Restriction of non-coding elements in the thymus by Aire

Shahan Mamoor¹, MS

¹Thomas Jefferson School of Law

San Diego, CA 92101

correspondence: mamoorsk@tjssl.edu

Aire is a unique transcriptional molecule that functions in immunological tolerance by promoting promiscuous expression of tissue-restricted genes. The expression of these otherwise tissue-restricted genes in the medullary epithelial cells of the thymus (mTECs) allows the presentation of self antigens to “teach” cells of the immune system what it means to be “self”. To understand the role of Aire in shaping the mTEC transcriptome, I performed global differential gene expression analysis of wild-type mTECs and mTECs deficient in *Aire*. I identified nineteen non-coding molecules whose expression were significantly modulated by the absence of Aire. These included pseudogenes, antisense RNA, small nucleolar and small nuclear RNA, and long non-coding RNA. This finding reveals that Aire controls the expression of both coding and non-coding genes, and further suggests that non-coding RNA might play a role in Aire’s instruction of immunological tolerance.

Keywords: Aire, non-coding RNA, lncRNA, snoRNA, snRNA, pseudogene, antisense RNA, medullary thymic epithelial cell (mTEC).

Introduction

Distinguishing the self from non-self is essential for the precise function of the immune system. In addition to the ability to discriminate between foreign and self molecules, the ability to tolerate the molecules, cells and tissues of the self is critical for the prevention of autoimmune disease. In order to teach developing lymphocytes what it means to be “self”, medullary epithelial cells of the thymus, or mTECs express genes that code for antigens whose expression is otherwise restricted to tissues in the periphery (tissue-restricted antigens). This promiscuous expression of tissue-restricted antigens by Aire and their subsequent presentation is a mechanism by which tolerance of tissue-specific antigens is established centrally in the thymus.

Accordingly, humans that have mutations in *Aire* experience autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, or APECED (also known as autoimmune polyendocrine syndrome type 1 (APS-1)) (2). These patients possess multi-organ autoimmune pathology and a classical triad of mucocutaneous candidiasis, autoimmune adrenocortical insufficiency and autoimmune hypoparathyroidism. Over 42 separate mutations affecting Aire have been discovered in patients with APECED/APS-1 (2); the underlying mechanism by which mutations in Aire lead to APECED is unknown.

To understand that transcriptional changes that accompany Aire expression in the mTECs, I performed global gene expression profiling and compared the transcriptomes of mTECs with high expression of the class II major histocompatibility complex (mTEC^{hi}) that possess and lack Aire (1). Surprisingly, of the most differentially expressed genes between Aire ^{-/-} and Aire ^{+/+} mTECs, I identified nineteen non-coding

RNA. These non-coding species include long and long intergenic ncRNA, small nuclear RNA, small nucleolar RNA, pseudogenes and antisense RNA. The discovery that non-coding RNA represent a major element of the Aire-induced transcriptome is unprecedented and suggests that Aire may function in immunological tolerance via non-coding mechanisms.

Methods

The GEO2R platform was utilized to compare samples from dataset GSE102527 (1) and identify the two hundred and fifty most differentially regulated transcripts between medullary thymic epithelial cells from wild-type and Aire knockout mice (n=2 samples each). No adjustment was made to *p*-values, the data was not log-transformed in any manners. Unpaired Welch's t-test with correction was performed for each comparison to determine the statistical significance of the difference between the raw sample values (PRISM 8).

Results

I mined a pre-existing transcriptome dataset of mTECs expressing high levels of the class II major histocompatibility complex (mTEC^{hi}), both wild-type and Aire-deleted (1), to determine the most significant transcriptional changes induced by the absence of Aire. I identified nineteen non-coding transcripts that were among the most differentially expressed genes when comparing the transcriptomes of Aire +/+ and Aire -/- mTEC^{hi}. Ten of the non-coding transcripts were up-regulated in the absence of Aire while nine of the non-coding transcripts were down-regulated in its absence.

Four of the transcripts were identified to be pseudogenes (Figure 1), nine were identified as long or long intergenic non-coding RNA (lncRNA or lincRNA, respectively; Figure 2), three were identified to be small RNA of either nuclear (snRNA) or nucleolar (snoRNA) types (Figure 3), and three were identified as antisense transcripts (Figure 4).

The Aly/Ref gene encodes machinery involved in the nuclear export of mRNA. The Aly/Ref pseudogene transcript, encoded on Chromosome 1 was one of the most differentially expressed genes when comparing the transcriptomes of mTEC^{hi} with and without Aire. The Aly/Ref pseudogene is expressed at significantly lower levels in Aire^{-/-} mTEC^{hi} (Figure 1; $p=0.0009$). Expression of the Aly/Ref pseudogene is nearly ablated in mTEC^{hi} lacking Aire.

GM7609 is a non-coding RNA and predicted pseudogene transcript encoded on Chromosome 1 that is annotated by NCBI as Sp100-rs and also among the genes most differentially expressed in Aire^{-/-} mTEC^{hi} compared to their wild-type counterparts. ncRNA GM6709 was expressed at higher levels in Aire^{-/-} mTEC^{hi} (Figure 1; $p=0.0339$).

Gm9782/NM_001270426 is a predicted pseudogene transcript encoded on Chromosome 3. Pseudogene Gm9782 was expressed at significantly lower levels in mTEC^{hi} lacking Aire than in wild-type mTEC^{hi} (Figure 1; $p=0.0158$).

ENSMUST00000070435 is a pseudogene transcript encoded on Chromosome 10 and also known as fatty acid binding protein 3, muscle and heart. Pseudogene transcript ENSMUST00000070435 was expressed at significantly lower levels in Aire^{-/-} mTEC^{hi} than in Aire^{+/+} mTEC^{hi} (Figure 1; $p=0.0487$).

NR_003647/Gm5039, encoded on Chromosome 12, is a 1641 bp lncRNA and one of the most differentially expressed genes in mTEC^{hi} lacking Aire. lncRNA

NR_003647/Gm5039 was expressed at significantly lower levels in *Aire* ^{-/-} mTEC^{hi} (Figure 2; p=0.0217).

Gm9999, encoded by a gene on Chromosome 7, was also one of the most differentially expressed transcripts between the transcriptomes of mTEC^{hi} and *Aire* ^{-/-} mTEC^{hi}. While MGI and Ensemble gene models have annotated this gene as protein-coding, NCBI has designated Gm9999 as a non-coding RNA. Gm9999 was expressed in significantly lower quantities in *Aire*-deleted mTEC^{hi} (Figure 2; p=0.0131).

Transcript NR_040392, or 1700063D05Rik, is a long non-coding RNA encoded by a gene on Chromosome 9. Data from the mouse ENCODE project indicates that lncRNA NR_040392 is expressed at highest levels in the testis and cerebellum. lncRNA NR_040392 was expressed at significantly lower levels in mTEC^{hi} lacking *Aire* (Figure 2; p=0.0211).

Gm5/NR_024513 is a 470 bp long non-coding RNA encoded on Chromosome 5. lncRNA Gm5/NR_024513 was expressed at significantly lower levels in *Aire* ^{-/-} mTEC^{hi} (Figure 2; p=0.0028).

ENSMUST00000147509, also known as GM15391, is a 257 basepair long intergenic non-coding RNA (lincRNA) and its gene, located on Chromosome 1 is one of the most differentially expressed when comparing the transcriptomes of mTEC^{hi} that do or do not express *Aire*. lincRNA ENSMUST00000147509/GM15391 is expressed at significantly higher levels in mTEC^{hi} in the absence of *Aire* (Figure 2; p=0.0390).

A930001A20Rik / AK019124 is a lncRNA encoded by a gene on Chromosome 3 and was among the most differentially expressed genes when comparing the global gene expression profiles of mTEC^{hi} sufficient and deficient for *Aire*. lincRNA

A930001A20Rik was expressed at significantly higher levels in *Aire*-deleted mTEC^{hi} (Figure 2; p=0.0357).

NR_033483, a 522 bp lncRNA also known as U90926, is encoded on Chromosome 5 and was one of the most differentially expressed genes when comparing the transcriptomes of *Aire* ^{-/-} and *Aire* ^{+/+} mTEC^{hi}, NCBI annotates NR_033483 as a putative TNF-resistance related protein mRNA. NR_033483 was expressed in *Aire* ^{-/-} mTEC^{hi} at lower levels than in *Aire*, and this decreased expression approached statistical significance (Figure 2; p=0.0514).

NR_046041, also known as GM11240, is a 759 base pair long non-coding RNA encoded on Chromosome 4. lncRNA NR_046041 was expressed at higher levels in *Aire* ^{-/-} mTEC^{hi} when compared to *Aire* ^{+/+} mTEC^{hi} and this increased expression trended towards statistical significance (Figure 2; p=0.0651).

NR_040328 is an 881 bp long-non coding RNA encoded by a gene located on Chromosome 18. lncRNA NR_040328 was among the genes most differentially expressed in *Aire*-deleted mTEC^{hi} and expressed at significantly higher levels in *Aire*-deleted mTEC^{hi} than in wild-type mTEC^{hi} (Figure 2; p=0.0123).

NR_003647/Gm5039, encoded by a gene located on Chromosome 12, is a 1641 bp lncRNA and one of the most differentially expressed genes in mTEC^{hi} lacking *Aire*. lncRNA NR_003647/Gm5039 was expressed at significantly lower levels in *Aire* ^{-/-} mTEC^{hi} when compared to wild-type mTEC^{hi} (Figure 2; p=0.0217).

ENSMUST00000083030 snRNA is a 105 bp small nuclear RNA (snRNA) encoded by a gene located on Chromosome 2. snRNA ENSMUST00000083030 was among the genes most differentially expressed in *Aire* ^{-/-} mTEC^{hi} when compared to

Aire ^{+/+} mTEC^{hi} and expressed at significantly higher levels in *Aire*^{-/-} mTEC^{hi} (Figure 3; p=0.0205).

snoRNA ENSMUST00000158627, or Gm25715-201 is a 125 bp small nucleolar RNA encoded by a gene on Chromosome 16. snoRNA ENSMUST00000158627/Gm25715 was expressed at significantly higher levels in mTEC^{hi} lacking *Aire* (Figure 3; p=0.0001).

snoRNA ENSMUST00000104164, encoded by a gene located on Chromosome 6, was also one of the most differentially expressed genes when comparing the transcriptomes of mTEC^{hi} with and without *Aire*. snoRNA ENSMUST00000104164 was expressed at significantly higher levels in *Aire*-deleted mTEC^{hi} (Figure 3; p=0.0197).

While only two snoRNA were identified among the genes whose expression was most differentially regulated in mTECs that lack *Aire*, the expression of both increased significantly after deletion of *Aire*. This might suggest that *Aire* restricts the expression of snoRNA in cells of the thymus that possess high expression of the class II major histocompatibility molecule (MHC Class II).

Antisense 2510003D118Rik corresponds to the opposite strand transcript from the ankyrin repeat and SOCS box-containing 17 gene located on Chromosome 3. Antisense 2510003D118Rik was expressed at significantly lower levels in *Aire*^{-/-} mTEC^{hi} (Figure 4; p=0.0152).

ENSMUST00000153522, also known as Gm15600, is encoded by a gene located on Chromosome 9 and measures 856 basepairs long. Antisense transcript ENSMUST00000153522/Gm15600 (or Gm15600-AS) was among the most differentially

expressed transcripts between *Aire* +/+ and *Aire* -/- mTEC^{hi}. Gm15600-AS was produced at significantly higher levels in *Aire* -/- mTEC^{hi} (Figure 4; p=0.0134).

Finally, ENSMUST00000138402, also known as Gm12089 is an antisense transcript encoded on Chromosome 11 and was among the genes most differentially expressed between mTEC^{hi} that possess a functional copy of the *Aire* gene and those that do not. Antisense Gm12089 was expressed at higher levels in *Aire* -/- mTEC^{hi} and this higher expression approached but did not reach statistical significance (Figure 4; p=0.0611).

Discussion

Tolerance occurs in the periphery and centrally; *Aire* is a critical molecule for the execution of central tolerance and loss of *Aire* function results in autoimmune disease in humans (1, 2). To understand the transcriptional effects of *Aire* on a global scale, I compared the transcriptomes of thymic medullary epithelial cells possessing or lacking *Aire* (1). I found that nineteen of the two hundred and fifty genes whose expression was most differentially regulated between mTECs deficient and sufficient for *Aire* were non-coding RNA transcripts. These included four pseudogenes, nine long non-coding RNA, three small nuclear or nucleolar RNA and three antisense RNA transcripts. These findings demonstrate that non-coding RNA are represented among the set of transcripts most differentially expressed in *Aire* -/- mTECs, and suggests that *Aire* is involved in the transcriptional control of non-coding RNAs. Future work is required to determine the role that non-coding RNA play (if any) in the establishment of tolerogenic mechanisms.

References

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Figure 1: Pseudogenes are among the transcripts most differentially expressed by mTECs deficient in *Aire*.

The mean RNA expression value of the specified non-coding transcripts in mTEC^{hi} and in *Aire*-deleted mTEC^{hi} (n=2, each) is depicted in graphical form with the error bars representing the standard deviation. The exact p-value of the comparison between the two mean RNA expression values, determined by an unpaired Welch's t-test, is indicated on each respective graph.

Figure 2: Long and long intergenic non-coding RNA are among the transcripts most differentially expressed by mTECs deficient in *Aire*.

The mean RNA expression value of the specified non-coding transcripts in mTEC^{hi} and in *Aire*-deleted mTEC^{hi} (n=2, each) is depicted in graphical form with the error bars representing the standard deviation. The exact p-value of the comparison between the two mean RNA expression values, determined by an unpaired Welch's t-test, is indicated on each respective graph.

Figure 3: Small nucleolar and small nuclear non-coding RNA are among the transcripts most differentially expressed by mTECs deficient in *Aire*.

The mean RNA expression value of the specified non-coding transcripts in mTEC^{hi} and in *Aire*-deleted mTEC^{hi} (n=2, each) is depicted in graphical form with the error bars representing the standard deviation. The exact p-value of the comparison between the two mean RNA expression values, determined by an unpaired Welch's t-test, is indicated on each respective graph.

Figure 4: Antisense non-coding RNA are among the transcripts most differentially expressed by mTECs deficient in *Aire*.

The mean RNA expression value of the specified non-coding transcripts in mTEC^{hi} and in *Aire*-deleted mTEC^{hi} (n=2, each) is depicted in graphical form with the error bars representing the standard deviation. The exact p-value of the comparison between the two mean RNA expression values, determined by an unpaired Welch's t-test, is indicated on each respective graph.