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MicroRNA 184 Regulates Expression of NFAT1 in Umbilical Cord Blood CD4$^+$ T-cells

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ABSTRACT

The reduced expression of NFAT1 protein in Umbilical Cord Blood (UCB)-derived $CD4^+$ T-cells and the corresponding reduction in inflammatory cytokine secretion following stimulation in part underlies their phenotypic differences from adult blood (AB) $CD4^+$ T-cells. This muted response may contribute to the lower incidence and severity of high-grade acute graft-versus-host disease (aGVHD) exhibited by UCB grafts. Here we provide evidence that a specific microRNA, miR-184, inhibits NFAT1 protein expression elicited by UCB $CD4^+$ T-cells. Endogenous expression of miR-184 in UCB is 58.4-fold higher compared to AB $CD4^+$ T-cells, and miR-184 blocks production of NFAT1 protein through its complementary target sequence on the *NFATc2* mRNA without transcript degradation. Furthermore, its negative effects on NFAT1 protein and downstream IL-2 transcription are reversed through antisense blocking in UCB and can be replicated via exogenous transfection of precursor miR-184 into AB $CD4^+$ T-cells. Our findings reveal a previously uncharacterized role for miR-184 in UCB $CD4^+$ T-cells and a novel function for microRNA in the early adaptive immune response.

INTRODUCTION

Numerous reports spanning two decades have confirmed umbilical cord blood (UCB) as a clinical source of hematopoietic progenitors for allogeneic transplantation in the treatment of hematologic malignancies[1,2]. Despite the primary drawback of slower kinetics of myeloid engraftment as a result of limited graft cell dose[3], UCB has several advantages over bone marrow (BM) in a therapeutic setting, particularly the observed lowered incidence of acute Graft-Versus-Host Disease (aGVHD) despite the infusion of HLA disparate grafts[4]. aGVHD remains a major obstacle to the broader application of allogeneic stem cell therapy and is characterized by donor $CD4^+$ T-cell activation in response to self antigen presented by Class II MHC on host antigen presenting cells (APC). The clinical manifestation of aGVHD closely mimics the pathophysiology of autoimmune disorders with early secretion of pro-inflammatory cytokines including IFNγ, TNFα[5], GM-CSF, and IL-1, as well as later secretion of IL-2 by donor-derived T-cells. These stimulate inflammatory cell proliferation, upregulate MHC expression, NK and cytotoxic $CD8^+$ T-cell recruitment, and widespread tissue damage particularly in the skin, large intestine, and liver[6,7].

A key transcription factor in $CD4^+$ T-cell activation and the downstream target of Cyclosporine A (CsA) treatment, Nuclear Factor of Activated T-cells 1 (NFAT1, mRNA: *NFATc2*) influences the expression of a wide array of cytokines[8], surface receptors[9], and cell cycle regulators[10] associated with normal and auto-immune responses[11]. Tandem interactions with other transcription factors, particularly the AP1 (fos/jun) complex occur at adjacent DNA binding sites (WGGAAAWN for NFAT and TGAGTCA for AP1) located in the promoter regions of the genes encoding such factors as IFNγ, TNFα., IL-2, IL-4, IL-5, CTLA-4, GM-CSF, and CD40L. Expression of many of these genes, specifically those associated with a Th2 or allergic response, is not severely diminished (and in some cases enhanced) in NFAT1-null mice, suggesting some level of redundancy among members of the NFAT family and their binding

partners[12]. However, NFAT1 has been shown to be required for the sustained production of IFN-γ[13], GM-CSF, IL-3, IL-4, IL-2 (with AP1)[8,14], and TNF-α[8], indicating a critical role for NFAT1 in the initiation of a productive Th1 immune response. In the absence of AP1, NFAT1 heterodimers may activate an alternate, anergic repertoire of T-cell gene expression[15,16].

Previous work by our laboratory has demonstrated significantly reduced NFAT1 protein expression in resting and stimulated UCB $CD4^+$ T-cells compared to adult blood (AB)[17]. This observation correlates with the severe reduction in IFNγ, TNFα, and other cytokine production by UCB after primary stimulation. Microarray gene expression profiling revealed markedly lower expression of nearly all NFAT1-associated transcripts, but importantly, not the mRNA for NFAT1 (*NFATc2*) itself. Notably, UCB $CD4^+$ T-cells express lower levels mRNA encoding GM-CSF, IFNγ, TNFα, IL-3, IL-4, IL-5, IL-13, MIP-1α as well as the inflammatory surface markers CD40L, CTLA-4, and the IL-2 receptor alpha chain (CD25)[18]. As secretion of the aforementioned cytokines is strongly associated with the non-self recognition of recipient APC by donor $CD4^+$ T-cells in aGVHD, the mechanisms by which expression of these factors is restrained in UCB may underlie peripheral tolerance exhibited by UCB $CD4^+$ T-cells and further the identification of risk factors or prophylactic treatments relevant to allogeneic transplantation. Following in these observations, our studies have led us to implicate a specific microRNA in the mechanism underlying restrained NFAT1 protein expression in UCB $CD4^+$ graft T-cells.

microRNA (miRNA) comprises a specialized subset of small cytoplasmic non-coding RNAs between 19-24 nucleotides in length. These highly-processed RNAs most frequently bind to regulatory sequences in the 3' untranslated region (UTR) of target mRNAs thus blocking gene expression by mediating mRNA degradation or translational repression. The latter scenario is thought to involve the RNA-induced silencing complex (RISC) and accumulation in translation-deficient but non-degradative complexes known as P-bodies[19]. mRNAs sequestered into these complexes can be later re-targeted to translation initiation complexes or degraded through the

decapping (Dcp) pathway[20], a choice thought to be in part dependent on sequence complementarity, RNA secondary structure, and other signals. The activity of specific miRNAs has been associated with a wide variety of cellular differentiation pathways including hematopoiesis (reviewed in [21]) and disease states such as cancer, diabetes[22,23], and neurodegenerative diseases[24,25]. Notably, expression of miR-155 has been implicated in the regulation of mature T-cell lineage fate through c-Maf[26,27] and is associated with FoxP3-mediated upregulation in T_{reg} cells[28].

Here we provide evidence that a specific microRNA, miR-184, appears to contribute to the reduced NFAT1 protein in UCB-derived $CD4^+$ T-cells. Expression of miR-184 is increased in human UCB $CD4^+$ T-cells as compared to AB controls, and specifically represses activity in luciferase reporter assays. A converse relationship is observed between miR-184 activity and NFAT1 protein expression in both antisense interference and miRNA precursor "knock-in" models. This study comprises the first known demonstration of miR-184 expression and activity in primary human lymphocytes and suggests a novel role for cellular RNA interference in regulating the $CD4^+$ T-cell immune response in UCB.

MATERIALS AND METHODS

Cell isolation and culture. Acquisition of both AB and UCB units conformed to and was approved under University Hospital Case Medical Center IRB protocol nos. 02-00-34/CASE 11Z05 (rev. 11/13/07). Whole blood was obtained from umbilical cords immediately after delivery or by venipuncture from healthy adult donors and informed consent was obtained in accordance with the Declaration of Helsinki. Mononuclear cells were isolated following centrifugation through Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) per manufacturer's instructions. $CD4^+$ T-cells were then isolated via magnetic bead-labeling and separation (AutoMACS, Miltenyi Biotec, Auburn, CA) by first depleting the sample of $CD14^+$ monocytes and then positively selecting for $CD4^+$ cells, per manufacturer's recommendations. $CD45RA^+$ naïve T-cells were isolated via the Naïve Human T-cell Isolation kit (Miltenyi). Purity as measured by flow cytometry was >90%. $CD4^+$ T-cells were cultured in RPMI 1640 medium containing 10% Fetal Bovine Serum (Gibco, Carlsbad, CA) supplemented with 2mM L-glutamine (Gibco). Where applicable, T-cells were stimulated in wells containing 1μg/mL plate-bound αCD3 antibody and 5μg/mL soluble αCD28 antibody (BD Biosciences, San Jose, CA).

Western Blot. Cells were pelleted and lysed with radio-immunoprecipitation assay (RIPA) buffer. Following centrifugation, lysate supernatants were assayed for total protein content by modified Bradford assay (BioRad, Hercules, CA) per manufacturer's instructions. The lysates were standardized for protein concentration, diluted with 4X SDS loading buffer containing β-mercaptoethanol and heated to 95°C for 5 min. Equal volumes of samples were loaded into the wells of a 7.5% polyacrylamide SDS gel and run per standard protocol. The gel was then transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA) via standard wet-transfer protocol. Membrane was blocked with 5% dry milk, cut, and probed with primary antibodies to NFAT1 (BD Transduction Laboratories, San Jose, CA, #610703), β-Actin (Sigma Aldrich, St. Louis, MO, #A5441), and HRP-conjugated α-mouse secondary antibody (Sigma,

#A9044) per standard procedures. Bands were illuminated by ECL-Plus Visualization System (GE Healthcare) and exposed to film per standard procedure and digitized. Protein expression was quantified by integration of the relevant band intensity with Image J software (NIH, Bethesda MD) and normalized to β-actin control.

miRNA candidate determination. The Sanger miRBase Targets v5 database was queried as described through the MicroCosm interface located online at http://microrna.sanger.ac.uk/ .[29] Complete computational protocol details are available therein and in the references. Briefly, this system uses the miRanda algorithm[30] to determine and score sites of complementarity between mRNA 3' UTR sequences and known human miRNA species. Predicted interactions are favored which exhibit a high degree of complementarity at the 5' end of the miRNA, predicted thermodynamic stability by the Vienna RNA folding routines[31], and occur in UTR sequences conserved across multiple species.

PCR and Luciferase vector construction. RNA was obtained from UCB MNC or isolated $CD4^+$ T-cells with the PureLink Micro-to-Midi RNA Isolation kit (Invitrogen, Carlsbad, CA) and then DNase treated (DNA-*free*™, Ambion, Austin, TX). Following DNase removal, cDNA was generated from RNA as described below for RT-PCR and subjected to 30 rounds of PCR per standard protocol with the following primers to verify the presence of the predicted targeted sequence: 5'-TTACTATTTGGACGGAACACC-3' (Reverse, both reactions), 5'-TATGAAACAGAATGACTGTGATC-3' (Forward, NM_012340 reaction), and 5'-CTACTTGGATGATGTTAATGAAAT-3' (Forward, NM_173091 reaction). These PCR reactions additionally generated 3' UTR sequences containing the full-length intervening sequence between the stop codon and the predicted miR-184 interacting site. This sequence (NM_012340 reaction only) was cloned into the pMIR-Report plasmid at the 3' UTR position following the luciferase gene per manufacturer's instructions (Ambion). Additionally, short (38-mer) synthesized DNA sequences matching the plus and minus-strand including and surrounding the

predicted targeted site were dimerized and cloned into the same vector. "Control" denotes pMIR-Report without either insert. Sequences of each vector are shown in Figure S1.

CD4$^+$ Cell Transfection. CD4$^+$ T-cells were transfected with luciferase vectors, synthetic precursor miRNA (Ambion, #17000/17010), and/or antisense miRNA inhibitor (Ambion, #17100/17110) via Amaxa Nucleofector (Amaxa, Gaithersburg, MD) per manufacturer's protocol for Unstimulated Human T-cells provided in the Human T-cell kit (#VPA-1002) using program U-14. Approximately 1µg plasmid or DNA sequence was transfected per $1x10^6$ cells. Typical efficiencies in control GFP plasmid transfections were approximately 50%.

Luciferase assay. Cells were transfected with the aforementioned constructs alongside a consistent quantity of pGL4.71[hRlucP] plasmid (Promega, Madison, WI) to control for transfection efficiency. Cells were lysed and prepared per manufacturer's instructions (Promega, Dual-Luciferase Reporter Assay System #E1910) and Luciferase activity was measured via fluorescence on a BMG Labtech (Durham NC) NOVOStar plate reader.

Quantitative RT-PCR. cDNA was generated from whole cellular RNA by MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) per manufacturers' instructions. TaqMan® RT-PCR assays were prepared per manufacturer's instructions (Applied Biosystems) utilizing probes for either *NFATc2* (#Hs00234855_m1) or *IL2* (Hs00174114_m1). Endogenous control for all relative mRNA quantifications was GAPDH (#Hs99999905_m1) and reactions were run in at least triplicate per experiment. Quantitative RT-PCR (qRT-PCR) was carried out on an Applied Biosystems 7500 thermocycler per manufacturer's instructions. Relative expression was quantified from the amplification curves using the 7500 Fast System Software. For relative mature miRNA quantification, small RNA was enriched with the Mission® Small RNA Isolation Kit (Sigma) and qRT-PCR carried out using the TaqMan miRNA Reverse Transcription kit

(Applied Biosystems) and primers for hsa-miR-184 (#4373113) or U47 snoRNA control (#4380911) and run per manufacturer's instructions[32].

Statistical analysis. qRT-PCR error bars represent standard error of the mean (s.e.m.) assuming a 95% confidence interval. Luciferase error bars represent sample standard deviation (S.D.) of the representative experiment. Quantified Western blot error bars represent s.e.m. for the stated number of experiments and p-values were calculated by Student's *t*-test and one-way ANOVA analysis.

RESULTS

UCB CD4$^+$ T-cells express significantly less NFAT1 protein but not mRNA compared to AB CD4$^+$ T-cells

To determine the nature of the mechanism underlying reduced NFAT1 protein expression in UCB CD4$^+$ T-cells, an *in vitro* stimulation time course of UCB and AB CD4$^+$ T-cells was performed and expression of NFAT1 protein and its mRNA transcript were compared. Western blot comparing NFAT1 protein expression in UCB CD4$^+$ T-cells versus AB during primary stimulation confirmed reduced baseline expression and attenuated upregulation in UCB. This discrepancy is evident throughout 48h of αCD3/αCD28 stimulation *in vitro* (Figure 1A). *NFATc2* transcript levels from multiple donors were measured by quantitative real-time PCR (qRT-PCR) as described and compared between UCB and AB samples (Figure 1B). These findings reveal only modest differences in relative *NFATc2* mRNA quantity which are insufficient to account for the NFAT1 protein expression discrepancy in UCB versus AB CD4$^+$ T cells.

Due to the dramatic differences in protein expression without significant corresponding differences in mRNA quantity, potential mechanisms of NFAT1 post-transcriptional regulation were investigated. Through proteasome inhibition and cellular fractionation experiments translocation of the *NFATc2* mRNA into polysomes by UCB CD4$^+$ cells was observed to lag behind AB CD4$^+$ T-cells by at least 6h during *in vitro* stimulation (Figure S2). Because similar effects have been observed in other confirmed miRNA-mRNA interractions[33,34], and our previous microarray studies had failed to reveal significant differences in translational initiation or elongation factors we focused our subsequent work on identifying potential miRNA species that could specifically affect the translation of NFAT1 in UCB CD4$^+$ T-cells.

miR-184 is predicted to interact strongly with the 3' UTR of the *NFATc2* mRNA

A search to determine specific miRNAs that may contribute to the observed differences in UCB $CD4^+$ T-cell NFAT1 protein expression was conducted. Putative miRNA regulators were determined by querying the Sanger MicroCosm resource and miRBase targets registry. Many miRNA sequences predicted to bind to the 3' UTR of NFAT1 were identified by this computational analysis (highest-scoring candidates shown in Table 1). Of the 58 and 35 predicted micro-RNA binders (for each transcript variant, NCBI accession numbers NM_012340 and NM_173091, respectively) identified by this query, the strongest predicted binder to the 3' UTR (both variants) was miR-184, a recently characterized miRNA present in a variety of tissues[35] and suggested to be of importance in DNA methylation pathways[36] and a potential antagonist of miR-205[37]. Conversely, the strongest predicted mRNA target of miR-184 (based on a reciprocal analysis of the miRNA sequence) was the previously identified sequence within the NFAT1 3' UTR (Table 2). The NM_173091 variant lacks a 3' exon containing the NM_012340 stop codon, but both variants show full homology downstream of that region. The complementary miR-184/*NFATc2* sequences are diagrammed in Figure 2, with the predicted interaction occurring 399 and 299 nucleotides downstream of the stop codons respectively.

miR-184 is more highly expressed in UCB than in AB $CD4^+$ and decreases through early stimulation timepoints

The expression of miR-184 in unstimulated UCB $CD4^+$ T-cells was on average quantified 58.4 times higher than in AB $CD4^+$ T-cells by qRT-PCR (Figure 3A). Notably, neonatal $CD4^+$ T-cells are known to contain a higher proportion of naïve recent thymic emigrants than AB[38], although NFAT1 expression is lacking in both RA^+ and RO^+ subsets UCB and not expressed differently in either subset in AB[17]. The expression of miR-184 was observed to be much more highly skewed towards the naïve ($CD45RA^+$) $CD4^+$ subset in AB than in UCB (Figure S3).

The expression of miR-184 over a time course of *in vitro* simulation was also measured (Figure 3B). Subjecting isolated and stimulated UCB $CD4^+$ T-cells to the same qRT-PCR assay at early timepoints revealed a modest decline in miR-184 quantity (40% of original by 6h). Later timepoints at which the eventual upregulation of NFAT1 protein expression is observable in UCB, but still dramatically lower compared to AB (Figure 1A) exhibit an eventual rebound in miRNA expression by 16h and modest upregulation through 48h. Conversely, detectable miR-184 expression in AB $CD4^+$ cells is observed to drop dramatically over the same timepoints following stimulation. (Figure 3C).

miR-184 affects protein expression through its predicted binding site on the *NFATc2* mRNA

To confirm the presence of the predicted binding site in the *NFATc2* transcript, whole cell mRNA was isolated from UCB MNC and selected $CD4^+$ T-cells, transcribed into cDNA as described in the methods for qRT-PCR, and subjected to PCR using primers directed to sites adjacent to the stop codons and overlapping the 3' end of the predicted binding sequence (Figure 4A). The gel bands verify the actual presence of the predicted target site within the 3' UTR of both transcript variants. Complete sequencing of the insertions within our generated luciferase vectors further confirms this observation (Figure S1).

To determine whether miR-184 indeed interacts with the corresponding *NFATc2* sequence as predicted (Figure 2), expression vectors designed to transcribe a luciferase-encoding mRNA containing either a short synthetically-prepared sequence matching only the predicted miR-184 binding site from *NFATc2* ("184-only") or the *NFATc2* (NM_012340 variant) 3' UTR through and including the aforementioned sequence ("cloned UTR") were constructed. The full-length *NFATc2* 3' UTR has not been fully cloned or sequenced, and we wished to avoid the effects of any possible variations in UTR length between samples or cell types. These vectors were

introduced into UCB (Figure 4B) and AB (Figure 4C) selected $CD4^+$ T-cells. Luciferase assays indicate only 38% and 60% expression compared to control when luciferase expression is influenced by the 184-only sequence and the cloned UTR, respectively (Figure 4B). This effect is almost completely reversed when a blocking antisense sequence to miR-184 is co-transfected. However in AB, the 184-only insertion has no effect on luciferase activity (middle column, white bar) while insertion of the cloned UTR results in a 58% reduction in expression (Figure 4C). Transfection of an exogenous precursor to miR-184 results in decreases of 61% and 23% respectively (middle bars). This effect is again attenuated when a blocking antisense sequence is co-transfected with the miR-184 precursor (rightmost bars). This data indicates that miR-184 does indeed modulate protein expression through its predicted binding site, and suggests significant endogenous miR-184 activity in UCB, but not AB $CD4^+$ T-cells.

To further confirm these observations, we mutated the "cloned UTR" luciferase expression vector insert used previously to disrupt either the seed region by removing the four 3'-most nucleotides from its predicted binding site (denoted "-seed region"), or the entire predicted binding site (denoted "-184 site"). Base-pair binding within the "seed" region has been shown to often be of importance to translational repression by miRNA[39]. We observed between a 4- and 3-fold induction, respectively, of luciferase expression in UCB $CD4^+$ cells when either the seed region or the miR-184 binding site was removed (Figure 4D). However, we only observed a comparatively slight (1.5-fold) increase in expression when the same vectors were transfected into AB $CD4^+$ cells (Figure 4E). Failure to observe a significant change in lucifierse expression with inhibition of miR-184 activity in UCB or introduction of miR-184 in AB (grey bars) confirmed the specificity of the observed effect.

miR-184 activity directly affects NFAT1 protein quantity in unstimulated $CD4^+$ T-cells

To determine whether endogenous miR-184 can directly repress NFAT1 protein expression in UCB $CD4^+$ T-cells, Western blot for NFAT1 was performed on unstimulated selected $CD4^+$ T-cells following transfection with either control or blocking antisense to miR-184 (Figure 5A, right). Band intensities were quantified and normalized to β-Actin and relative NFAT1 expression under the influence of each treatment was compared. Aggregate data (Figure 5A, left) reveals an 86% increase in NFAT1 protein expression when the cells were treated with antisense to miR-184. *NFATc2* mRNA levels (Figure 5B) were however unchanged between samples. Likewise, when unstimulated AB $CD4^+$ T-cells are transfected with a synthesized precursor to miR-184 (Figure 5C), NFAT1 protein levels as quantified by Western blot are reduced by approximately 31%. However, interference with miR-184 in AB $CD4^+$ cells failed to yield an observable change in NFAT1 protein expression (Figure 5D). This series of experiments indicates negative regulation of NFAT1 protein through the microRNA pathway by miR-184 in UCB $CD4^+$ T-cells and further suggests a non-degrading mechanism of action.

miR-184 activity conversely affects production of the NFAT-associated IL-2 transcript

To determine whether manipulation of NFAT1 protein levels through interference with the activity of miR-184 is sufficient to result in an increase in transcription of NFAT1-target genes, the transcription of Interleukin 2 (IL-2), a gene strongly activated by NFAT1 binding to its promoter following stimulation[14], was assayed by qRT-PCR. UCB $CD4^+$ cells were transfected with blocking antisense to miR-184, assayed for upregulated NFAT1 expression by Western blot (data included in Figure 5A) after 16h and then stimulated *in vitro* as described previously. Data reflects a significantly greater amount of IL-2 mRNA in the miR-184 antisense-treated samples by 6h of stimulation which is maintained through 16h (Figure 6A). Conversely, AB $CD4^+$ T-cells transfected with an exogenous miR-184 precursor exhibited dramatically reduced IL-2 transcription through the same stimulation timepoints (Figure 6B). These findings indicate that

miR-184 interference with NFAT1 in UCB $CD4^+$ T-cells is indeed sufficient to influence both NFAT1 protein levels and transcription of the known NFAT1 target gene IL-2.

DISCUSSION

Our findings comprise the first observation of miR-184 activity in immune cells and a characterization of its activity on a key transcriptional regulator of inflammation specifically known to exhibit decreased activity in UCB $CD4^+$ T-cells. We identified miR-184 as a strong predicted regulator of NFAT1 and confirmed its interaction with the observed complementary binding site within the *NFATc2* mRNA 3' UTR. UCB $CD4^+$ T-cells were shown to exhibit significantly more miR-184 RNA and miR-184-mediated repressive activity than AB $CD4^+$ T-cells. We additionally confirmed through blocking and gain-of-function analyses that manipulation of miR-184 is sufficient to influence NFAT1 protein as well as its known downstream target, IL-2.

miRNA regulation of various myeloid and lymphoid lineage differentiation steps, as well as key signaling components including transcription factors within specific immune cells has been recently described by multiple groups. Notable targets of miRNAs involved in lymphocyte activation include SHP-2 and multiple phosphatases associated with TCR signal transduction[40], TRAF6, IRAK1[41], and c-Myb[42]. Additional studies have revealed dramatic immune phenotypes in specific miRNA-knockout mice, such as impaired T-cell dependent antibody response and Th2 skewing[27]. Herein we present the first known evidence of NFAT targeting by a miRNA. Although the full implications of our findings with respect to immune cell function remain to be fully elucidated, particularly with respect to the naïve and immunotolerant phenotype exhibited by UCB lymphocytes and the associated clinical observations for allogeneic stem cell transplantation in humans, it is clear that an understanding of miRNA targeting mechanisms will further a comprehensive understanding of immune cell activation.

miR-184 was first observed by Lagos-Quintana et. al. in the murine eye[43] and later its expression was shown to be localized to basal and suprabasal regions of the corneal

epithelium[44]. miR-184 was later described in the human brain as being regulated by the methyl-CpG binding protein MeCP2[36]. More recent studies have linked its overexpression in squamous cell carcinoma of the tongue and regulation of c-Myc in those cell lines[45], suggesting that a dysregulation of miR-184 leading to its overexpression may be associated with an increase in cellular proliferation. This hypothesis would agree with our earlier findings of increased *in vitro* proliferation of UCB $CD4^+$ compared to AB $CD4^+$ cells in response to antigen, as well as reports suggesting reduced apoptosis in UCB $CD4^+$ T-cells in response to primary stimulation[46]. Yu et. al. have recently identified a role for miR-184 in antagonizing miR-205, leading to inhibition of blocking SHIP2 expression by miR-205[37], suggesting a possible pro-apoptotic role for miR-184 as well. However, the contribution of miR-184 to the proliferative phenotype observed in UCB $CD4^+$ cells, either through NFAT1 inhibition or other mechanisms, remains to be investigated.

The mechanism we describe for miR-184 regulation of NFAT1 in UCB $CD4^+$ T-cells suggests a relatively high sustained expression of the miRNA in the absence of stimulation. miR-184 is located on chromosome 15 and how miR-184 expression is regulated remains unclear, however earlier reports in neural tissue link its expression to the release of promoter-bound MeCP2 due to phosphorylation following polarization in mice[36]. Although DNA methylation patterns in the neonatal immune system are still largely unstudied, it is important to note that DNA methylation has been linked with the expression of many other miRNA species in humans[47-49]. As expected, we observed a decrease in levels of miR-184 relative to *NFATc2* mRNA at early timepoints following stimulation. Although this decrease is relatively modest, we observed a significant effect on both NFAT1 protein and downstream target gene expression following antisense blocking of miR-184. The mechanism underlying increased NFAT1 protein by 24-48h of stimulation in UCB in the midst of rebounding miR-184 expression at those timepoints remains an open question, although the relative increase in NFAT1 protein expression by these timepoints generally exceeds the relative observed increase in miR-184 expression and the

kinetics of protein upregulation are significantly delayed compared to AB $CD4^+$ cells. Data from sucrose-gradient fractionation experiments (Figure S2) do suggest *NFATc2* translocation to polysomal fractions by 16h. It is possible that miR-184 later assumes another function in the presence of NFAT1 protein and its downstream transcriptional targets, and it is likely that additional microRNAs predicted to interact with the *NFATc2* mRNA 3' UTR additionally play a role in these processes. Various groups have published data suggesting "rheostat" mechanisms for fine-tuning protein expression which are highly dependent on the expression of other miRNAs and other competitive mRNA targets over time (reviewed in [50]), resulting in dynamic changes in the relative regulatory function of each individual miRNA-mRNA interaction. Thus, it may be hypothesized that any one of a number of mechanisms may permit eventual upregulation of NFAT1 protein, even in the midst of sustained miR-184 expression in UCB $CD4^+$ cells. Multiple miRNA species are predicted to bind sites proximal to and overlapping with the miR-184 binding site, therefore a yet-to-be-identified miRNA may modulate the initiation of NFAT1 protein translation at later timepoints. In AB $CD4^+$ cells, it is foreseeable that the already higher expression of NFAT1 protein (Figure 1A) and the relatively lower expression of miR-184 at rest (Figure 3A) and its decrease in expression over stimulation (Figure 3C), combined with increasing expression of *NFATc2* mRNA at early stimulation timepoints (Figure 1B) may be sufficient to permit efficient NFAT1 translation without miR-184 exhibiting a major regulatory role. Future investigations into the expression of additional UCB-associated miRNAs may elucidate potential miRNA expression thresholds in those cells with consequences for NFAT1 protein expression. Additionally, although other pathways of post-transcriptional regulation have been implicated for NFAT1 in other cell lineages, specifically protein degradation through ubiquitin-mediated proteasome targeting in breast cancer cells[51], our work has not identified enhanced NFAT1 protein degradation to account for reduced expression in UCB $CD4^+$ T-cells.

We have not observed *NFATc2* transcript degradation mediated by miR-184. Translational regulation of mRNA transcripts through microRNA interference as an alternate mechanism to transcript degradation is a subject of growing interest and inquiry. Multiple mechanisms have been suggested, including blocking of the initiation factor eIF4E binding to the 5' m^7G cap[52], cap-independent blocking of initiation[53], and co-translational mechanisms[53-55] such as inhibition of ribosome recycling, elongation, favoring early termination, or influencing co-translational degradation of the nascent protein. Importantly, recent studies have also revealed that miRNA may also positively regulate translation under specific conditions[56], further suggesting multiple complex mechanisms by which miRNA may play a role in translation. It is clear that some of these mechanisms involve "pseudo-polysomes" and/or P-bodies, sites of reversible sequestration of targeted transcripts in association with miRNA and other ribonucleoprotein complexes[19]. In light of these complex and sometimes disparate observations, our studies provide insight into the contribution of the specific miR-184/*NFATc2* binding site on its own as well as in the context of the preceding UTR sequence. As is evident from our luciferase assays and other studies, the full 3' UTR sequence likely contains multiple features which positively and negatively influence protein expression at rest.

Additional predicted miR-184-interacting transcripts include the transcription factor LMO1 associated with T-cell leukemia and the extensively characterized oncogene MPL, as well as a host of genes not yet characterized in immune cells but in some cases not tightly restricted to particular cell lineages (Table 2). We have observed MPL to be expressed significantly lower in UCB $CD4^+$ cells compared to AB by microarray (unpublished data); however, due to the complex and poorly-understood nature of degradative versus non-degradative mechanisms involved in miRNA/mRNA interactions it is clear that analyses based on simple mRNA quantification may fail to elucidate the true role of miRNA. It is often assumed that weaker complementarity in general results in a less-degradative fate for the mRNA, but it is unlikely that

a discrete binding affinity threshold exists for transcript degradation across miRNAs, transcripts and cell types.

As specific miRNAs and miRNA families are currently being widely proposed as biomarkers for various cancers, future implications for this work may include graft selection, *ex vivo* graft manipulation and targeted therapy of hematologic malignancies. Pharmacological targeting of NFAT1 as a key regulator of the autoimmune/inflammatory response is well-described: CsA and FK506 (tacrolimus), which separately disrupt the calcineurin/calmodulin signaling interaction preventing NFAT1 nuclear translocation, have been used as therapeutic prophylaxis and treatment for aGVHD for decades, However, these treatments exhibit toxic calcineurin/NFAT-independent side effects, particularly in the nephritic and circulatory systems in addition to the traditional infection considerations of immunosuppressive therapy. GVHD still remains the major limitation to successful allogeneic transplantation, and directed molecular T-cell targeting therapies could potentially alleviate some significant toxicities of pharmacologic immunosuppressive calcineurin inhibitors.

In summary, miR-184 is capable of regulating NFAT1 protein expression without causing transcript degradation through its predicted complementary binding site within the *NFATc2* mRNA 3' UTR. This process in UCB $CD4^+$ T-cells may comprise one mechanism underlying the relatively low levels of expressed NFAT1 protein compared with AB $CD4^+$ cells at rest and early stimulation timepoints, resulting in their characteristically lower expression of pro-inflammatory cytokines upon activation. We have observed that UCB $CD4^+$ T-cells endogenously express significantly greater amounts of miR-184, and our studies have elucidated a previously uncharacterized role for miR-184 in the early adaptive immune response. Taken together, we have identified a key molecular difference between $CD4^+$ T-lymphocytes derived from adults and neonates with potential implications for our further understanding of autoimmunity and GVHD.

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AUTHOR CONTRIBUTIONS

R.P.W. designed and performed the experiments, analyzed data and wrote the manuscript; M.L.L. and P.H. analyzed data and provided vital reagents; S.K. designed experiments and helped write the manuscript; P.L. designed experiments and provided vital reagents; N.J.G and M.J.L. secured funding, designed experiments, supervised the research, and helped write the manuscript.

CONFLICT OF INTEREST DISCLOSURE

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FIGURE CAPTIONS

Figure 1: Relative NFAT1 protein and mRNA expression in stimulated UCB and AB $CD4^+$ T-cells. (A) Approximately $2x10^6$ isolated UCB and AB $CD4^+$ T-cells were stimulated *in vitro* as described for each of the designated timepoints and Western blotted. Data representative of five independent experiments. (B) *NFATc2* (NFAT1-encoding) mRNA was assayed in stimulated UCB and AB $CD4^+$ T-cells by qRT-PCR as described and normalized to UCB at 0h. Each bar indicates mean +/- s.e.m. of 2-5 independent data points.

Figure 2: Diagram of the predicted *NFATc2* 3' UTR/hsa-miR-184 interaction. The sequence of miR-184 was retrieved from the Sanger database and the sequences for *NFATc2* were retrieved from NCBI Entrez Nucleotide Sequence Viewer. Diagram indicates position of the stop codon relative to the first indexed base of each transcript and the position of the predicted 3' UTR base pairing region relative to the stop codon.

Figure 3: miR-184 in UCB $CD4^+$ T-cells. (A) miR-184 was quantified in AB (n=8) and UCB (n=10) samples by qRT-PCR as described. P-value obtained from unpaired, two-tailed Student's T-test. (B) miR-184 was quantified in UCB $CD4^+$ T-cells stimulated *in vitro* as described for the designated timepoints (representative of 3 experiments). (C) miR-184 was quantified in AB $CD4^+$ T-cells stimulated *in vitro* as described for the designated timepoints (representative of 2 experiments)

Figure 4: Interaction between the *NFATc2* 3' UTR and miR-184. (A) PCR amplification of the predicted target region from UCB MNC and $CD4^+$ cDNA. Sequences of primers are specified in Materials and Methods. (B) Expression of luciferase in transfected UCB $CD4^+$ cells under the influence of minimal 3' UTR (left column), the predicted miR-184 binding site from *NFATc2* (middle column), or the cloned 3' UTR from *NFATc2* (right column) with antisense sequence to miR-184 or irrelevant control DNA sequence. (representative of 3 independent experiments) (C) Expression of luciferase in transfected AB $CD4^+$ cells under the influence of the aforementioned 3' UTRs, exogenous pre-miR-184, and antisense to miR-184. (representative of 3 independent experiments) N.D. denotes data not determined. (D)

Expression of luciferase in transfected UCB $CD4^+$ cells under the influence of the cloned *NFATc2* 3' UTR (left column), the same UTR with the predicted miR-184 seed region (4nt) removed (middle column), and the same UTR with the entire predicted miR-184 binding site removed (right column) with and without antisense to miR-184. (representative of 2 independent experiments). (E) Expression of luciferase in transfected AB $CD4^+$ cells under the influence of the aforementioned 3' UTRs, with and without precursor to miR-184. (representative of 2 independent experiments)

Figure 5: miR-184 negatively effects NFAT1 protein synthesis. (A) Quantification and representative blot of NFAT1 protein expression in UCB $CD4^+$ T-cells 16 hours following transfection with antisense to miR-184. (n=3). (B) qRT-PCR analysis of samples in (a), confirming no significant change in *NFATc2* mRNA quantity. (C) Quantification and representative blot of NFAT1 protein expression in AB $CD4^+$ T-cells under the following transfection with pre-miR-184. (n=4). Blot bands were quantified using ImageJ software. (D) Western blot of NFAT1 protein expression in AB $CD4^+$ T-cells 16 hours following transfection with antisense to miR-184.

Figure 6: (A) *IL2* transcription in stimulated UCB $CD4^+$ T-cells under the influence of miR-184 antisense. (B) *IL2* transcription in stimulated AB $CD4^+$ T-cells following transfection of exogenous miR-184 precuror. Cells were stimulated *in vitro* 16 hours post-transfection and *IL2* mRNA was assayed by qRT-PCR as described from $1x10^6$ cells per data point. Data representative of two independent experiments.

Table 1: micro-RNAs predicted to interact with the 3' UTR of each *NFATc2* transcript. The Sanger Micro-RNA database was queried for the *NFATc2* transcript as described. Table reflects all miRNAs scoring higher than 17.0. Last accessed July 15, 2008.

Table 2: Genes predicted to interact with miR-184 in human. The Sanger Micro-RNA database was queried for human transcripts with predicted complementary to miR-184. Table reflects the top twenty results with duplicates removed. Last accessed July 15, 2008.

Table 1: Predicted NFATc2-interracting miRNAs

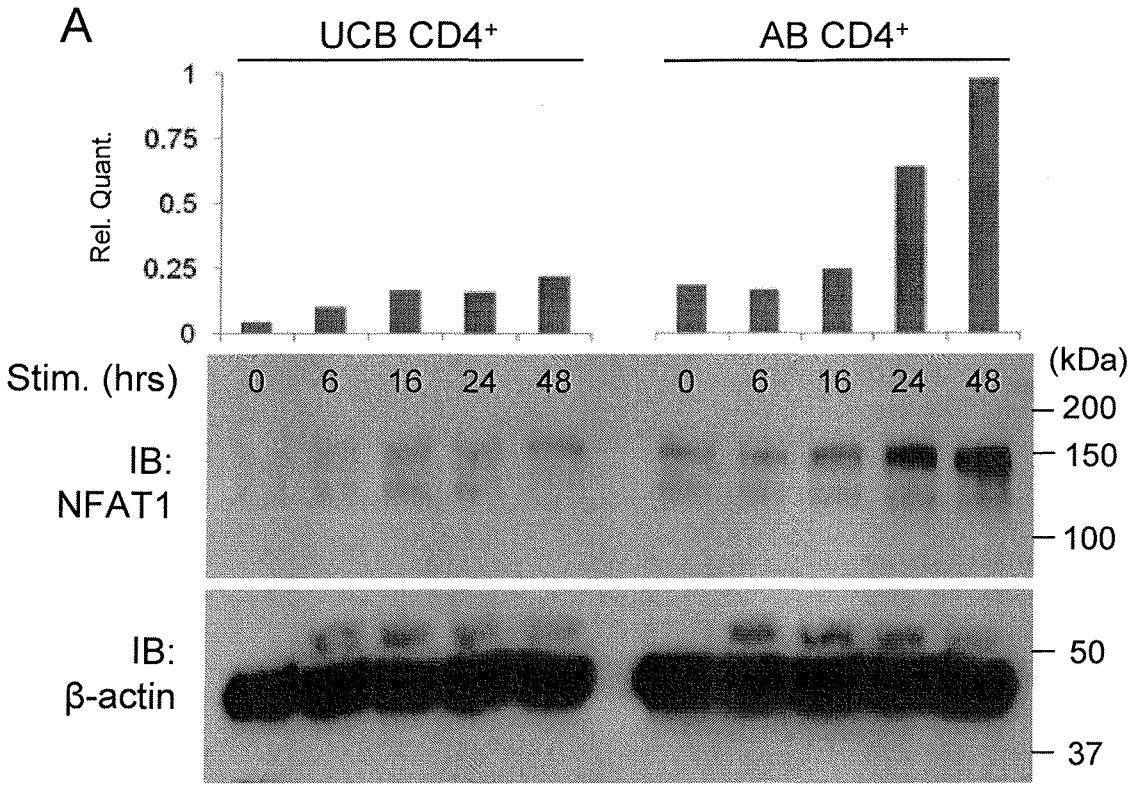
Transcript Variant (NCBI Acc. No.)

NM_012340						NM_173091					
micro-RNA ID	***Score***	***Energy***	***Base P***	***Start***	***End***	***micro-RNA ID***	***Score***	***Energy***	***Base P***	***Start***	***End***
hsa-miR-184	19.7351	-25.74	6.81E-04	329	350	hsa-miR-184	20.1255	-25.74	4.53E-04	229	250
hsa-miR-135a	18.3064	-14.69	7.16E-03	89	111	hsa-miR-494	18.6473	-14.34	6.44E-03	185	206
hsa-miR-494	18.2857	-14.34	8.75E-03	285	306	hsa-miR-765	18.5179	-25.71	1.69E-02	59	79
hsa-miR-765	18.1568	-25.71	2.32E-02	159	179	hsa-miR-23a	18.1925	-26.38	1.51E-02	29	50
hsa-miR-23a	17.8397	-26.38	2.06E-02	129	150	hsa-miR-30b	18.0788	-23.31	2.43E-02	73	94
hsa-miR-30b	17.7282	-23.31	3.38E-02	173	194	hsa-miR-29c	17.9651	-17.7	7.70E-03	224	244
hsa-miR-29c	17.6167	-17.7	1.05E-02	324	344	hsa-miR-29a	17.9651	-17.64	7.41E-03	224	244
hsa-miR-29a	17.6167	-17.64	1.01E-02	324	344	hsa-miR-342-5p	17.9463	-20.89	8.91E-03	205	225
hsa-miR-342-5p	17.5964	-20.89	1.27E-02	305	325	hsa-miR-30c-2	17.624	-17.22	1.91E-02	73	94
hsa-miR-135b	17.4188	-13.41	1.57E-02	89	111	hsa-miR-30c-1	17.624	-18.77	2.22E-02	73	94
hsa-miR-30c-1	17.2822	-18.77	3.15E-02	173	194	hsa-miR-29b	17.4222	-16.53	1.37E-02	223	244
hsa-miR-30c-2	17.2822	-17.22	2.72E-02	173	194	hsa-miR-21	17.3748	-14.3	1.06E-02	223	244
hsa-miR-302b	17.1707	-9.66	1.71E-02	24	45	hsa-miR-801	17.3388	-26.53	7.57E-03	96	119
hsa-miR-302d	17.1707	-13.13	1.34E-02	24	45	hsa-miR-452	17.2829	-13.26	1.75E-02	236	257
hsa-miR-29b	17.086	-16.53	1.85E-02	323	344	hsa-miR-369-5p	17.2829	-17.61	2.56E-03	212	233
hsa-miR-29a	17.0592	-16.65	3.67E-02	74	95	hsa-miR-135a	17.196	-11.51	2.24E-02	1	11
hsa-miR-21	17.036	-14.3	1.45E-02	323	344	hsa-miR-330-5p	17.1692	-19.06	4.48E-02	195	217
hsa-miR-801	17.0057	-26.53	1.09E-02	196	219						

Table 2: Predicted miR-184 mRNA Binding Partners

mRNA	*Score*	*P Value*
NFATC2	20.1255	4.53E-04
SMPDL3B	19.557	8.20E-04
GPBAR1	19.1021	1.05E-03
LMO1	19.0877	1.34E-03
MPL	18.9884	1.48E-03
PSMA4	18.6473	1.19E-04
ABO	18.5336	2.38E-03
THOP1	18.3249	5.10E-05
TPM3	18.1925	3.40E-03
GAS6	18.1856	3.42E-03
ANKRD54	18.1508	3.55E-03
CXYorf3	18.1496	8.49E-04
TCEAL4	18.0965	3.75E-03
GAS6	18.0921	3.77E-03
PZP	18.0788	3.82E-03
C20orf196	18.056	3.92E-03
SIDT2	17.997	3.20E-05
ZBED3	17.9705	4.28E-03
FAM72B	17.9651	4.30E-03
TFF3	17.9651	6.29E-04

Figure 1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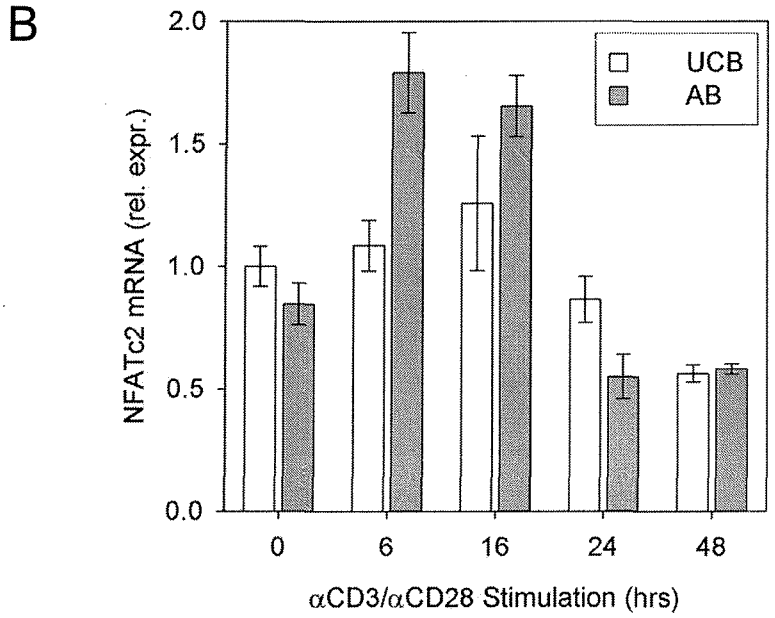
AB CD4+
Rel. Quant.
1
0.75
0.5
0.25
0
Stim. (hrs)
0 6 16 24 48
0 6 16 24 48
(kDa)
200
150
100
IB:
NFAT1
IB:
β-actin
50
37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2.0
1.5
1.0
0.5
0.0
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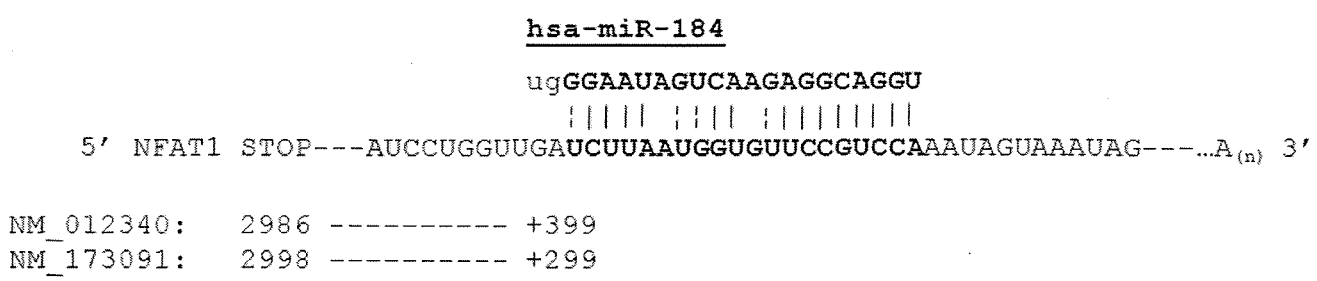
UCB
AB
0
6
16
24
48
αCD3/αCD28 Stimulation (hrs)

Figure 2



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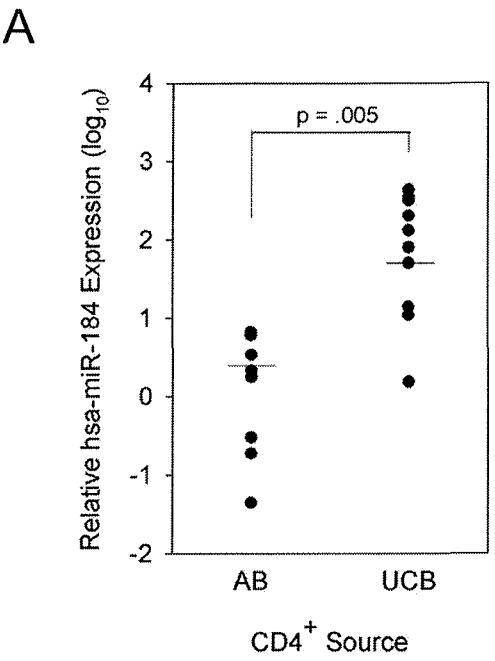
NM_173091: 2998 ---------- +299

Figure 3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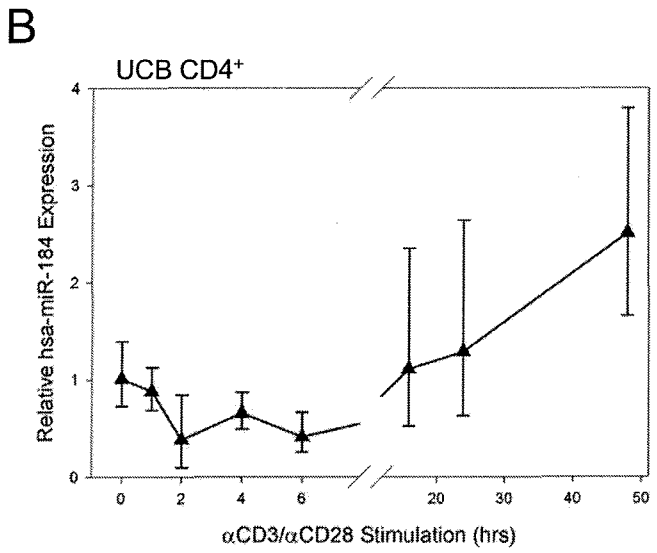
3
2
1
0
-1
-2
AB
UCB
$CD4^+$ Source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UCB CD4+
Relative hsa-miR-184 Expression
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3
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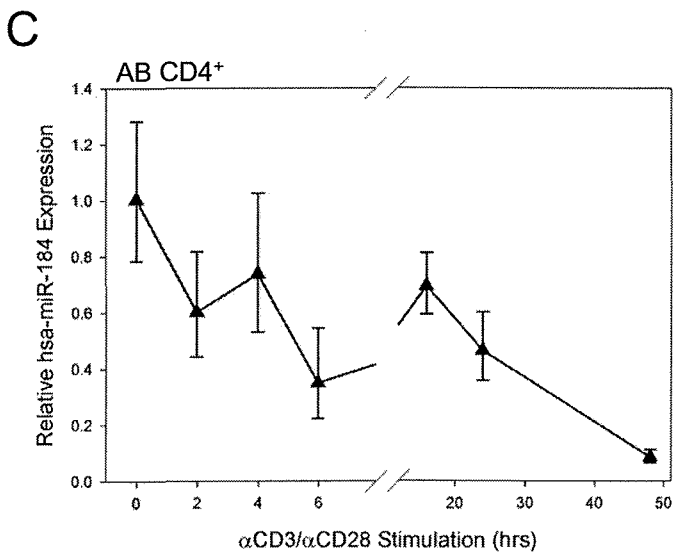
6
20
30
40
50
αCD3/αCD28 Stimulation (hrs)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AB CD4+
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4
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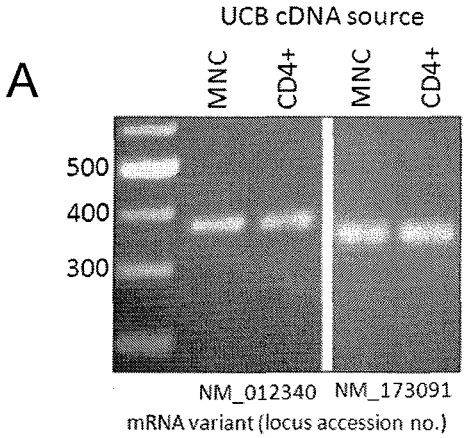
20
30
40
50
αCD3/αCD28 Stimulation (hrs)

Figure 4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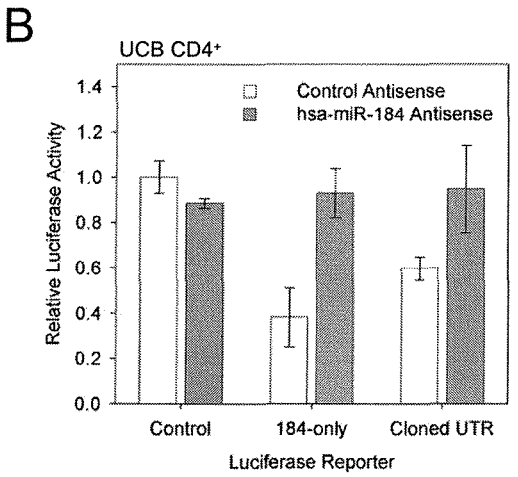
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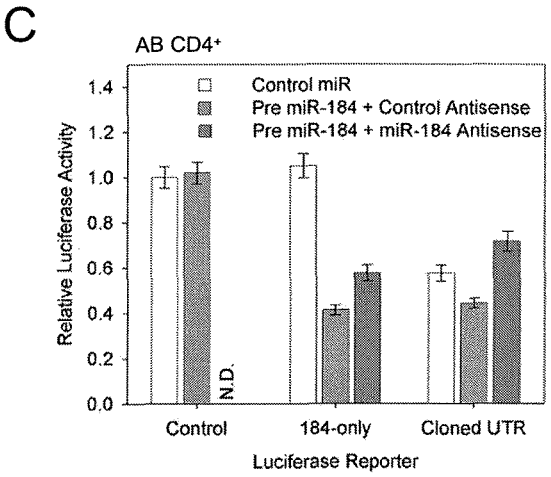
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AB CD4+
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Pre miR-184 + miR-184 Antisense
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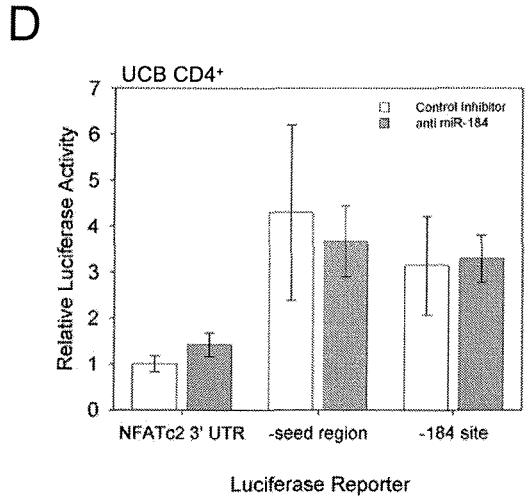
Cloned UTR
Luciferase Reporter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Control Inhibitor
anti miR-184
Relative Luciferase Activity
NFATc2 3' UTR
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-184 site
Luciferase Reporter

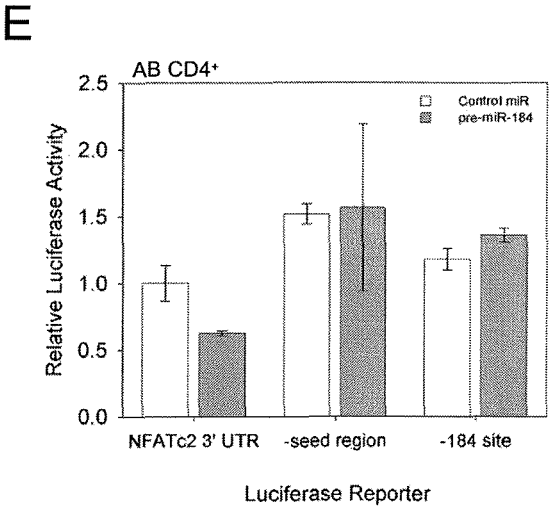

E
AB CD4+
Control miR
pre-miR-184
Relative Luciferase Activity
NFATc2 3' UTR
-seed region
-184 site
Luciferase Reporter

Figure 5

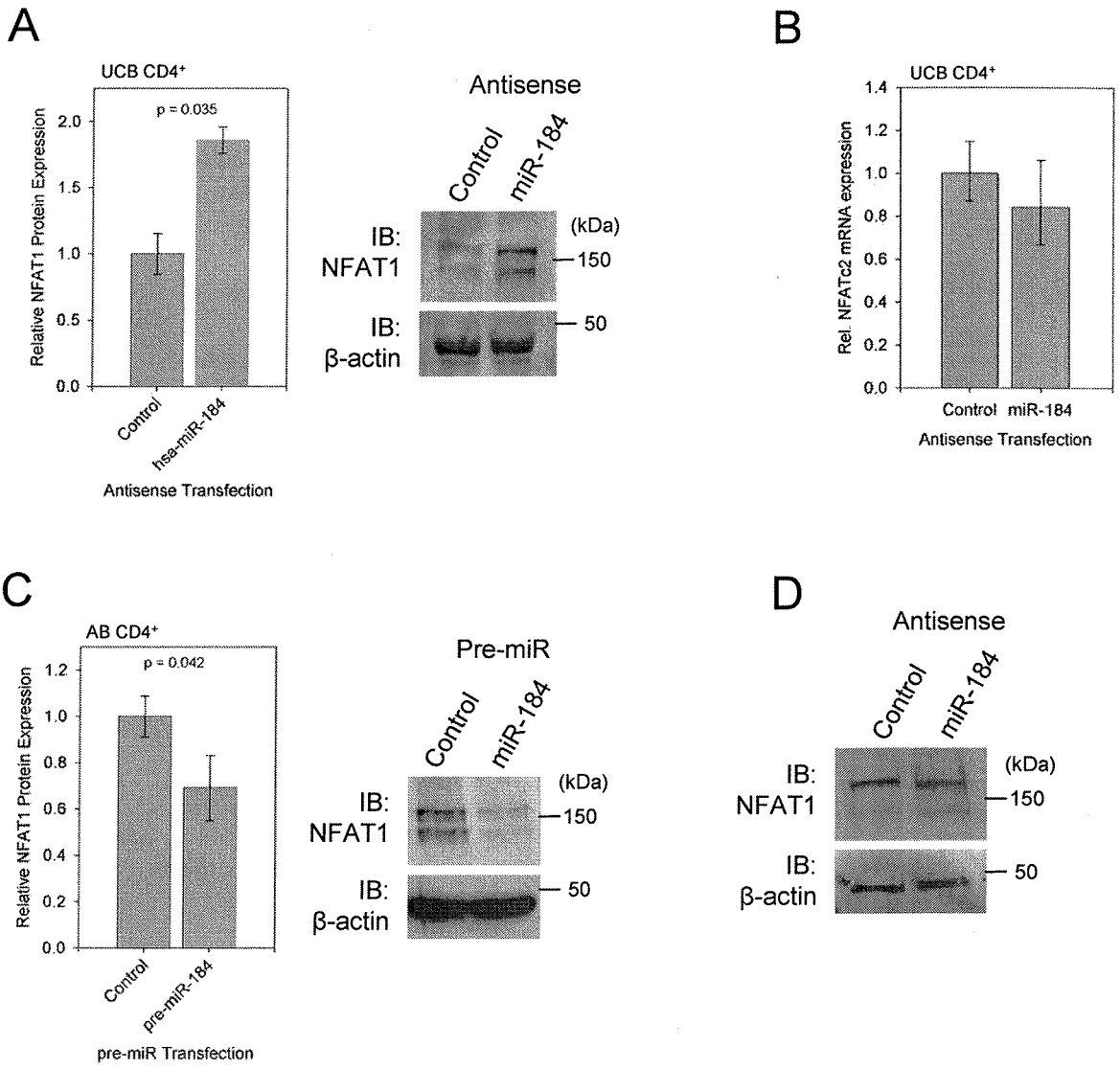
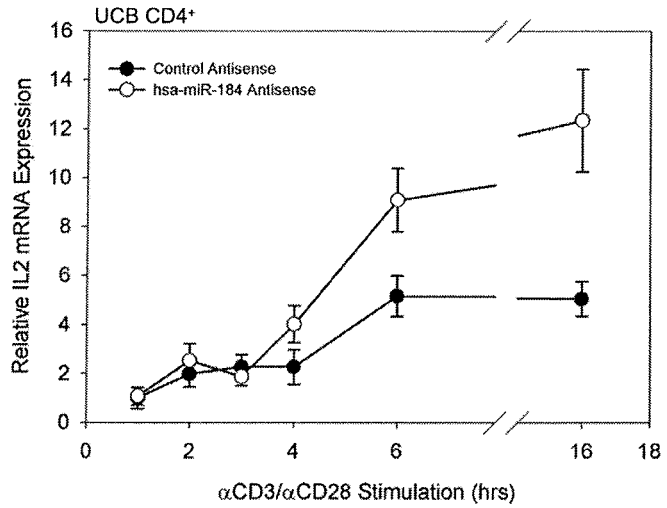
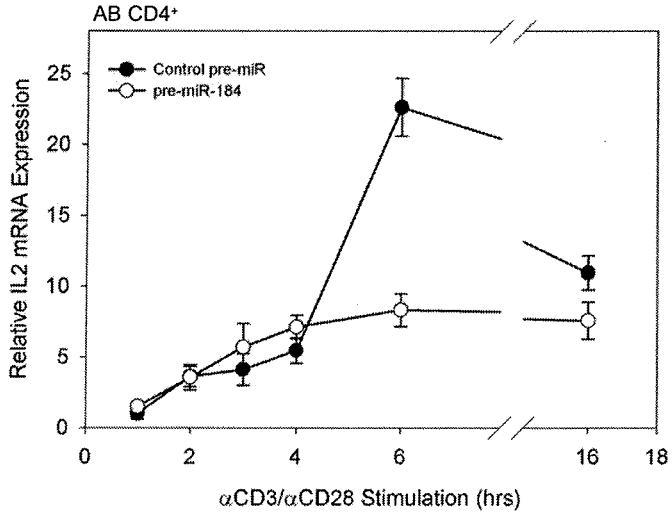

A
UCB CD4+
p = 0.035
Relative NFAT1 Protein Expression
2.0
1.5
1.0
0.5
0.0
Control
hsa-miR-184
Antisense Transfection
Antisense
Control
miR-184
IB:
NFAT1
(kDa)
150
IB:
β-actin
50
B
UCB CD4+
Rel. NFATc2 mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
miR-184
Antisense Transfection
C
AB CD4+
p = 0.042
Relative NFAT1 Protein Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
pre-miR-184
pre-miR Transfection
Pre-miR
Control
miR-184
IB:
NFAT1
(kDa)
150
IB:
β-actin
50
D
Antisense
Control
miR-184
IB:
NFAT1
(kDa)
150
IB:
β-actin
50

Figure 6

A


UCB CD4+
Control Antisense
hsa-miR-184 Antisense
Relative IL2 mRNA Expression
0
2
4
6
8
10
12
14
16
0
2
4
6
16
18
αCD3/αCD28 Stimulation (hrs)

B


AB CD4+
Control pre-miR
pre-miR-184
Relative IL2 mRNA Expression
0
5
10
15
20
25
0
2
4
6
16
18
αCD3/αCD28 Stimulation (hrs)