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Age-Associated Changes in miRNA Expression Profiles in Thymopoiesis

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Abstract

During adult life, the thymus involutes and thymic output of mature T cells drastically declines. The molecular events underlying this process are not well understood. Here, we present evidence of the importance of miRNAs in regulating T cell differentiation in the aged. miRNAs are a wide-ranging regulatory element influencing gene expression throughout the lifetime of the organism. To establish whether they play a role in the age-specific thymic decline, the miRNA expression pattern was examined in TN subsets of young and aged mice. Fifty-two percent of the miRNAs exhibited elevated expression levels in the aged TN1 cells. This expression profile leads us to hypothesize that the large number of highly expressed miRNAs, indicative of rigidly controlled protein expression, limits the developmental potential of this population and results in the age-induced decline in thymopoiesis.

The differentiation of mature functional $\alpha\beta$ -TCR T lymphocytes within the thymus is a complex, multi-step process (Godfrey and Zlotnik, 1993; Kisielow and von Boehmer, 1995; Anderson et al., 1996; Fehling and von Boehmer, 1997; Rodewald and Fehling, 1998). The bone marrow derived progenitor cells entering the thymus are characterized by the absence of the mature T cell markers, CD3, CD4 and CD8 (triple negative, TN). These develop into CD4/CD8 double-positive cells that finally undergo selection to produce CD4 or CD8 single positive cells. The TN thymocytes progress through a series of 4 developmental stages, each of which can be functionally and phenotypically distinguished by CD44 and CD25 expression. Thymopoiesis is initiated by the entry of the blood-borne, multi-potential CD44⁺CD25⁻ (TN1) population originating from the bone marrow. The TN1 cell gives rise to the CD44⁺CD25⁺ TN2 subset following up-regulation of CD25. The TN2 cells undergo extensive proliferation before proceeding to the TN3 subset, which is marked by the loss of CD44 (CD44⁻CD25⁺). Cell surface expression of CD25 is lost in the transition to TN4 (CD44⁻CD25⁻) another proliferative step (Godfrey and Zlotnik, 1993). During this developmental progression, the TN thymocytes lose their multilineage plasticity such that the TN2 subset are more restricted than the TN1 cells in their lineage commitment. By the TN3 stage, the population is totally committed to the T cell lineage (Schmitt et al., 2004).

During the lifetime of the individual, the thymus shrinks or involutes and thymic output drastically declines (Santisteban, 1960; Hirokawa, 1977; Kay et al., 1979). Coincident with the involution, thymic- dependent T cell lymphopoiesis is reduced and is accompanied by changes in the distribution of the thymocyte subsets indicating that a limited number of age-

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sensitive “checkpoints” inhibit thymopoiesis. In aged mice there is a significant increase in the number of TN1 cells indicating that the percentage of cells making the TN1-TN2 transition is reduced in the involuting thymus. In addition, the proliferation of the diminished TN2 population is greatly decreased (Thoman, 1995, 1997; Rodewald and Fehling, 1998; Li et al., 2003). The combination of these two age-related checkpoints results in the significantly lower percentage of CD25⁺ cells, a hallmark of the aged thymus.

The underlying molecular events regulating thymic involution and the drastic reduction in thymic output are not well understood. Our research has focused on understanding the molecular mechanisms contributing to the diminished capacity of T cell progenitors to initiate differentiation in the aged thymus. Recent data illuminating the critical role of microRNAs (miRNAs) in development led us to hypothesize that miRNAs may be a regulator of thymopoiesis in adult animals. MicroRNAs are small noncoding RNAs that exhibit distinct spatial and temporal expression patterns; as a result, tissues in developing and mature organisms are characterized by unique profiles of miRNA expression (Chen et al., 2004; Kim, 2005; Lim et al., 2005). MiRNAs function by controlling gene expression during cellular processes such as differentiation and development. Through the direct interaction of the miRNA with an individual messenger RNA (mRNA), microRNAs specify mRNA degradation, translational repression or both; consequently, the level of protein produced from the target mRNA is decreased (Lim et al., 2005; Baek et al., 2008; Selbach et al., 2008; Bartel, 2009). A single miRNA can directly or indirectly repress protein synthesis from thousands of genes establishing miRNAs as global regulators of gene expression (Chen et al., 2004; Kim, 2005; Lim et al., 2005).

It is becoming abundantly clear that miRNAs are a pervasive regulatory element influencing gene expression throughout the lifetime of the organism. Over the last several years, data has illuminated the importance of their tissue- and cell-specific expression profiles. Now it is evident that changes in miRNA expression are also critical during the aging process (Wang, 2007; Maes et al., 2008). In an effort to establish the importance of miRNAs in immunosenescence, we initiated a study to define their role in the decline of T cell development, focusing on the initial steps in differentiation.

To address this question, we identified the miRNA expression profile from lineage negative (Lin⁻), triple negative (TN) thymocytes of young and aged mice. Due to the low number of Lin⁻ TN thymocytes, it was necessary to pool cells from two young (3-months) and six aged (24-months) C57BL/6 animals. This approach enables us to obtain sufficient quantities of RNA while avoiding possible problems associated with a preamplification step. However, the use of pooled samples precluded the standard statistical analysis of the data. The Lin⁻ TN population, designated CD8⁻, CD4⁻, CD3⁻, CD11b⁻, B220⁻, Gr-1⁻ (Ly6G), and Ter-119⁻ (Ly- 76), was purified on a BD FACSARIA cell sorter. It should be noted that this population consists of all four subpopulations (TN1 – TN4). Total RNA was isolated from these cells with Trizol (Life Technologies, Carlsbad, CA). System Biosciences (Mountain View, CA) generated the microRNA expression profile using qRT-PCR. The miRNA expression levels of 627 miRNAs were assayed and the fold difference ($\Delta\Delta C_T$) in expression determined. The $\Delta\Delta C_T$ for each miRNA was defined as the change in expression in the aged TNs relative to the young TNs using the comparative C_T method for relative quantification as calculated with the following equation:

$$2^{\Delta\Delta C_T} = \frac{(C_T \text{ aged miRNA} - C_T \text{ aged endogenous control}) - (C_T \text{ young miRNA} - C_T \text{ young endogenous control})}{C_T \text{ young miRNA} - C_T \text{ young endogenous control}}$$

. The geometric mean of the C_T values from three reference genes (U6 snRNA, RNU43 [snoRNA], U1 snRNA) was used to normalize the expression data. A 3- fold or greater difference was considered significant.

The profiling results for the total TN population are shown in Figure 1 and summarized in Table 1 and Figure 5. Thirty-two percent of the miRNAs (199 miRNAs) exhibited a 3-fold or greater increase in the aged animals. Only 5% (34 miRNAs) were down regulated by 3-fold or more in the older mice relative to the young population.

The magnitude of the differences in miRNA expression between the young and aged TN cells was unexpected. The TN population encompasses at least four identifiable subpopulations, representing distinct steps in differentiation, and these subpopulations differ in miRNA profile (Monticelli et al., 2005; Neilson et al., 2007; Merkerova et al., 2008). Since the relative proportion of the TN subsets changes with aging, some of the miRNA expression differences may reflect the dissimilarity in the makeup of the populations used for RNA isolation. To rule out this trivial explanation for our results, we examined the miRNA expression in the individual thymic subsets of both young (2 months) and aged (22 – 24 months) C57BL/6 mice. The scarcity of cells comprising these subsets again necessitated the pooling of thymocytes from multiple animals. By means of FACS sorting, TN1, TN2/3 and TN4 were separately purified as described (Figures 2, 3, and 4). System Biosciences determined the miRNA expression of the thymocyte subpopulations and the $\Delta\Delta C_T$ was calculated as above. In this instance, the $\Delta\Delta C_T$ for each miRNA was defined as the change in expression in the aged subset relative to the young subset. The results were quite striking. Most notably, the expression levels of a majority of the miRNAs were elevated in the aged TN1 cells (Figures 2 and 5, Table 1). Fifty-two percent of the miRNAs profiled were up regulated more than 3-fold. On the other hand, the expression of only 0.8% (5 miRNAs) was down regulated in this aged subpopulation.

Our profiling results indicate that there is a significant up regulation of miRNA expression in the aged TN1 cells. Such major changes in miRNA levels could have extreme consequences on gene expression in this subset. Other laboratories have shown that elevation in the expression of just a few miRNAs caused widespread changes in the cellular protein profile (Lim et al., 2005; Selbach et al., 2008). Indeed, a 50% decrease in the level of a single miRNA can result in significant changes in development (Cordes and Srivastava, 2009). It would follow that the drastic increase in miRNA expression in the aged TN1 population would result in considerable changes in the protein production in these cells. Our data suggest that protein expression in the TN1 cells of aged animals may be very highly regulated. As a result, the TN1 cells may have a more difficult time initiating and proceeding through the developmental pathway thus creating the initial age-related block in thymopoiesis. Those TN1 cells able to complete the transition to the TN2 subset and beyond are more young-like in their miRNA profile (as shown in Figures 3 – 5) and, therefore, in their gene expression patterns.

The aged TN2/3 (Figures 3 and 5, Table 1) and the TN4 (Figures 4 and 5, Table 1) populations exhibited fewer changes in their miRNA expression pattern. Neither of these subsets had the drastic increase in miRNA expression identified in the TN1 cells. Only 6 (0.96%) miRNAs in the TN2/3 and 9 (1.4%) in the TN4 cells were elevated in the aged. These data indicate that the majority of the up regulation of miRNAs detected in the entire aged TN population resulted from elevated miRNA expression in the TN1 subset. More importantly, the results show that in aged murine thymocytes, the least mature developmental subset (TN1) exhibits a significant increase in miRNA expression.

It should be noted that the aged TN2/3 cells had a less young-like miRNA profile than that exhibited by the TN4 subset. There were a moderate number of miRNAs whose expression in the aged TN2/3 cells was reduced 3-fold or greater (81 miRNAs or 12.8%). Finally, the most mature developmental population (TN4) had the fewest differences in miRNA expression when aged was compared to young.

An intriguing feature of the aged TN2/3 miRNA profile was the number of miRNAs whose expression was down regulated compared to the young subpopulation. One potential implication of this finding is that gene expression in the aged TN2/3 cells may be less controlled and, consequently, these cells may be more developmentally plastic than their younger counterparts. While the TN1 population is capable of generating multiple hematopoietic cell types upon appropriate stimulation, TN2 thymocytes are more restricted in their developmental potential. Full commitment to the T cell lineage does not occur until the TN3 stage (Schmitt et al., 2004). As the cells undergo lineage commitment the transcription profiles clearly indicate that gene expression is down regulated. Many of these differentially expressed genes are associated with non-T lineage developmental programs indicating that T cell specification requires the repression of multi-lineage-affiliated genes rather than the induction of a set of T cell specific genes (Kawazu et al., 2007). Other evidence shows that transcriptional regulation during early T cell development represses expression of “prethymically inherited genes.” (David-Fung et al., 2009). In light of these recent reports, we propose that the failure of TN2/3 cells in aged mice to up regulate specific miRNAs results in cells that are less committed to T cell specification than those in the young population. Our data identifies several miRNA species in the young that display the requisite profile of expression up regulation as the cells transit to the TN2/3 population. A subset of this group of miRNAs is not up regulated in the aged. Four exhibit that unique pattern of expression (mmu-miR-18a, 382, 493 and 688). Several potential target genes for these miRNAs were identified using Targetscan (Lewis et al., 2003; Lewis et al., 2005), MiRanda (Betel et al., 2008), MicrCosm Targets (Griffiths-Jones et al., 2008) and TargetSpy (Sturm et al., 2010) prediction algorithms. Examples of those candidates recognized by two or more algorithms include: Runx1 (miR-18a and 382), a master regulator of the hematopoietic system as well as an enhancer of B cell development (Maier et al., 2004; Michaud et al., 2008), Runx3 (miR-18a, 382, and 493), a transcription factor involved in both B cell and NK cell differentiation, (Whiteman and Farrell, 2006; Ohno et al., 2008), and Shh (miR-18a and 493), a morphogen differentially expressed during young and aged thymopoiesis (Virts et al., 2006). Further exploration of the role these miRNAs play in T lineage commitment is underway.

Our analysis reveals that there are many age-associated differences in miRNA expression profiles in developing T cells. The altered miRNA expression pattern and the corresponding change in overall protein expression in the aged TN1 cells may account, in part, for the decline in thymic output during aging. Those aged thymocytes that did progress through the thymic developmental pathway exhibited a more young-like miRNA profile. The data suggests another intriguing possibility: a major change in miRNA regulation has occurred in the TN1 subpopulation with age. Alterations in miRNA processing are a likely candidate. Overall we hypothesize that the large number of highly expressed miRNAs, indicative of rigidly controlled protein expression, limits the developmental potential of this population and results in the age-induced decline in thymopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fold difference in miRNA expression in aged relative to young total TN thymocytes
 Two thymuses from 3 month old mice were dissected and the thymocytes pooled for the isolation of young total TN cells. Thymocytes from six animals (24 months old) were pooled for the isolation of aged total TN cells. Panel A. Each vertical bar corresponds to the fold difference in expression of a single miRNA. The columns extending above the horizontal line represent the miRNAs whose expression level is greater than 3 fold in the aged TN thymocytes. Panel B. The pie chart shows the distribution of miRNA's whose fold expression is up regulated, down regulated or not significantly changed in aged compared to young TN thymocytes. The percentage comprising each group is indicated.

For the isolation of the young TN1 subpopulation, thymocytes were pooled from five animals (2 months in age). Ten animals, 22 to 24 months old, were used for the purification of the aged thymic subsets. The Lin⁻ TN1 cells were selected by cell sorting as CD8⁻, CD4⁻, CD3⁻, CD11b⁻, B220⁻, Gr-1⁻ (Ly6G), and Ter-119⁻ (Ly-76), CD44⁺, and CD25⁻. Total RNA was isolated and the miRNA profiles generated as described for the TN population. Panel A. The setup of the graph is as detailed in Figure 1. Panel B. The organization of the pie chart is described in Figure 1.

Figure 3. Fold difference in miRNA expression in aged relative to young TN2/3

The cells were isolated as in Figure 2. The TN2/3 subset was generated during sorting by pooling the TN2 cells, CD8⁻, CD4⁻, CD3⁻, CD11b⁻, B220⁻, Gr-1⁻ (Ly6G), Ter-119⁻ (Ly-76), CD44⁺, CD25⁺ and the TN3 cells, CD8⁻, CD4⁻, CD3⁻, CD11b⁻, B220⁻, Gr-1⁻ (Ly6G), Ter-119⁻ (Ly-76), CD44⁻, CD25⁺. RNA isolation and miRNA profiling is as detailed for the TN population. Panel A. The graph organization is described in Figure 1. Panel B. The pie chart is as detailed in Figure 1.

Figure 4. Fold difference in miRNA expression in aged relative TN4 thymocytes

The thymocytes were obtained as described in Figure 2. The TN4 cells were purified by choosing the CD8⁻, CD4⁻, CD3⁻, CD11b⁻, B220⁻, Gr-1⁻ (Ly6G), Ter-119⁻ (Ly-76), CD44⁻, CD25⁻ population during cell sorting. RNA isolation and miRNA profiling is as detailed for the TN population. Panel A. The graph organization is described in Figure 1. Panel B. The set up for the pie chart is explained in Figure 1.

Figure 5. Comparison of the total number of miRNAs up or down regulated in all cell types analyzed

Panel A. The columns represent number of miRNAs whose expression level is greater than 3 fold in the aged. Panel B. The columns indicate the number of miRNAs whose expression is down regulated 3 fold or more in aged cells.

Table 1

Percentage of up regulated or down regulated miRNAs in aged thymocyte populations. The actual number of miRNAs whose expression levels were changed in the aged thymocytes is given in the parenthesis.

Thymocyte population	Up regulated in Aged	Down regulated in Aged
Total TN's	32.7% (199)	5.4% (34)
TN1	52.5% (329)	0.80% (5)
TN2/3	0.96% (6)	12.8% (81)
TN4	1.4% (9)	1.28% (8)