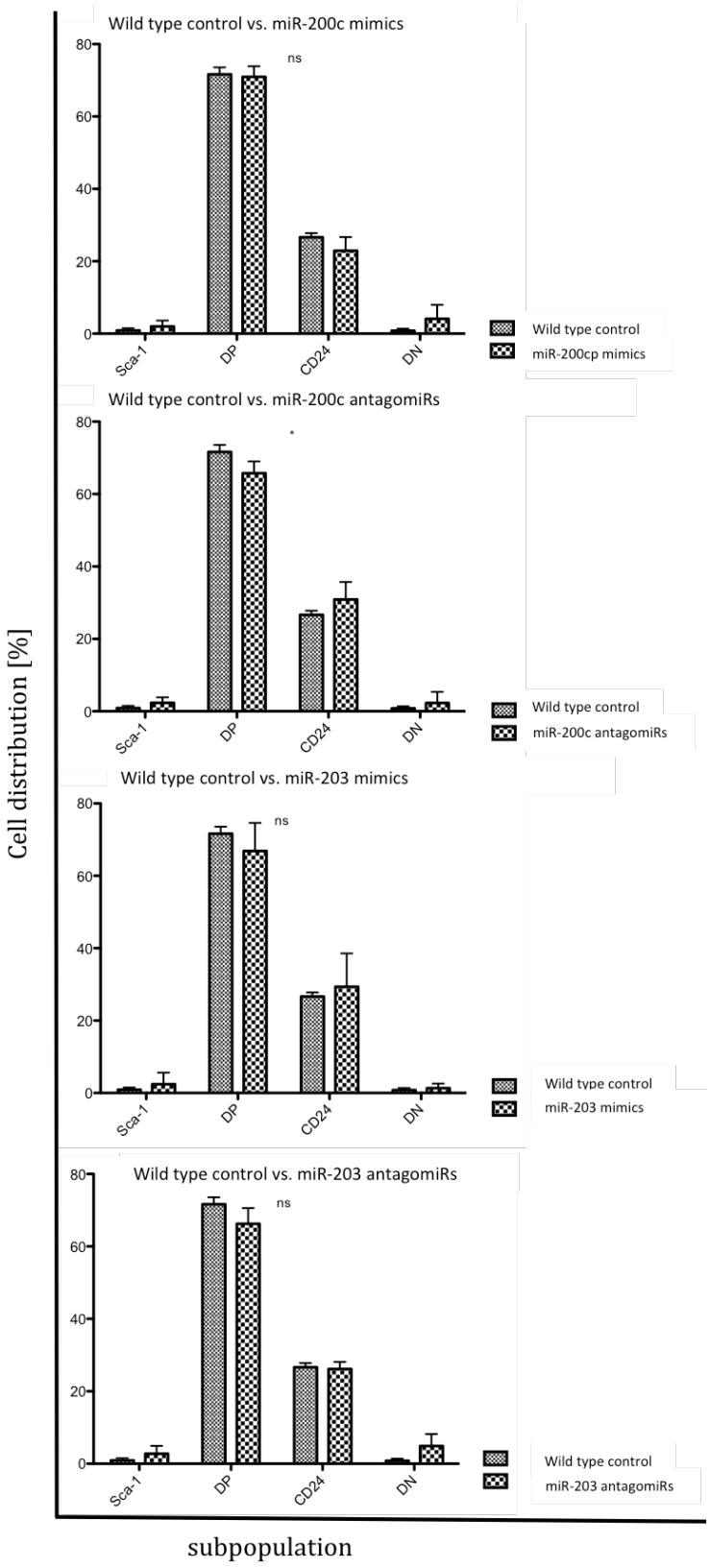

Figure 41 Effect of an overexpression and a downregulation of miR-200c and miR-203 Based on the t-test we saw either no statistical significance (ns) p>0.05 and * = p<0.05.

miR-205c and miR-29a

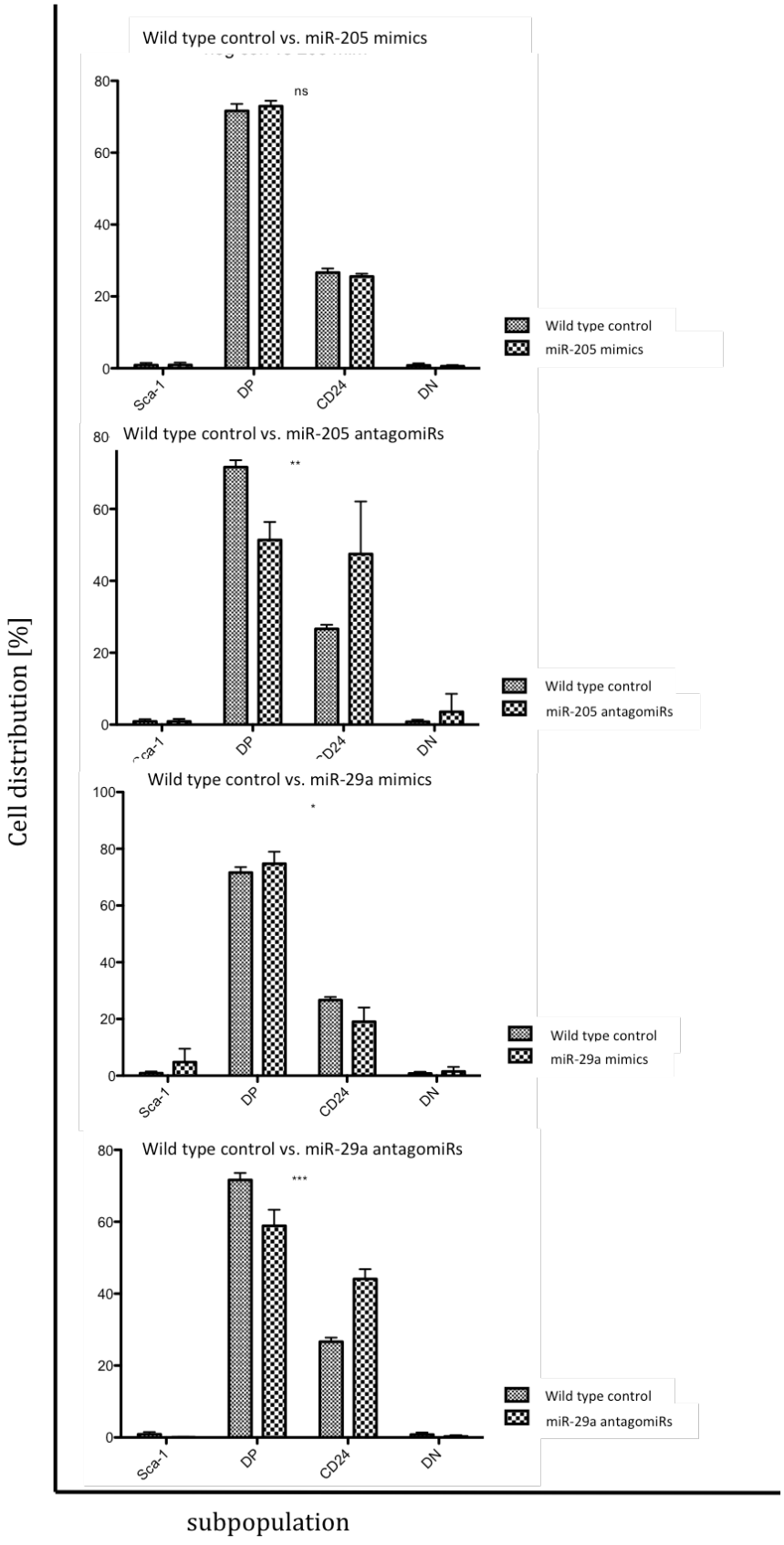

Figure 42 Effect of an overexpression and a downregulation of miR-205 and miR-29a Based on the t-test we saw either no statistical significance (ns) p>0.05, * = p<0.05 and ** = p=<0.01.

miR-34a and miR-34b

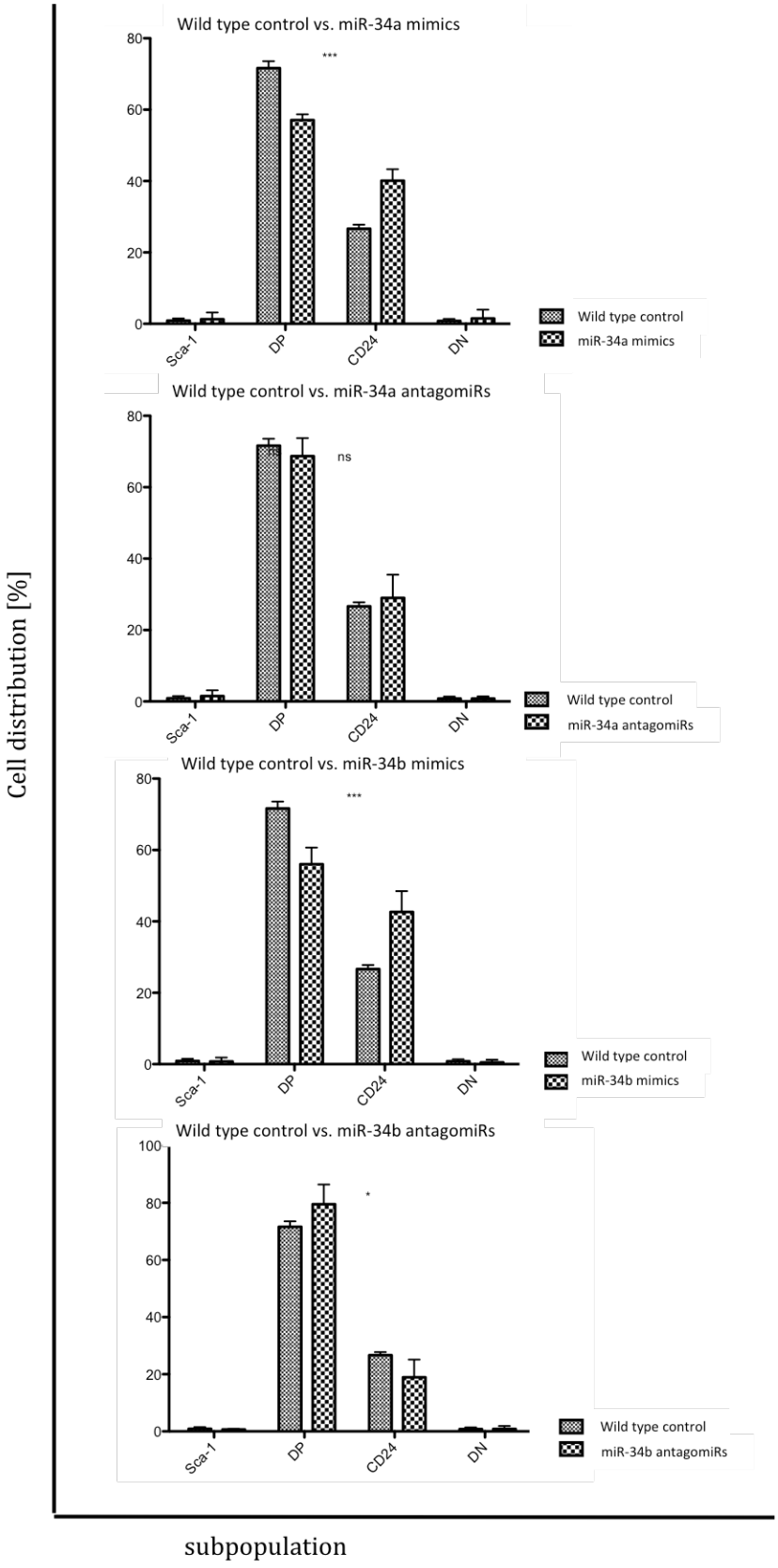

Figure 43 Effect of an overexpression and a downregulation of miR-34a and miR-34b Based on the t-test we saw either no statistical significance (ns) p>0.05, * = p<0.05 and *** = p<0.001.

miR-494 and miR-709

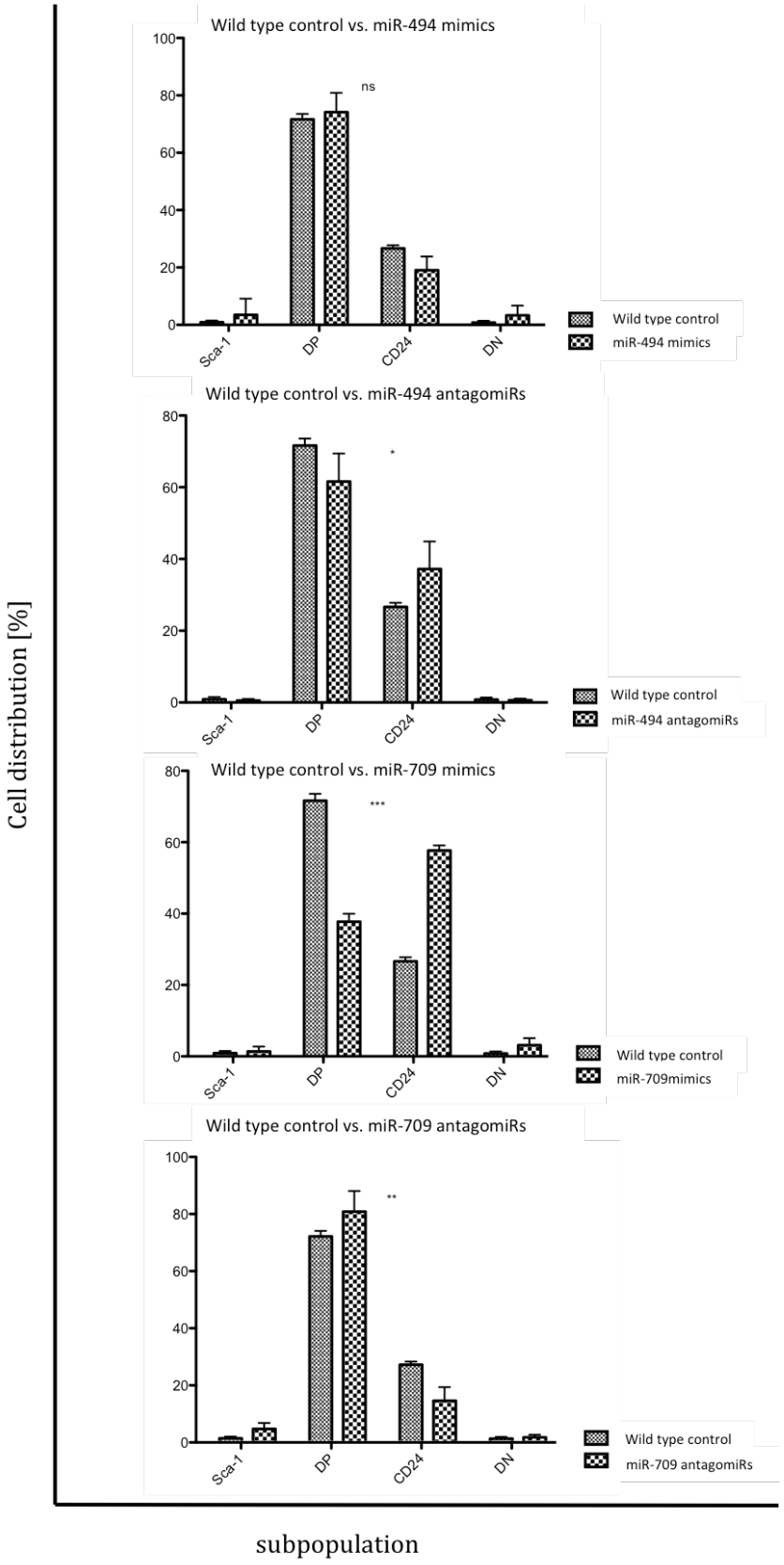

Figure 44 Effect of an overexpression and a downregulation of miR-494 and miR-709 Based on the t-test we saw either no statistical significance (ns) p>0.05, * = p<0.05, **= p< 0.01 and *** = p<0.001.

miR-761 and miR-877

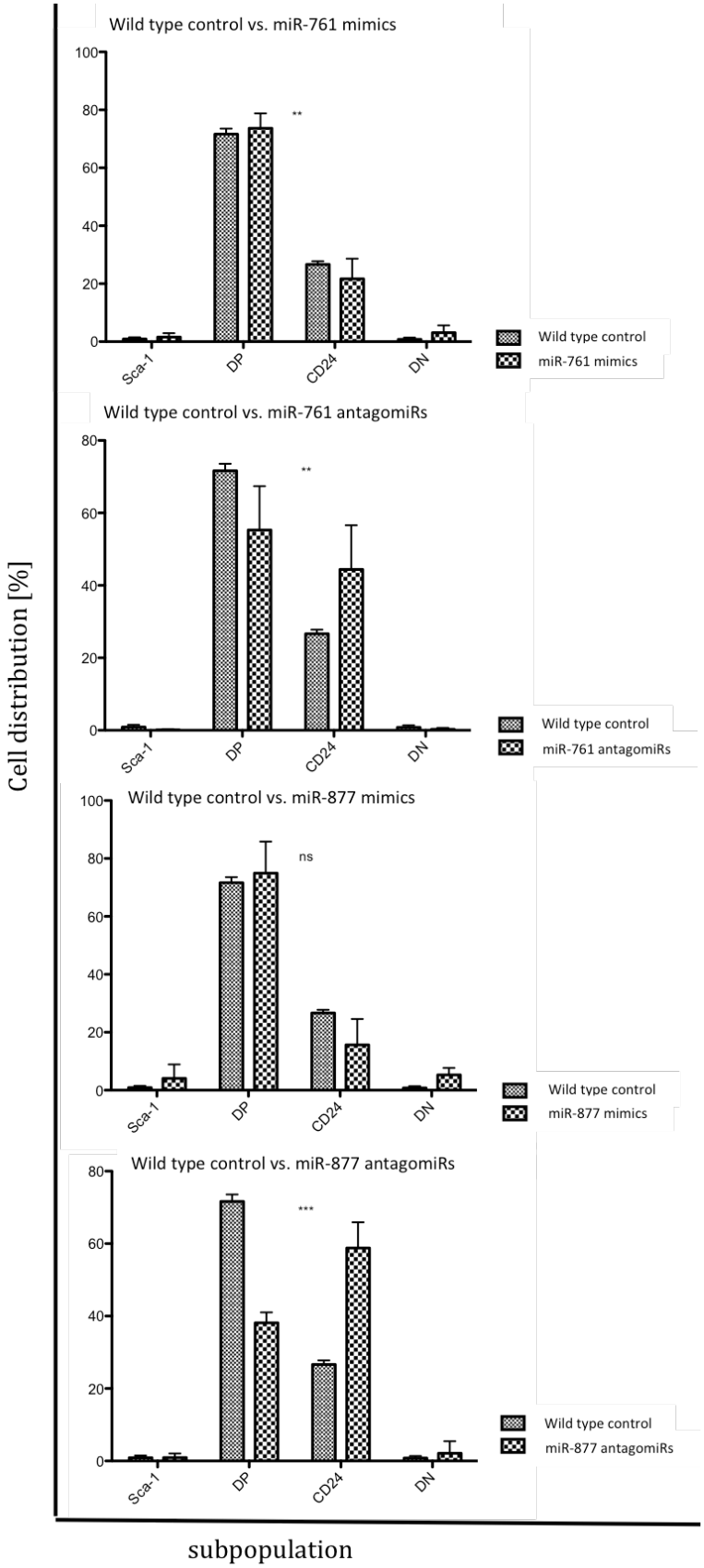

Figure 45 Effect of an overexpression and a downregulation of miR-761 and miR-877 Based on the t-test we saw either no statistical significance (ns) p>0.05, **= p< 0.01 and *** = p<0.001.

miR-92a

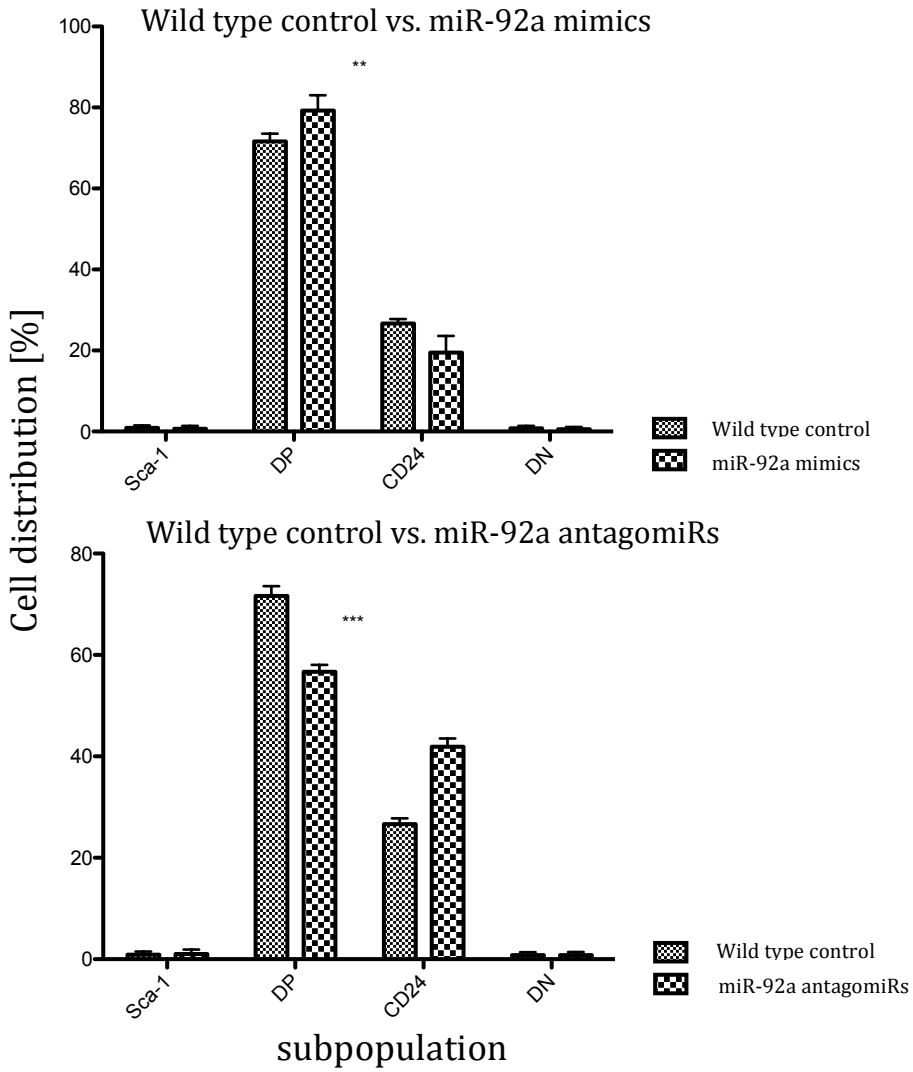

Figure 46 Effect of an overexpression and a downregulation of miR-92a Based on the t-test we saw either **= p< 0.01 and *** = p<0.001.

3.16 Search of mRNA targets

We have chosen the following miRNAs based on their high significant differences between wild type control and the manipulated miRNA: miR-125a-3p, miR-125b-5p, miR205, miR-29a-3p, miR-34a-5p, miR-34b-3p, miR-709, miR-761, miR-877-3p and miR-92a-5p. For those miRNAs our statistic collaborators from UCLA (Dr. M. Pellegrini and Dr. W. Yan), have generated a list of mRNAs, which are negatively correlated to our miRNAs. Dr. Yan has generated a further list where the miRNA candidates and its negatively correlated targets have been verified and/or published, thus to diminish false

outcomes. For example miR-125b-5p has been already published targeting Bak1 in prostate cancer (Shi et al. 2007) and Mapk14 in Salmonella infection pathway (Yao et al. 2016). Based on those two lists we have selected for each miRNA 2-4 negatively correlated targets, which have a negative correlation score between -0.60 until -0.99 (Tab.4).

miRNA	candidate 1	candidate 2	candidate 3	candidate 4
miR-125a-3p	Psmb11	Ubac1	Fam83e	
miR-125b-5p	Apln	Bak1	Mapk14	Arid3b
miR-205	Dhcr24	Grtp1		
miR-29a	Dcx	Col4a4	Fgf23	Sirt3
miR-34a-5p	Bcl6	Rab11b	Foxp1	Sall4
miR-34b-3p	Tbx5	Fbxo45	Sirt1	Abcb1b
miR-709	Egr2	Jun	Zfp113	Sgms2F
miR-761	Rps15a	Acsbg2		
miR-877	Tirap	Rab11b	Fli1	Dusp7
miR-92a	Btg2	Trp63	Zfp300	

Table 4 Selected mRNA targets: For each miRNA we selected based on several criteria their potential mRNA targets.

In total we selected 34 mRNA targets and have ordered for those designated targets adequate siRNAs.

3.17 Quick screening of mRNA targets

To be able to tell that a certain mRNA target has an influence on the development of the four subpopulations, all 34 mRNA targets are under scrutiny. For all 34 mRNA targets siRNA have been tested twice in freshly isolated tomato-red labelled immature mTECs.

After 34 mRNA targets have been screened for their functional impact on the development of the four subpopulations we found only four mRNA targets which show a change in the distribution of the four subpopulations. Whereas the subpopulation in control populates 82.4% cells in DP, the positive control (let-7c antagomiRs) reduced the DP subpopulation to 20.9%, Bak1 siRNA to 61.9%, Foxp1 to 59.9%, Mapk14 to 49.0% and Fam83e to 44.4% (Fig.47).

None of the mRNA targets can reduce the DP subpopulation as much as a let-7c antagomiRs, but still we found in Fam83 a reduction of DP up to 50%.

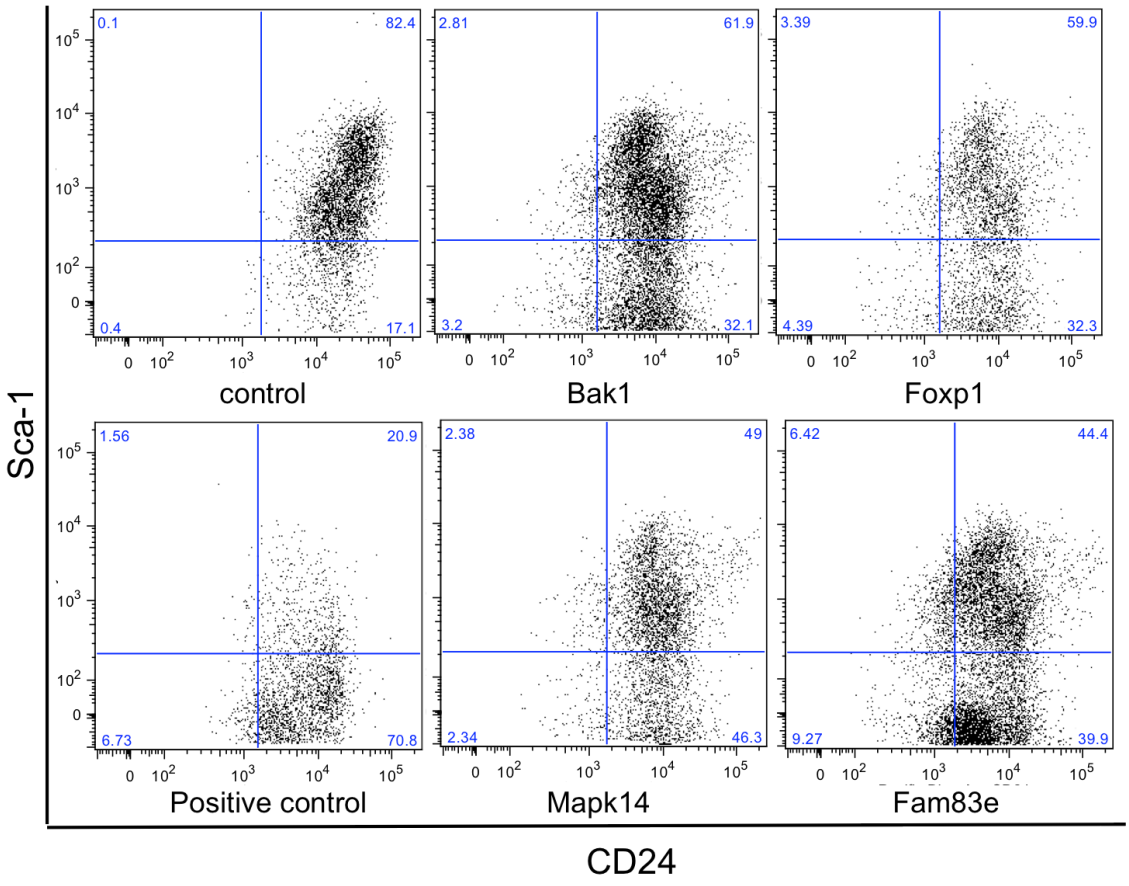

Figure 47 potential mRNA targets: Immature tomato red cells were transfected with PBS (control), with let-7c antagomiR (positive control), Bak1 siRNA, Foxp1 siRNA, Mapk14 siRNA and Fam83e siRNA. The expression of the four subpopulations changes differently.

As all four show a significant difference in the four subpopulations after an inhibition of the mRNA targets (Fig.48), we continued to verify their binding capability to their predicted miRNA targets via a luciferase assay. To amplify the gene of interest with the binding site of our miRNA candidate we generated primers with additional restriction enzyme sites for PmeI and XbaI. We also generated mutated binding sites to disrupt the binding of the miRNA to the candidate target. The inserts were ligated to a pmirGLO dual-luciferase vector, which has both luciferase and *renilla* luciferase. Plasmid were transfected to HEK293T cells and incubated to 48 hours. Afterwards the luciferase and *renilla* luciferase signals were measured and normalised. The results are indicating that Bak1 and Mapk14 are regulated by miR-125b-5p, while Foxp1 by miR-34a-5p and Fam83e by miR-125a-3p (Fig.49).

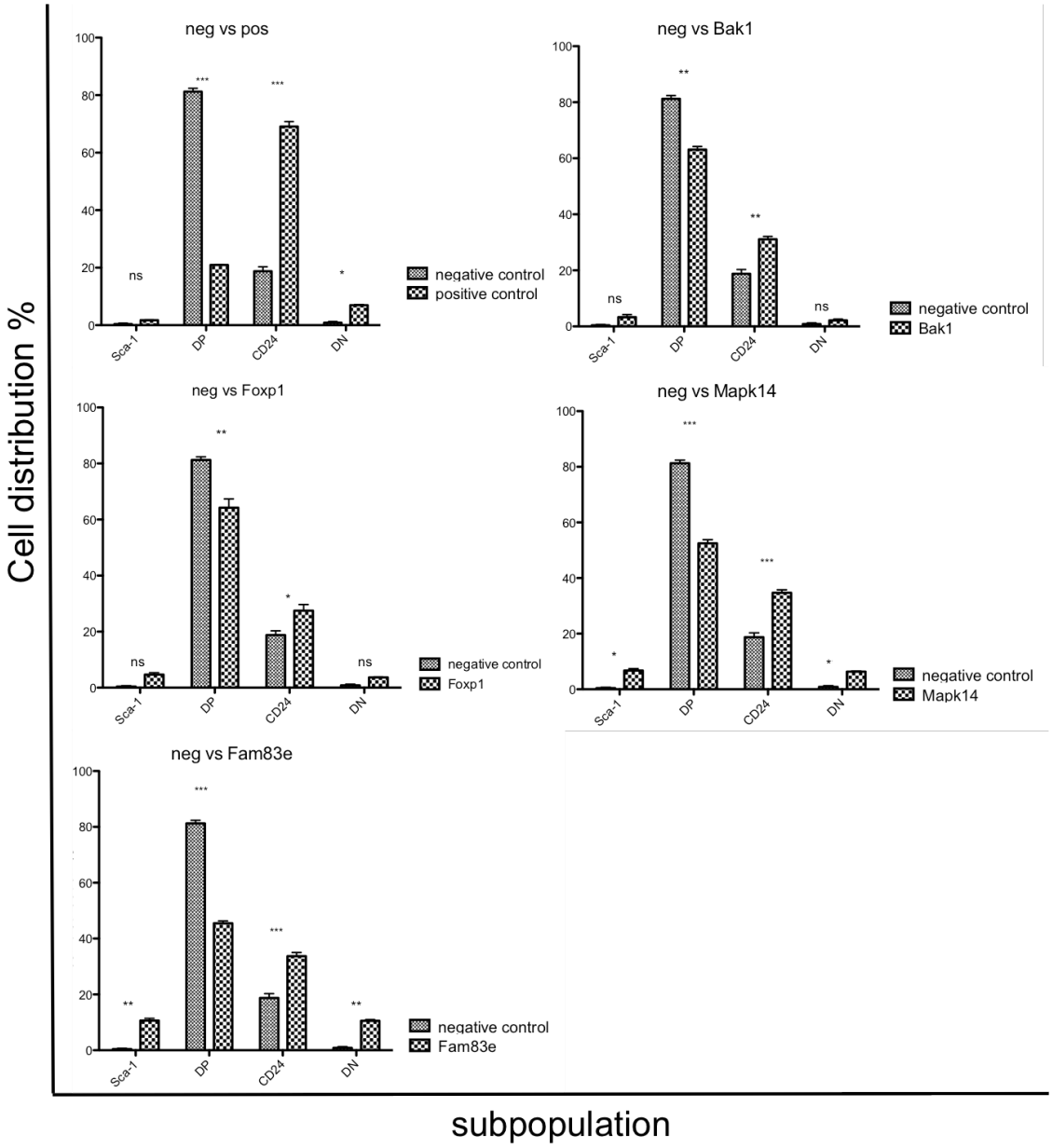

Figure 48 Statistical analyses between negative control and mRNA candidate: Each pair has been tested for their statistical significance (two-tailed t-test). Each experiment has been performed three times with three independent replicates. The negative control was viromer Green transfected with PBS and the positive control a transfection of viromer Green with let-7c antagomiR. NS = not significant, *= p<0.05, **= p<0.01 and ***= p<0.001.

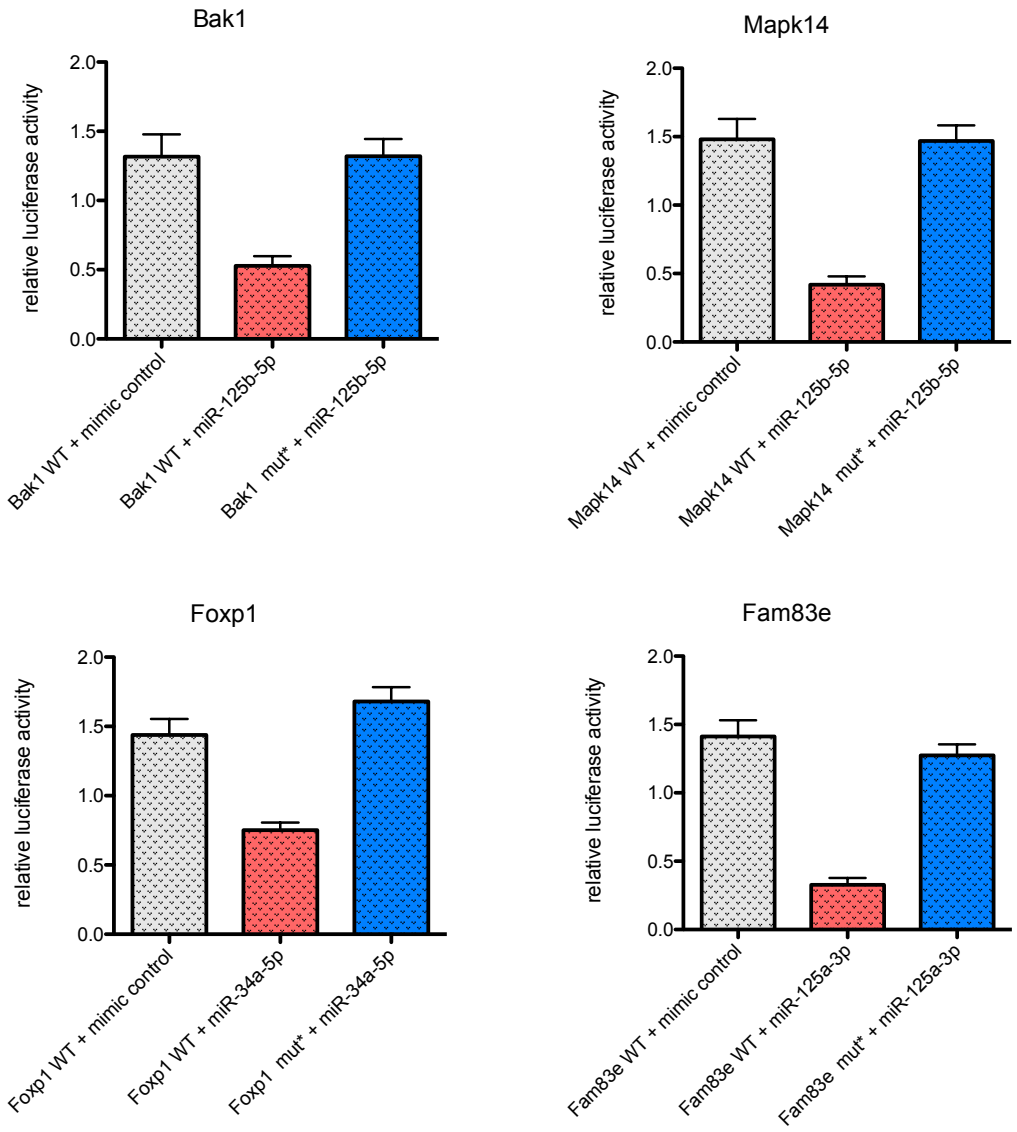

Figure 49 Luciferase signal: We have tested the luciferase signals between the mRNA gene + mimic control (empty vector), mRNA gene + potential miRNA candidate and mRNA gene mutated + potential miRNA candidate. The signals were normalised based on the renilla signal.

To verify these findings we isolated immature mTECs and transfected them either with no transfection reagent, a negative control, miRNA antagomiR or mRNA siRNA. After 2 days we checked their expression levels using qPCR (Fig.50). The results show the negative correlation between our miRNA and its mRNA candidate.

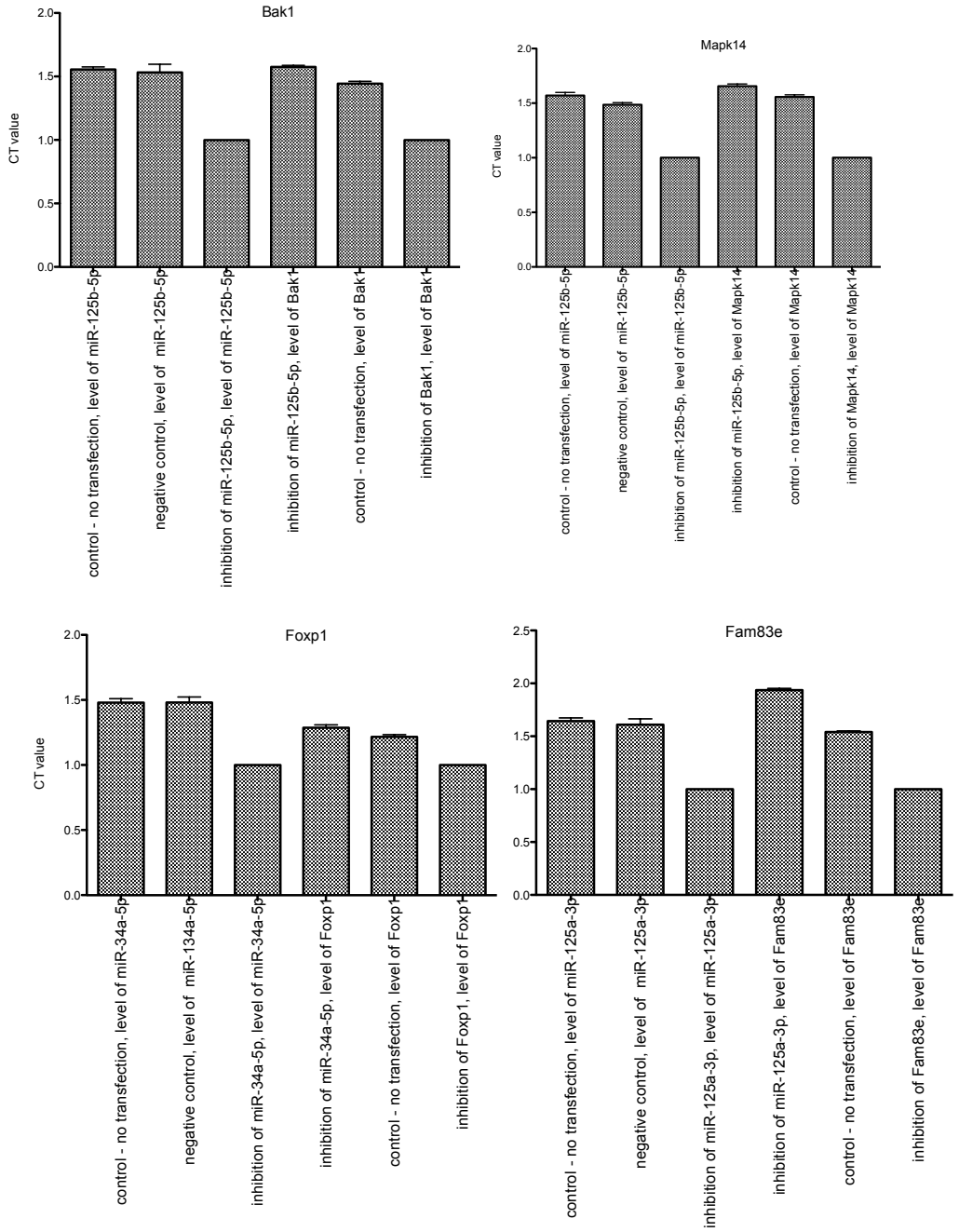

Figure 50 Negative Correlation miRNA and mRNA expression levels: To proof the efficacy of the transfections with mimics, antagomiRs or siRNAs respectively, we isolated the RNA from different samples and checked their mRNA and miRNA levels: Control - no transfection (only viromer Green and PBS) detects the candidate miRNA expression level. Negative control (viromer Green and scramble miRNA) detects the expression level of candidate miRNA. Inhibition of candidate miRNA (viromer Green and antagomiR) detects expression level of candidate miRNA. Inhibition of candidate miRNA detects the expression level of target mRNA. Control – no transfection (viromer Green and PBS) detects the target mRNA expression level. Inhibition of target mRNA (viromer Green and siRNA) detects the expression level of target mRNA.

4 Discussion

The proper maturation of mTECs is an essential process for the T cell development and selection of auto-reactive T cells. Nonetheless, the molecular mechanism, which influences the development of mTECs is still poorly understood. Previous studies reported stem cell like cells, called thymospheres, which have the ability to give rise to bipotent thymic epithelial cells (Ucar et al. 2015), either develop further to mTECs or cTECs. The development of immature mTECs to mature mTECs is well known. Recently our group described the separation of immature mTECs into four new subpopulations by using expression markers such as CD24 and Sca1 to further distinguish the immature mTEC population and to category their developmental status. Those four subpopulations have been studied in embryos as well as in newborns but not in adulthood (Klug's dissertation 2013). The results have shown that all four subpopulations are distinct with respect to their ontogeny and their ability to give rise to mature mTECs (unpublished, Klug). In the recent years microRNAs received an important status in the field of thymus and mTECs (Papadopoulou et al. 2012; Khan et al. 2015). In addition, it has been reported that Dicer knockout mice resulted to a disruptive medulla organization, loss of pGE and a shifted distribution of mTECs from $mTEC^{high}$ to $mTEC^{low}$ (Ucar et al. 2013).

This work aimed for a further understanding of the involvement of miRNAs in the maturation of mTECs, in particular the development of the four subpopulations from the immature mTEC population pool. To discover potential miRNAs related to mTEC maturation, immature mTECs, segregated into four subpopulations, and mature mTECs were analysed using microarray and bioinformatics analyses as well as different mouse models. These approaches led to the discovery of new miRNAs and their target mRNAs, specially needed during the development of immature mTECs and have a functional role to drive the development within the immature subsets, which would not have been obvious just by comparing the difference between $mTEC^{low}$ and $mTEC^{high}$ populations.

4.1 Identifying a lineage progression within the four subpopulations

Initially the four subpopulations were discovered in embryonic and neonatal stages but have never been defined in adulthood. Thus we looked closer to the distribution of the four subpopulations in Black6 mice at the age of 6-8 weeks. Furthermore other mouse strains were under scrutiny, especially those that have an impaired mTEC development such as SCID and nude mice so see if we can find a hint for their impairment. We expected to see a difference in the distribution of the four subpopulations within nude, SCID and Dicer KO and Black6 mice. The reason for this assumption is the following. Nude mice have absent/vestigial thymus thus resulting to no mature T-lymphocytes and harbour only limited numbers of mTECs. SCID mice have an impairment of the recombination of antigen receptor genes, which causes an arrest of T-lymphocytes, therefore T-lymphocytes are missing to migrate to the thymus, while the thymus is existing but is impaired to due the lack of a thymus-crosstalk. Whereas Dicer KO mice have a functional thymus and a proper T-lymphocyte development, but those mice are lacking of mature miRNAs in the thymus. Due to the fact that all three mouse strains have a deficiency from different sites, therefore it would not be surprising to see a difference in their distribution of the four subpopulations. Surprisingly the distribution of the nude, SCID and Dicer KO mice have a similar allocation of all four subpopulations. Whereas the biggest population in Black6 mice is represented in DP, the major subpopulation DP in the other three mouse strains decreased dramatically and shifted to CD24.

To understand why populations are shifting and to decipher the relevance of the four subpopulations in terms of a potential lineage development of mTECs, which have been observed in embryonic and neonatal mice (Klug, unpublished), it needed to be figured out how those subpopulations relate to each other. Hence all four subpopulations (Sca-1, DP, CD24 and DN) and mature mTECs (MHC II high, referred as high) were isolated, their RNA extracted and microarray analysis on mRNA and miRNA levels were done. By

taking the mRNA expression data from all four subpopulations and mature mTECs our collaborator was able to create a lineage progression sequence with all five populations. The sequence based on the dendrogram is: Sca-1 → DP → CD24 → DN → high. It is clearly that Sca-1 is very different compared to the other subpopulations, while DP and CD24 as well as DN and high are more related to each other. Sca-1 might be the earliest population and DN is latest stage before becoming mature mTECs. To verify this hypothesis each of the four subpopulations were placed individually into a RTOC system and analysed after 7 days. The rational behind this experiment was to see if the lineage sequence we have gathered could be true. Unfortunately all four subpopulations, when placed individually into the RTOC system was able to give rise to the remaining three populations (not shown here). That could be the reason that the gates, which were set during the cell sorting was not stringent enough or an alternative could be that our two markers Sca-1 and CD24 are not enough to exclude other subpopulations within the immature mTECs which also have the potential to give rise to mature mTECs. Thus more markers are needed to get a better picture in how many different stages immature mTECs undergo before they become mature mTECs. Although the sequence could not be verified through in vivo experiments we used two other approaches to show that the sequence we have gathered could be real. The first approach was to look into known markers, which have been linked to the mTEC development to see if the sequence: Sca-1 → DP → CD24 →DN → high matches with the mTEC maturation. All eight markers CD80, FoxN1, Aire, Gad1, Csnb, Pcp4, Sirt1 and CCR4 should increase during the mTEC development, while the four markers Pdpn, Meis1, Px1 and Trp63 should decrease during the maturation. Indeed we see a lineage progression when we checked the levels of mRNA expression of those 12 markers in our five populations. We also used a FoxN1 eGFP mouse to detect the levels of FoxN1 expression by using flow cytometry. The results are showing the same outcome that the expression of FoxN1 follows the same sequence as we found in our microarray analysis.

4.2 Clusters of miRNA sets in the four subpopulations

The data of the miRNA microarray analysis revealed three different miRNA sets. One identical miRNA set shared by DP and CD24 and another one by Sca-1 and DN, while high has its own miRNA set. It is already known in the literature (J. Shim and J. Nam, 2016), that changes in the miRNA expression will lead to mRNA changes as well as it can lead to differentiation of cells.

The first set of miRNA is activated potentially to drive Sca-1 cells to become DP. As DP and CD24 share the same miRNA set and their development goes from DP to CD24, it could be the reason that DP is just a short intermediate stage and cells are moving quickly to CD24 without activating any miRNA sets to drive differentiation. To differentiate CD24 cells to DN another miRNA set needs to be activated. Then again the miRNA set " Sca-1 and DN" needs to be activated to move those cells further to become mature mTECs. Therefore we believe that in those three mouse strains (Dicer KO, nude and SCID mice) miRNAs are not properly expressed due to an impaired mTEC development or even being absent as Dicer is missing to process immature miRNAs to mature miRNAs, thus we see no cells accumulating in Sca-1, an arrest of cells halted in CD24, as well as an almost non existing number of mature mTECs, because those internal miRNA switches are not activated. Certainly a specific set of miRNAs is needed to drive the bipotent progenitor cell to become Sca-1 positive cells, as the developmental stage between the bipotent progenitor cell to become a mTEC is still unknown, no early stage before Sca-1 could be used to manifest this hypothesis that miRNA might play a role. As it has not been investigated how the expression of those miRNA sets are regulated and whether feedback loops are integrated within the four subpopulations to ensure a constant flow of immature mTECs to replenish the mature mTEC pool, this would be something which needs to be done to get a better understanding of the whole differentiation process.

Hypothesizing that mTEC lineage progression occurs in a miRNA-dependent manner. It would make sense that when a certain set of miRNA is upregulated during development that it cause changes in the mRNA expression of the next subsequent population for instance when the miRNA set in CD24 is changing

due to external signals/factors, that might cause mRNA expression changes leading to a conversion of CD24 cells to DN cells, when assuming the sequence lineage progression we are claiming is in that order. It is known in the literature (Winter et al. 2009) that miRNA expression changes would proceed to mRNA changes, which then leads the cell to differentiate and that is what we believe what we are observing here. These results are suggesting that the miRNAs in the four subpopulations might be part of the molecular drive to move mTEC lineage progression forward.

Taken all mRNA and miRNA data into consideration we are proposing the lineage progression sequence where Sca-1 is probably the most progenitor cell population from all four and develops to DP. DP is the population, which suffers the most severe loss of cells when T cell development is hampered or miRNAs are missing. This population populates the most cells within the four subpopulations. According to our analysis DP develops further to CD24. In CD24, cells are arrested in this stage when the maturation of T cells and miRNAs are impaired. Normally CD24 inhabits the second biggest population within the four subpopulations, but when signals from T cells and miRNAs are missing, cells tend to get stuck in this stage. The explanation on the miRNA level could be that as DP and CD24 are sharing the same sets of miRNAs, which is missing in Dicer KO mice. The progress of DP cells to become CD24 is not disturbed, but from CD24 on another miRNA set is needed to process the CD24 subpopulation to DN, which explains why the population is accumulated in CD24. Certain miRNAs from the Sca-1 and DN miRNA set are probably needed to let the CD24 cells to move forward to become DN cells, as well as cells from the DN population to become mature mTECs. It could be the reason that certain miRNAs are regulating specific checkpoint molecules or targets in diverse signalling pathways, which have an influence for the mTEC development. And as immature mTECs have to replenish the mature mTEC population, cells need to be ready for the maturation. Theoretically it could be that the DP population normally gets a signal to progress further to become CD24 and that this progress is regulated. Then the CD24 cells get another signal to progress further to DN. As cells are arrested in CD24 and

DN it would be that it gives a certain signal that there are enough maturing cells in the thymus and that there is no need for a replenishment of immature cells that could be the reason why the Sca-1 population is fully missing. It is known that miRNA act as a positive or negative feedback loop as well as switches in differentiations. Thus it would make sense, that missing miRNA will lead to an arrest of the development of immature mTECs to mature mTECs. As the shift of the four subpopulations also occurs in mice lacking T cells, it could be that certain signals coming from the T cells are needed to activate gene expression of particular miRNAs, which are needed for the checkpoints on Sca-1, DP, CD24 and DN. Thus finding the miRNAs, which are playing an important role to drive the maturation of immature mTECs to mature mTECs as well as the miRNAs inducing the differentiation of one stage to the other might answer the questions.

4.3 Usage of different approaches to find potential miRNAs

To find potential miRNAs, which might be relevant for the development of immature mTECs to mature mTECs we first generated a list of 80 miRNAs, which shows the four subpopulations with the highest variance difference within their miRNA expressions. Afterwards we did a principle component analysis (PCA) where we could see in the first PC, PC1, that 63% of that component provides the uppermost difference. We picked 25 miRNAs from that narrowed list, thus increasing our chance to find miRNAs, which have a functional role in the mTEC development. To understand the importance of those miRNAs in our four subpopulations we lined those miRNAs according to our lineage sequence and found that most of them are increasing or decreasing during lineage progression, while some are fluctuating during the development. We also verified the existence of those miRNA candidates with qPCR, comparing wild type Black6 to Dicer KO mice. The results revealed that our miRNA candidates are non-existing or have a minimal expression level in Dicer KO mice. Thus those miRNAs we have picked are worth investigating.

The potential miRNA candidates, which we found as the most prominent ones, are the results of two steady-state models. However the mTEC development is a dynamic process. To investigate on steady state miRNAs is a good start, as it hasn't been done before, but to go further in exploring dynamic miRNAs in the mTEC development would be more promising, as those will be influencing the development directly. Thus we will search for dynamic miRNAs by using a SCID mouse model and re-establishing the thymic crosstalk. As outlined before, SCID mice have an intact thymus but exhibit a blockade of mTEC development due to the lack of functional T-cells. A reconstitution of SCID mice with wildtype bone marrow can actually reverse this blockade. This approach has many advantages over other approaches, which have been used to study mTEC regeneration, such as use of irradiation or cyclophosphamide treatment all of which involve cytoablative techniques and therefore generate possible systemic effects or defects. In contrast, bone marrow reconstitution in SCID will be performed under physiological conditions, allowing us to measure the unperturbed response of the mTEC compartment to T cell-derived signals as well as to follow the expression of miRNAs during the reconstitution. Another important feature of this model is that the reconstitution is done in the intact adult thymus by adult bone marrow-derived cells, resembling the adult thymus and their miRNA pool as the measured outcome will therefore reflect the dynamics of mTEC maintenance and lineage progression in the adult thymus, which might differ from the events taking place during embryonic development.

We isolated bone marrow from wild type mice and injected them i.v. in SCID mice. Always a pair of 12 SCID injected and SCID wild type mice are kept under surveillance. The time slot for the FACS analysis and RNA isolation was 3, 4, 5, 7 and 8 weeks post transplantation. The time periods were selected based on experiments to determine the time period where mTECs might start developing and when the mTEC development process becomes stable (not shown). CD4 and CD8 T cells are generating already after 4 weeks post bone marrow transplantation while the four subpopulations can only be seen in week 5. Therefore it is interesting that immature mTECs need roughly

a week to be generated as the time shift between T cell generation and immature mTEC development was around a week. After we have isolated the RNA during the different time points we checked for the miRNA expression levels of our miRNA candidates. Interestingly we saw a very dynamic change of miRNA expression during the maturation process from the beginning until week 5. The levels between week 5 and week 8 did not show any differences (not shown). Thus we believe that the miRNAs we have picked might be relevant for the maturation process of immature mTECs especially the generation of the four subpopulations within the immature mTEC pool. Due to the low number of RNA in Sca-1, DP and DN, it was necessary to pool Sca-1 with DP and CD24 with DN respectively. It was enough to see that those miRNAs we picked are not stable during the development and therefore could exclude non-relevant miRNAs. Ideally would be to have four separate samples but it would have been too pricy and too many animals to collect the amount needed.

4.4 Discovery of a new reliable transfection method

So far researcher have failed to find a liable method to transient transfect freshly isolated mTECs. Most researchers are using either a mouse line to check the loss and gain function of a certain miRNA or using mTEC cell lines to investigate miRNA functions. None of those two methods are practical as generating a mouse line with a certain miRNA is not cost effective and time consuming. Furthermore, cell lines do not resemble the microenvironment of a mouse itself. Therefore a new method needs to be established to do a wide range screening of miRNAs on mTECs in a short time manner. We tried lentivirus as a transfection method but did not proceed further even though we produce a high percentage of positive cells, around 80%, but the amount of living cells were extremely poor, around 25%, thus those cells were not capable to generate RTOCs or there were not enough cell numbers left to run further analysis. Afterwards we started to test with different transfection reagents and only one reagent was able to produce high numbers of positive cells. Not only we reached 73% of positive cells, the viability of mTECs was

around 88%. The next step was to transfect all mimics and antagomiRs individually in freshly isolated mTECs and then checked for their functionality by qPCR. An incubation of 14 hours was enough to increase miRNA level by mimics or decrease the miRNA level to a non-detectable level by antagomiRs. A shorter time than 14 hours were not checked as normally mimics and antagomiRs are recommended in the protocols to be incubated for at least 24 hours. In our case we needed to reduce the incubation time as freshly isolated mTECs are not surviving for longer than 24 hours if not placed into a RTOC system. Though the incubation time was shorter than suggested the results we have achieved was evidently effective to induce miRNA changes.

4.5 MicroRNA level changes influence the development of the four subpopulations

Red-labelled immature mTECs were transfected with antagomiRs and mimics, respectively to see their relevance in the development of the four subpopulations. When we compared transfected cells with a negative control we indeed we saw significant shifts in the four subpopulations when we transfected immature mTECs with the following miRNA mimics and antagomiRs, respectively: miR-125a-3p, miR-125b-5p, miR-200c, miR205, miR-29a-3p, miR-34a-5p, miR-34b-3p, miR-709, miR-761, miR-877-3p and miR-92a-5p. The only miRNA, which failed to show any significant changes was miR-203. Surprisingly as miR-203 has been linked to epithelial differentiation along with miR-200 family and miR-205, but only miR-200c and miR205 showed a distribution change in the four subpopulations (Yi et al. 2008), (Khan et al. 2015). The average distribution of the subpopulation DP was around 71.6% when cells were transfected with a negative control and 23,7% transfected with a positive control that is a drop of cells in the DP population of 67%. When comparing the negative control and the DP population we could observe the lowest difference of 3% induced by miR-761 mimic. The highest difference, 47%, is shown between the negative control and miR-709 mimic and miR-877 antagomiR. Five miRNAs did show a significant change when transfected either with a miRNA mimic or

antagomiRs, respectively. These were miR-29a, miR-34b-3p, miR-709, miR-761 and miR-92a-5p. The distribution changes which each of the miRNA candidates have caused could only be seen as a whole and not how each of the miRNA interfered the development of each subpopulation. Due to the limited numbers of cells it was not possible to do such experiments. Therefore it would be worth investigating in those miRNA candidates generating a mouse line with the respective miRNA and to see the lost and gain function. There we could see the impairment of the development of each individual subpopulation, the generation of TRAs and mTEC development.

4.6 Search for relevant mRNA-miRNA pairs

Our collaborator in UCLA generated the list of mRNAs with their negatively correlated miRNA candidates. They used two different approaches to narrow down the list of potential candidates by looking first at the most negatively correlated miRNA-mRNA pairs. Then we searched if the pair has been already verified or published. In the list the miRNA-mRNA pairs have been mostly published such as Bak1 is reported to be a direct target of miR-125b (Chen et al. 2015), miR-125b targeting Arid3b (Akhavantabasi et al. 2012) or miR-34a regulates Bcl2 (Wang et al. 2017). The list was then further analysed and narrowed through an intensive search in genecards.org to see which mRNA might target important signalling pathways or are involved or reported to be involved in development, maturation or differentiation. All 34 mRNA targets are highly negatively correlated to our candidate miRNAs. Though it seems biased to select from a list of known miRNA-mRNA pairs but the chances to find functional miRNA-mRNA pairs are still not guaranteed.

4.7 MiRNAs with functional effects

We tested all 34 mRNA targets from our list and check for any changes in the four subpopulations by using siRNAs to downregulate their expression levels. Quickly we found four targets, which have shown a variation in the distribution of the four subpopulations. Therefore the entire experiment was done another time to verify the findings. Indeed those four mRNAs are showing a significant

change when compared to the negative control. The mRNA targets are Bak1, Mapk14, Foxp1 and Fam83e. Based on the p-value of Foxp1, which was 0.04. But Bak1, Mapk14 and Fam83e have a p-value below 0.007, which give us more confident that those three mRNA targets are playing a significant role in the development of the four subpopulations. Nevertheless we did for all four targets a luciferase assay to confirm the negative correlation between the miRNA-mRNA pairs. All four were positive and showed that miR-125b-5p interacts with Bak1 and Mapk14, miR-125a-3p with Fam83e and miR-34a-5p with Foxp1. MiR-125a-3p, miR-125b-5p and miR-34a-5p have shown already very promising results when we transfected immature mTECs with either mimics or antagomiRs. MiR-125a-3p antagomiR shrunk the DP population from 71.6% to 43.5%, which is a decline of 39%. Whereas the miRNA mimics of miR-125b-5p and miR-34a-5p decreased the DP subpopulation to 61.4% and 57.1%, respectively. Unfortunately we could not find any promising targets for miR-709 and miR-877 as those miRNAs have shown very promising results. Both miRNAs provoked very significant changes in the four subpopulations; a drop in the DP population compared to the negative control was 12% with miR-709 antagomiR, 47% with miR-709 mimics and 47% with miR-877 antagomiR. Therefore an intensive investigation of those two miRNAs is worth as those two miRNAs are playing an important role to keep the immature mTEC pool intact.

4.8 The new mRNA targets in immature mTECs and their potential involvement in the mTEC development

MiR-34 family has been reported to play a role in regulating important targets in cell cycle progression, regulation of cell growth and anti-apoptosis. Foxp1 is a bona fide target of miR-34a and has been linked to play a crucial role in B cell development, especially in the early B lymphoid development (Rao et al. 2010). Mir-125b-5p has been linked to repress its direct target Bak1, a pro-apoptotic gene (Bayoumi et al. 2018) and is known to play a role in myeloid cell proliferation (Surdziel et al. 2011). Another important target of miR-125b is Mapk14 (Yao et al. 2016), which belongs to the Map kinases and are known

to play important role such as development, proliferation, differentiation and transcription regulation. Fam83 family has been linked to the Mapk signalling and has been identified as a driving force for epithelial cell transformation. Fam83e was found to drive mammary epithelial cell transformation (Cipriano et al. 2014). Our outcomes have shown that we have searched in the right direction as all four mRNA targets are linked to differentiation and development. To understand the importance of the miRNAs and the mRNA targets mouse models need to be generated and looked closer to the changes of mTEC maturation and expression of tissue restricted antigens.

4.9 Interesting mRNA target for further investigation

As a positive control we used let-7c-5p as it has been reported to correlate negatively to HMGA2. HMGA2 is a non-histone, which is ubiquitously expressed and has the feature as a chromatin protein to change the gene expression through chromatin modulation. The rational for using let-7c as a positive control was that the gene expression in immature mTECs is different compared to mature mTECs as well as the expression levels within the four subpopulations. A disruption of gene expression can induce a halt in the development within the four subpopulations. Interestingly the induction of let-7c in the immature mTECs caused a significant change in the four subpopulations with a similar distribution in SCID, nude and Dicer KO mice. In this PhD we only investigated in the other miRNA candidates and used let-7c as a positive control as it has shown to induce changes in the development of the four subpopulations hence it makes sense to study further this miRNA in a mouse model to find their relevant targets in mTECs. Notably a group has found the importance of the miR-let-7 family in mTECs, especially in controlling the expression of tissue restricted antigens (Oliveira et al. 2016).

5 Concluding remarks and future perspectives

In overall our results could increase the understanding of the involvement of miRNAs in the development of immature mTECs to mature mTECs. In this study we have added novel aspects to the developmental sequence of TECs as well as novel miRNAs and mRNA targets in TECs:

1. In regards to the knowledge of the existence of the four subpopulations in neonatals and embryos, we expanded their lineage progression in adults (Sca-1 < DP < CD24 < DN < mature mTECs).
2. We developed a new method to induce miRNA changes in freshly isolated primary mTECs via transient transfection.
3. The miRNA and mRNA microarray data revealed numerous interesting miRNAs linked to the development of the four subpopulations to become mature mTECs. Those results were compared with the outcome of a dynamic SCID mouse model where we could track miRNAs during the development of mTECs, from the development of immature mTECs until mature mTECs. With the knowledge of both models we generated a list of miRNA and confirmed their relevance by overexpressing or downregulating those miRNA candidates. At the end we discovered 10 relevant miRNAs playing a role in the development of the four subpopulations.
4. From those 10 potential miRNAs we found 4 mRNA targets, which seem to play a major role in the maturation step of the subpopulations and negatively controlled by three of our miRNA candidates.

Mouse lines expressing candidate miRNAs should be generated to investigate the gain and loss of function of these miRNAs and the consequences in the thymus, on mTEC development and on the expression of tissue restricted antigens (TRAs). Importantly for the clinical perspective those miRNAs and

their mRNA targets should be verified and characterized in immature mTECs using the human mTECs operating with a similar experimental approach.

6 Reference

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