REPORT

Cell Cycle 9:20, 4213-4221; October 15, 2010; © 2010 Landes Bioscience

A LIF/Nanog axis is revealed in T lymphocytes that lack MARCH-7, a RINGv E3 ligase that regulates the LIF-receptor

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Key words: nanog, MARCH-7, E3-ligase, T lymphocytes, LIF

Abbreviations: (h)ESC, (human)embryonic stem cell; (h)iPS, (human)induced pluripotent cell; JAK, janus-activated kinase; LIF, leukaemia inhibitory factor; IL-6, interleukin 6; miR, microRNA; Q-PCR, quantitative polymerase chain reaction; STAT, signal transducer and regulator of transcription; Th, T helper cell; Treg, regulatory T cell; SOCS-3, suppressor of cytokine signaling-3

Nanog is a stem cell transcription factor required for self-renewal and for maintaining pluripotency, and Nanog itself is regulated at least in part by leukaemia inhibitory factor (LIF)—a pluripotent cytokine of the IL6 family. MARCH-7 is an E-3 ligase linked to regulation of the LIF-receptor in T lymphocytes and T cells from mice that lack expression of MARCH-7 are hyper-responsive to activation signals and show a five-fold increase in LIF activity. Here we ask, does MARCH-7 influence the expression profile of Nanog during the synchronized entry of T cells into the cell cycle? We discovered that lack of MARCH-7 was permissive for Nanog expression at both transcript and protein levels during G_1/S: moreover, addition of exogenous LIF to the MARCH-7 null cells caused a further 13-fold induction of Nanog; other measured transcripts including TGFβ, p53 and STAT3 were relatively unchanged. Since lack of MARCH-7 altered responsiveness to activation signals we sought evidence for pre-existing regulatory miR's that might correlate with MARCH-7 gene dose using head-to-head comparisons between MARCH-7 null, heterozygous and wt spleen cells. Thirty-four miRs were found including miR-346 that is known to target LIF transcripts and miR-346 is one of 16 miRs differentially expressed between hESCs and induced hiPSCs. Of the 34 miRs, 12 were known to be temporally regulated in embryonic nerve cells. In summary, in the absence of MARCH-7 a new signaling pathway is unmasked that involves Nanog expression in the T-cell lineage. This is the first demonstration that T cells retain responsiveness to a LIF/Nanog axis and that this axis is linked to MARCH-7.

Introduction

The peripheral naive T lymphocyte is quiescent, arrested in G_0/G_1 phase of the cell cycle. Upon activation by cognate antigen the cell enters a phase of rapid replication where initially the genome is plastic, then progessively moves through a series of epigenetic events guiding development of distinct T-cell lineages, either effector (Th1, Th2, Th17) or tolerogenic (Treg). Cues from the micro-environment are integrated with lineage-specific gene expression profiles, these eventually becoming hard-wired in the fully differentiated cell. These micro-environmental cues include cytokines and the discovery that LIF and IL6 counter-regulate development of the Treg and Th17 lineages[1] has revealed reciprocity in T cell fate determination during the early response phase.

MARCH-7, also known as "axotrophin," was identified as one of only eight genes specifically associated with immune tolerance in vivo by use of full subtractive kinetic gene arrays of 36,000 genes, comparing tolerance versus rejection.[2] Unpublished at the time and known only as a neural stem cell gene, bioinformatics revealed MARCH-7 as a RINGvariant E3-ligase (Dr. Toby Gibson, EMBL Heidelberg, personal communication) and a member of the MARCH family.[3] The introductory **Figure 1** summarises earlier data where, using the null mouse, MARCH-7 was discovered to have profound effects on T, but not B, lymphocytes [**Fig. 1A(a) and (b)**].[4] LIF activity in T cells was regulated by MARCH-7 with abnormally high LIF [**Fig. 1A(a)**] release and overexpression of gp190 [**Fig. 1B(a)**] in the null mouse.[1,4] Gp190 is the LIF-specific subunit of the heterodimeric gp190/gp130 LIF-receptor (LIF-R)[5-7] and our recent discovery that MARCH-7 is required for degradation of gp190 [**Figure 1B(b)**][1] revealed a novel regulatory component in the LIF signaling pathway that explains the observed effects of MARCH-7 on T cell LIF

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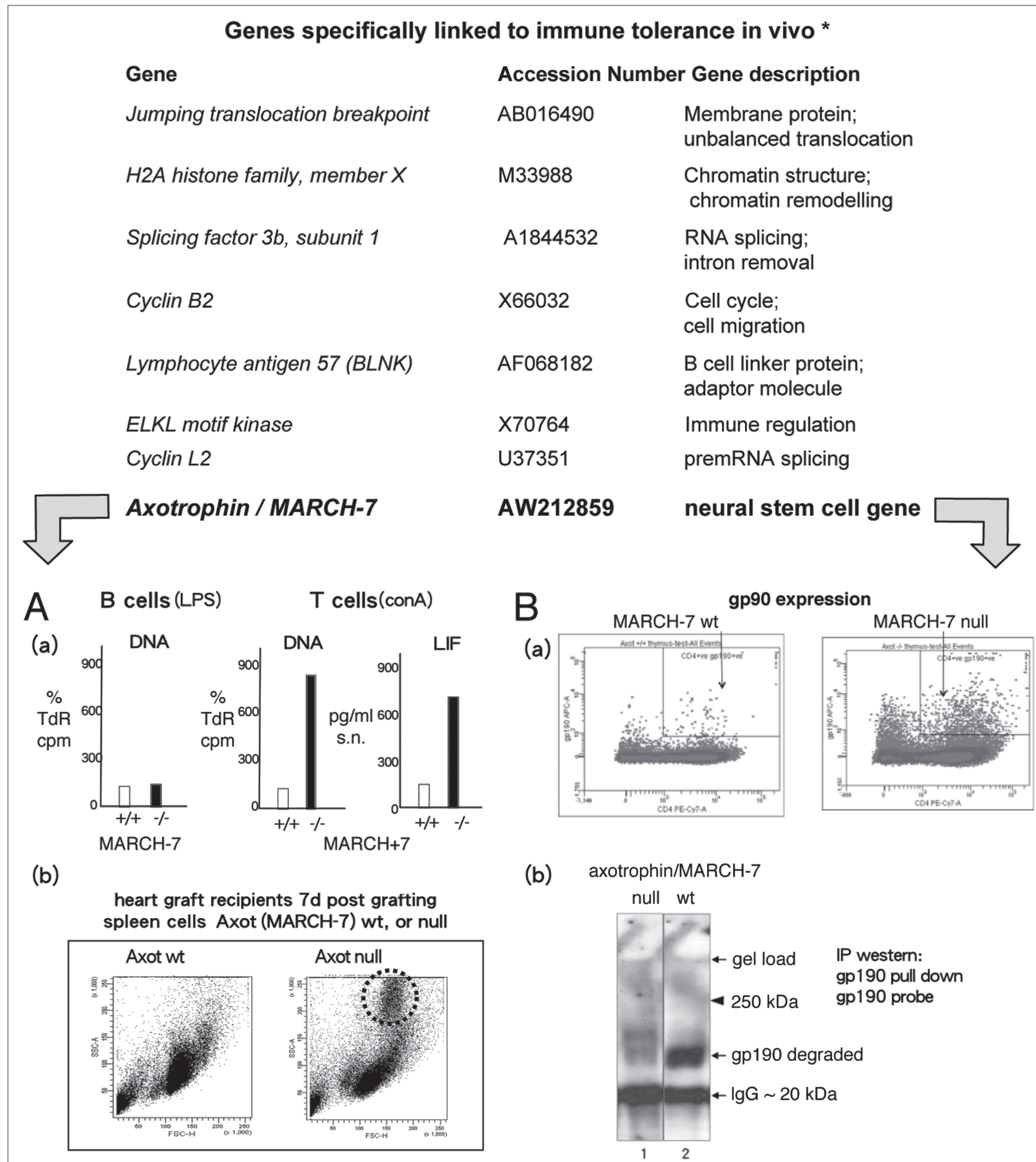
Submitted: 08/26/10; Accepted: 09/04/10
Previously published online: www.landesbioscience.com/journals/cc/article/13543
DOI: 10.4161/cc.9.20.13543



Genes specifically linked to immune tolerance in vivo *

Gene	Accession Number	Gene description
Jumping translocation breakpoint	AB016490	Membrane protein; unbalanced translocation
H2A histone family, member X	M33988	Chromatin structure; chromatin remodelling
Splicing factor 3b, subunit 1	A1844532	RNA splicing; intron removal
Cyclin B2	X66032	Cell cycle; cell migration
Lymphocyte antigen 57 (BLNK)	AF068182	B cell linker protein; adaptor molecule
ELKL motif kinase	X70764	Immune regulation
Cyclin L2	U37351	premRNA splicing
Axotrophin / MARCH-7	**AW212859**	**neural stem cell gene**

Figure 1. Discovery of MARCH-7. The table shows the 8/36,000 genes linked to immune tolerance in the in vivo/ex vivo model as revealed in a compound gene array of tolerance versus rejection.[2] Several genes encode RNA-binding proteins, others are involved in the cell cycle or apoptosis, whilst Axotrophin/MARCH-7 was identified as a RINGv E3 ligase of the MARCH family.[3] (A) (a) shows that lack of MARCH-7 causes T cell hyper-responsiveness in vitro,[4] and (b) in vivo following stimulation by heart allografting.[35] (B) shows data linking MARCH-7 to expression of gp190, the LIF-specific subunit of the LIF-R: (a) shows increased gp190 protein on the surface of MARCH-7 null thymocytes and (b) shows relatively low levels of gp190 breakdown products in the absence of MARCH-7 as detected by IP western for gp190.[1]

activity. By controlling levels of LIF-R protein through its degradation, MARCH-7 may function as a rheostat, able to increase or decrease levels of endogenous LIF signaling generated in response to LIF/LIF-R engagement at the cell surface. Importantly, by regulating the LIF receptor itself, MARCH-7-mediated regulation is mechanistically distinct from regulation involving downstream LIF signaling pathways, either JAK/STAT-3-driven or PI(3)K/Akt-drive,[8] where differential regulation may operate.

LIF itself functions as a pluripotent cytokine acting on many cell types, including embryonic stem cells (ESC) where LIF is involved in maintaining pluripotency and self-renewal, reviewed in reference 9. Since, in the absence of MARCH-7, LIF signaling is uncoupled from feedback control at the level of LIF-R degradation, we reasoned that novel regulatory pathways might become revealed due to high endogenous LIF signaling. Our attention turned to Nanog, a core stem cell transcription factor that, under the influence of LIF, choreographs pluripotency and self-renewal in ESC.[8–13] Moreover, induced pluripotent cells (iPS) are inhibited from lineage commitment by LIF-induced Nanog where Nanog again promotes self-renewal.[14]

In mouse ESC, Nanog, Oct3/4 and SOX2 make up the core circuitry of transcription factors required to maintain

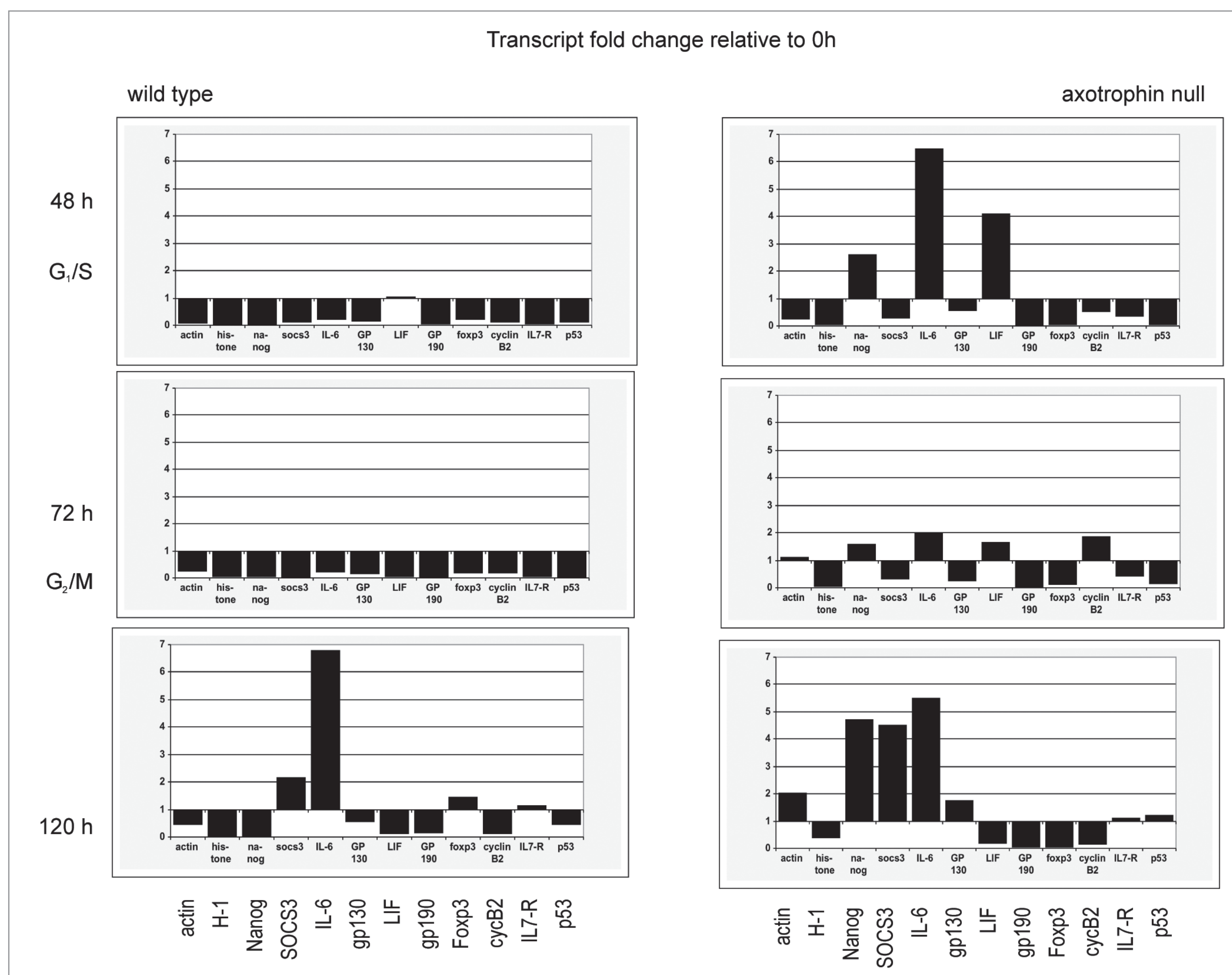


Figure 2. Nanog, LIF and IL6 are early T cell activation genes in the absence of MARCH-7. MARCH-7 wild-type and null spleen cells were activated by conA. Replicate flasks were harvested at 0 h, 48 h (G_1/S), 72 h (G_2/M) and 120 h and cells were snap frozen and processed for transcript expression profiles. Each time point for each gene is plotted relative to the 0 h background transcript level for that gene. The left-hand panels show spleen cells wild-type for MARCH-7; the right-hand panels show MARCH-7 null cells. In addition to those shown, other genes measured at the 48 h time point included Oct4, SIRT1, neuropilin-1, c-kit, histone-2AX and importin-7; in each case the Q-PCR signal was very low or below detection cut-off limit.

pluripotency. LIF signals to this core circuitry via two transcription factors (1) Klf4 that is activated by the JAK/Stat-3 pathway downstream of LIF and (2) Tbx3, activated by LIF-induced P(I)3K/Akt signaling: Niwa's group have shown that Tbx3 predominantly stimulates Nanog, whilst Klf4 preferentially activates SOX2.[8] Further, when investigating the role of Wnt versus LIF in maintaining pluripotency of ES cells, Wnt was found to enhance the effect of LIF but Wnt alone was not sufficient.[15]

As mentioned above, LIF may signal through the JAK/STAT pathway as do other members of the IL6 cytokine family,[5] where cytokine-specific response genes are qualified by the epigenetic state of the target cell. Importantly, in Drosophila—where a single JAK (Hopscotch) and a single STAT (STAT92A) function in a canonical JAK/STAT pathway—over-activation of JAK globally counteracts heterochromatic gene silencing, demonstrating that the JAK/STAT pathway is able to regulate epigenetic status:[16] thus JAK/STAT activity may open previously silenced genes for transcription. Although Nanog is a LIF-response gene normally limited to expression in ESC or iPS, we hypothesized that high endogenous LIF signaling in the absence of MARCH-7, associated with high JAK/STAT activity, and/or P(I)3K/Akt signaling will become permissive for Nanog gene expression.

To test this hypothesis we looked for an inter-regulated node involving MARCH-7, LIF and Nanog, asking (1) is Nanog expressed in the absence of MARCH-7; and (2) is Nanog induced by exogenous LIF in the absence of MARCH-7? We also asked, does MARCH-7 gene-dose influence expression profiles of short non-coding RNAs (miR), reasoning that miR's may reflect putative changes in chromatin state associated within an environment of increased endogenous LIF signaling. Each question was positively confirmed, unmasking a novel LIF/Nanog axis that is linked to MARCH-7 in the T cell lineage.

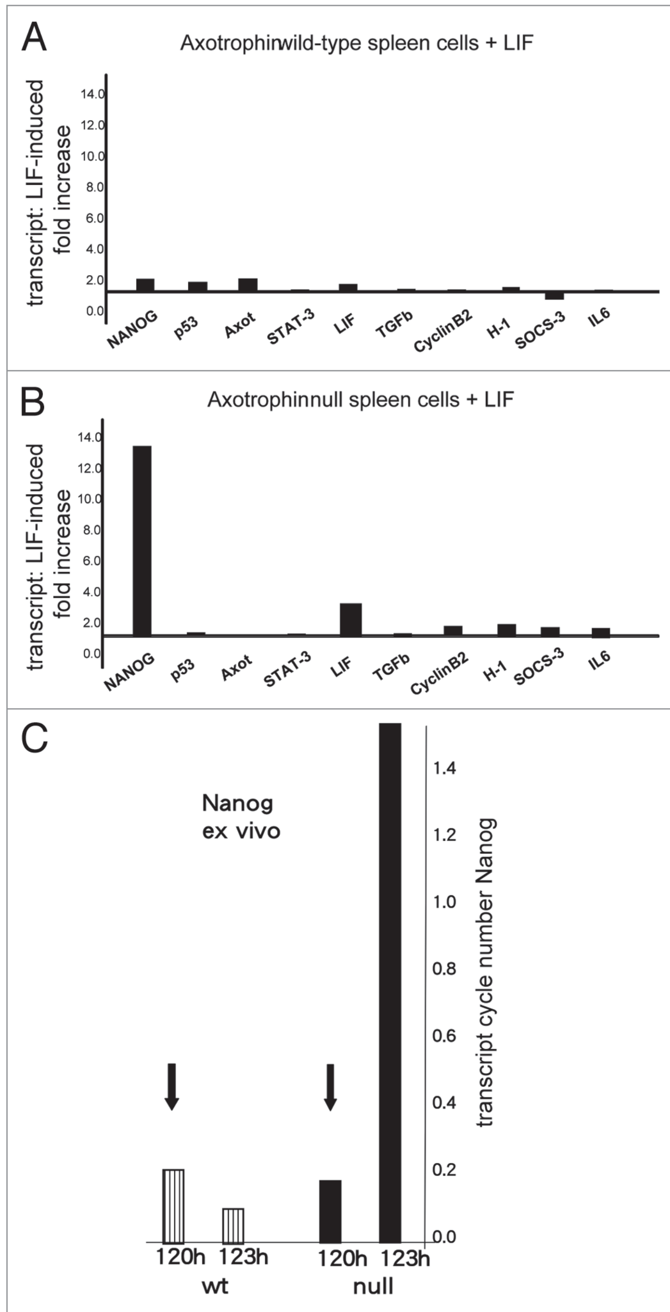


Figure 3. Nanog and LIF are LIF-response genes in T cells that lack MARCH-7. Comparison of transcript levels between MARCH-7 wild-type (A) and null (B) spleen cells with or without exogenous LIF (10 ng/ml) in serum-free GM 48 h after activation by anti-CD3 plus anti-CD28. The axis shows fold-increase in transcript due to added exogenous LIF relative to no LIF controls. (C) Nanog transcript levels following ex vivo allo-stimulation of in vivo primed tolerant spleen cells, wild-type (striped columns) or null (solid columns) for MARCH-7. Irradiated donor spleen cells were used to challenge the primed tolerant cells at 0 h and then at 120 h.

Results and Discussion

Splenic T cells express Nanog in the absence of MARCH-7. We first looked at gene expression during transit of T cells through the cell cycle, where endogenous LIF activity is normally coupled to T-cell activation. The experimental design used either wild-type (wt) mice or MARCH-7 null litter mates. Freshly isolated spleen cells were stimulated either by concanavalin A (ConA) or by anti-CD3 plus anti-CD28, for selective activation of T cells. Under these conditions naive T cells show synchronized entry into the cell cycle, progressing from G_0 though G_1/S (48 h) and then through G_2/M (72 h).[17] Expression of a range of genes including Nanog, LIF, IL6, actin, histone-1, SOCS-3, gp130, gp190, Foxp3, cyclin-B2, IL7-R and p53 was measured within aliquots of the same experimental samples. The samples were taken at 0 h, 48 h, 72 h and 120 h. Two identical series were analysed, one wt for MARCH-7, the other null. Kinetic determination of DNA synthesis and flow cytometric analysis of cell cycle stage confirmed both wt and null T cells were arrested in G_0/G_1 at 0 h.

The hallmark feature of the null cells was early expression of Nanog transcripts. This was present within 48 h of activation and continued through to 120 h, the last measured time point (**Fig. 2** right panels). We also found that expression of Nanog varied during cell cycle progression, being relatively high in G_1/S, decreasing in G_2/M, followed by a four-fold increase at 120 h. At 48 h, the only measured genes showing co-incident expression with Nanog were LIF and IL6. IL6 continued to be co-expressed with Nanog, and at 120 h SOCS-3 (the feedback inhibitor of IL6-type cytokines) had also become induced. In addition to Nanog and IL6, we noted that cyclin B2 activity was cell cycle linked, increasing in G_2/M, in accord with cyclin B2 protein function when its breakdown releases $p34^{cdc2}$ for G_2 to M transition.[18] Activity of Foxp3, known to be required for lineage development of Treg,[19-21] and gp190 remained low throughout whilst LIF gene activity, although initially high, tapered off over time. With respect to Nanog, the possibility that we were measuring Nanog pseudogene expression was discounted since flow cytometric analyses demonstrated induction of Nanog protein in $CD4^+$ spleen cells (data not shown).

In marked contrast to the null, spleen cells from MARCH-7 wt mice showed no activity of the measured genes at either 48 h or 72 h (**Fig. 2** left panels). At 120 h, IL6 alone showed major induction, this being six-fold: SOCS-3 was increased two-fold whilst the other genes showed little or no change.

The profound effect of lack of MARCH-7 on gene activity during activation of naive T cells revealed a novel pathway linked to expression of Nanog and we suspected increased endogenous LIF signaling might play a central role in the observed induction of Nanog.

Nanog is induced by exogenous LIF in peripheral T cells. We next asked, does exogenous LIF activate signaling in T cells lacking MARCH-7? Here we hypothesized that LIF-R is the limiting factor for responsiveness to LIF, and thus LIF-R will qualify signal strength that in turn will qualify signal outcome: this would be in accord with the relative lack of effect of exogenous LIF on wt spleen cells previously observed.[4] We speculated that signaling

to Nanog might be sensitive to high endogenous LIF signaling due to high endogenous LIF-R in the absence of MARCH-7, based on the known regulatory relationship between LIF and Nanog seen in stem cells, and on the potential of unmasking genes by over-activation of JAK.

Using a similar experimental design to that described in **Figure 2**, but in serum-free conditions to avoid serum-derived growth factors, we measured changes in transcript levels in response to 10 ng/ml exogenous LIF. **Figure 3A** shows wt spleen cells following 48 h activation by anti-CD3 plus anti-CD28, where gene expression due to added LIF is plotted relative to no LIF (baseline). **Figure 3B** again shows the effect of added LIF at 48 h, but here gene expression was in spleen cells that lack MARCH-7. In addition, to explore Nanog behaviour in vivo, we used an in vivo/ex vivo model of donor-specific tolerance: here both wt and MARCH-7 null BALB/c recipients of a vascularized CBA heart allograft were analyzed ex vivo following stimulation of recipient spleen cells with irradiated donor spleen cells (**Fig. 3C**).

Of the genes measured in the presence of MARCH-7 there was very little response to added exogenous LIF with the exception of minor increases in Nanog, p53, MARCH-7 and endogenous LIF (**Fig. 3B**). In marked contrast, cells lacking MARCH-7 responded to exogenous LIF with a 13-fold increase in Nanog transcript levels: this was specific to Nanog within the range of genes investigated (**Fig. 3C**). After Nanog, endogenous LIF showed the second highest response to exogenous LIF with a 3-fold increase, suggesting a LIF-driven autocrine effect. The remaining genes showed little or no response to exogenous LIF. The in vivo/ex vivo experiments measuring primed allo-reactivity revealed a link between lack of MARCH-7 and strong donor-driven induction of Nanog: this occured within 3 h and was in marked contrast to the wild-type controls where Nanog expression was minimal and unchanged in response to donor (**Fig. 3C**).

We propose that Nanog is a LIF-response gene in T cells, with a LIF/Nanog axis being qualified in a MARCH-7-dependent manner via expression levels of the LIF-R. We speculate that regulation via MARCH-7, by influencing degradation of gp190, is a natural mechanism for control of transcription of LIF-target genes. Such regulation will be sensitive to micro-environmental cues, since MARCH-7 is itself subject to modulation. For example, in our previous experiments where we demonstrated a LIF/IL6 axis in T cell lineage development,[1] we found that IL6 strongly *induced* MARCH-7 transcripts in naive CD4$^+$T cells, coinciding with both suppression of LIF transcription and induction of TH17 cells. Thus, differential gene silencing during lineage development of T cells appears to be sensitive to levels of MARCH-7 activity.

miRNA expression profiles link MARCH-7 to precursor cells. Having discovered profound effects of MARCH-7 on T-cell gene expression following activation, we were interested in identifying any pre-existing regulatory factors that could influence responses in a MARCH-7-dependent manner. We therefore asked, does MARCH-7 influence profiles of miR expression in spleen cells. Given the broad range of potential targets for a given miR, defining potential functions in terms of miR abundance is necessarily imprecise without highly detailed data to highlight the full profile of coding and non-coding RNA's within a given cell at a given stage of development or function. Our approach, exploring the impact of the MARCH-7 gene on miR profiles, reduced this complexity by simply measuring effect of gene dose.

By comparing MARCH-7 null, heterozygous and wt spleen cells, we discovered 34 miRs where expression showed significant correlations according to MARCH-7 gene dose as detailed in **Table 1:** Supplemental Table 1 shows miR transcript levels and statistical significance between expression relative to MARCH-7 gene dose.

Having found relatively few miR's where expression level changed in response to MARCH-7, we searched for potential functional correlates that might relate in someway to LIF or to Nanog. MiR-346 was increased 1.4 fold in the null compared to wt, in both spleen and thymus. MiR 346 belongs to a cluster core that is upregulated at ESC differentiation and is highly expressed in the brain.[22] MiR 346 is also noted as being differentially expressed between hESC's and hiPSC's,[23] again suggesting a regulatory function linked to stem cells/precursor cells. Differentiation of human mesenchymal stem cells from bone marrow is regulated at least in part by miR346 through its targetting of LIF transcripts[24] and we speculate that increased LIF activity associated with loss of MARCH-7 in T cells remains subject to miR 346-driven post-transcriptional control of LIF expression.

The second feature of note from the miR study was the correlation between miR's linked to MARCH-7 expression and miR's found to co-purify with polyribosomes in developing rat brain neurons.[25] One of these was miR-124 known to inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells.[26] MARCH-7 was first identified as a neural stem cell gene (Melissa Haendel, Ph.D. Thesis University of Wisconsin 1999) and future studies on the role of MARCH-7 in the central nervous system—including in the aging mouse—may reveal system-specific functions of relevance to neurodegenerative disease. Bioinformatic studies have shown that regulatory gene networks that define pluripotent stem cell lines do not include brain-derived neural stem cell lines:[27] it will be of interest to similarly explore the gene clustering networks of MARCH-7 null cells to ask if the null shows a pluripotent profile.

General Discussion

This study has tested our hypothesis that high endogenous LIF signaling due to the lack of MARCH-7 will be permissive for Nanog gene expression in T lymphocytes. We discovered that lack of MARCH-7 (1) was permissive for endogenous Nanog gene activity and (2) was associated with specific increases in Nanog transcription in response to exogenous LIF. Nanog is critical to the core circuitry that controls pluripotency and self-renewal in embryonic stem cells and here we speculate that Nanog also functions in T cells where it is subject to tight regulation by endogenous LIF signaling. We envisage a state of genomic plasticity in newly activated T cells during their amplification and lineage development in the context of micro-environmental cues, these cues influencing MARCH-7 and levels of endogenous LIF activity. In contrast to the effector T-cell lineages we also

Table 1. MARCH-7 Gene Dose-linked miRNA expression relative to wild type (+/+) = 1.0

mmu-mir	MARCH-7 gene dose			Theoretical targets include
	-/-	+/-	+/+	
miR levels showing correlation across null, heterozygous and homozygous MARCH-7				
7-1No1*	3.29	1.96	1.0	*Klf4, Rb1, synuclein*
26a-2No2	3.15	2.00	1.0	
375No1**	5.47	2.19	1.0	*meis1, Klf4, Bmpr1b, Cdk5r1 (p35), Pax6*
375No2	4.88	1.95	1.0	
376bNo1	4.43	2.00	1.0	*T cell invasion, cerebellar degen., bcl2-like 11, Cdk5r1 (p35)*
466No1	8.63	2.00	1.0	
miRs linked to neuronal development or homeostatic regulation of hematopoietic stem cells				
125a-prec***	0.46	0.54	1.0	*synaptotagmin, neuron specific 2, Bak1*
140s-prec*	0.38	0.47	1.0	*Pdgfr, Fgf9, noggin, Bmp2, Foxp2, Tgfb-R1, NFAT5, Klf6*
148bNo1*	0.27	2.42	1.0	
22No2*	0.88	0.08	1.0	
29b-1No1*	0.71	0.61	1.0	
29b-2No1*	0.72	0.58	1.0	
181-prec*	0.52	0.50	1.0	
138-1No1*	2.94	1.42	1.0	
124a-1No1*	2.12	0.68	1.0	
124a-1No2*	1.54	1.16	1.0	
98No1*	3.90	0.92	1.0	
138-1No2*	2.34	1.46	1.0	
miR known to target leukaemia inhibitory factor (LIF)				
346No1****	1.43	0.80	1.0	
Loss of MARCH-7 → increased miR level				
468No1	2.36	1.13	1.0	
297-2No1	2.95	1.25	1.0	
321No2	2.34	1.46	1.0	
410No1	2.67	1.74	1.0	
466No2	4.32	3.47	1.0	
Loss of MARCH-7 → reduced miR level				
379No2	0.12	0.27	1.0	
223-prec	0.39	0.27	1.0	
134-precNo1	0.64	0.15	1.0	
378No2	0.90	0.16	1.0	
030e-prec	0.41	0.27	1.0	
123prec1	0.48	0.32	1.0	
miR variably affected by MARCH-7 gene loss				
27aNo1	1.13	0.58	1.0	
470No2	0.78	2.15	1.0	
409No1	3.47	0.76	1.0	
27aNo2	1.01	0.18	1.0	

* May function in regulation of translation in mammalian neurons; temporally regulated and no-fractionate with polysomes [25]
** Pancreatic islet-specific miR375 regulates insulin secretion.[35]
*** Recently discovered to regulate hematopoietic stem cell pool size and to target Bak1[37]
**** LIF-specific in bone marrow stromal cells.[24]

speculate that Treg, required for antigen-specific immune tolerance, become epigenetically stable in a relatively plastic state, this being perpetuated by release of endogenous LIF in response to antigen: since LIF prevents further differentiation towards an immune aggressive phenotype, a stable feedback loop for antigen-specific tolerance is established and our current data further supports the previously published model of a "stemness" link to regulatory immune tolerance.[28]

As shown in **Figure 4** and discussed in the legend, we propose that during activation of naive peripheral T cells, LIF/LIF-R signaling primes the responding T cell for subsequent progression through clonal expansion and decreasing genetic plasticity towards lineage-specific gene expression profiles. Importantly, in addition to LIF, IL6 is also induced during activation (see **Fig. 2**), thus both LIF and IL6 will contribute to the priming micro-environment of the responding T cell. We envisage this priming will be influenced by receptor competition for gp130 and will be linked to the LIF/IL6 axis. Key to the outcome of this competition will be modulation of MARCH-7 activity. In the absence of MARCH-7, the steady state of gp190 protein is increased[1] presumably shifting the balance in favor of LIF signaling due to increased gp190/gp130 heterodimers (LIF-R) relative to gp130/gp130 homodimers (IL6-R). The proportional increase in LIF-R, versus IL6-R, may induce the observed new responsiveness to exogenous LIF.

Future studies will include other known factors that influence Nanog, including p53 and SIRT1, a p53 deacetylase. p53 inhibits Nanog gene transcription[29] whilst SIRT1 blocks nuclear translocation of p53, thus preventing p53 access to the genome.[30] Since LIF induces SIRT1, for example being associated with the high levels of SIRT1 that occur in ESC cells maintained in LIF-medium,[30] it is possible that MARCH-7 activity may influence this inter-linked node leading to regulation of Nanog. Comparison of SIRT1 levels in the presence or absence of MARCH-7 both in T cells and ESC, will be of interest. Another factor worth exploring in the absence of MARCH-7 is Chd1, a chromatin remodelling factor required to maintain open chromatin and pluripotency in mouse ESC.[31]

An important issue to be addressed in future work is, how selective is the effect of loss of MARCH-7 for Nanog gene activation? A global trend to relax heterochromatin structure would be relatively non-specific whereas our data shows that, in the absence of MARCH-7, Nanog was selectively induced amongst the genes tested, especially in response to exogenous LIF (**Fig. 3**). The concept of Nanog playing a functional role in specific cell types where phases of rapid multiplication combined with progressive differentiation are intrinsic to the cell's functional biology is attractive to us. Although normally occult, a role for a LIF/Nanog axis is suggested by the LIF/Nanog interactions in the absence of MARCH-7.

Materials and Methods

RNA extraction. Resuspended cell pellets, either fresh or snap frozen and stored at -20°C, were treated with 1 ml trizol

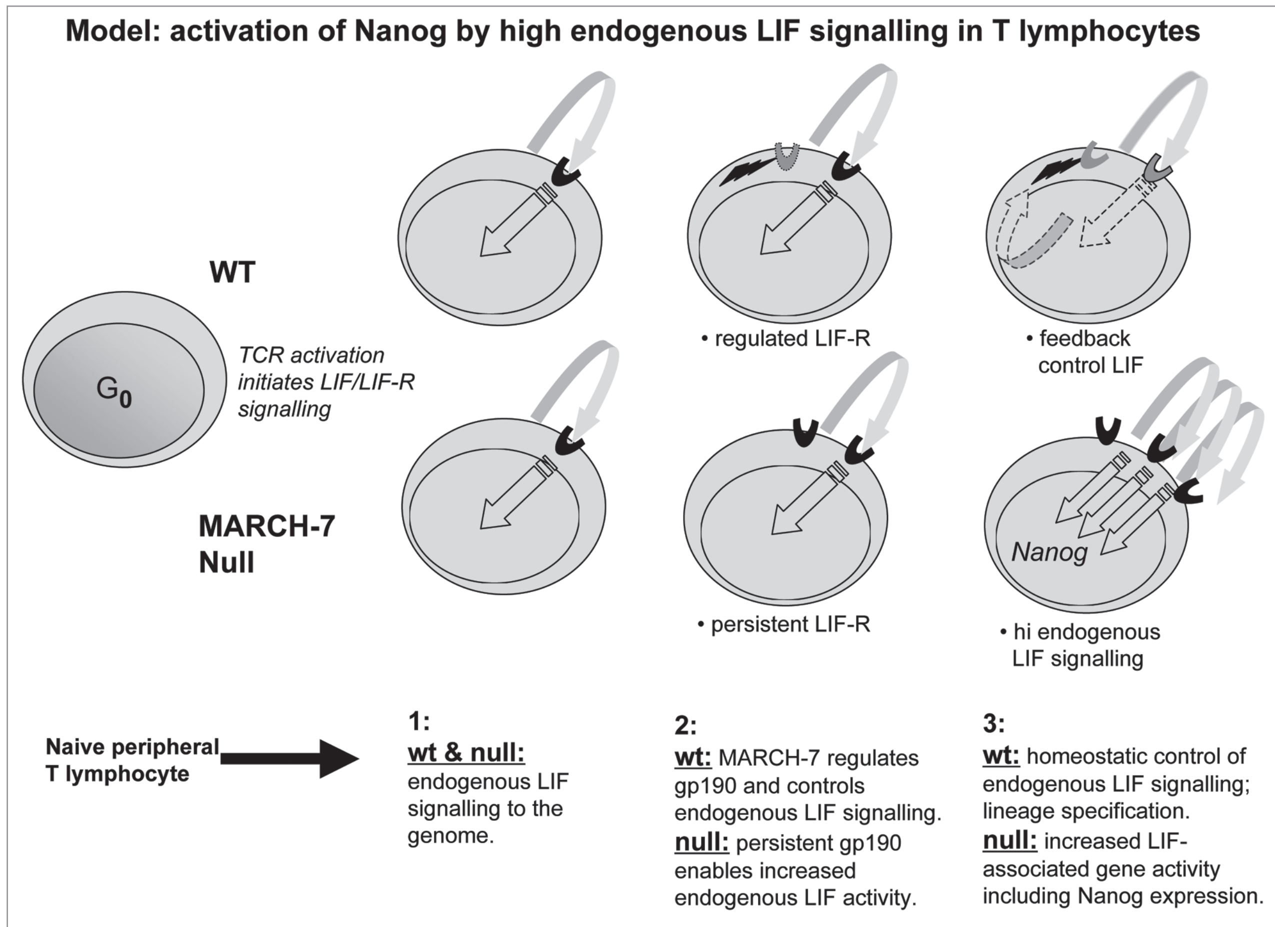


Figure 4. Model for regulation of both endogenous LIF and Nanog by MARCH-7 in T cells. We have previously shown that both LIF and LIF-R are induced upon activation of naive T cells.[1] We propose that LIF/LIF-R signaling primes the responding T cell for subsequent progression through clonal expansion and decreasing genetic plasticity towards lineage-specific gene expression profiles. In addition to LIF, IL6 is induced upon activation (see **Fig. 2**) thus both LIF and IL6 will contribute to the priming micro-environment of the responding T cell. We envisage this priming will be influenced by receptor competition for gp130 and that this will be linked to the LIF/IL6 axis. Key to the outcome of this competition is MARCH-7. In the absence of MARCH-7, gp190 protein is increased[1] and this will shift the balance in favor of LIF signaling due to increased gp190/gp130 heterodimers (LIF-R) relative to gp130/gp130 homodimers (IL6-R). The resultant increase in endogenous LIF activity may increase expression of Nanog that is normally occult in the wt. Further, the proportional increase in LIF-R, versus IL6-R, will induce new responsiveness to exogenous LIF. These predicted effects of loss of MARCH-7 are in accord with our experimental data (**Figs. 2 and 3**). The cartoon shows (1) LIF and LIF-R become activated in T cells that are wild type (upper panel) or null (lower panel), for MARCH-7. (2) Shows regulated expression of LIF-R in the presence of MARCH-7 (jagged symbol), due to controlled degradation of gp190: in the null, LIF-R accumulates due to persistence of gp190 in the absence of its normal degradation. (3) Shows the homeostatic control of endogenous LIF signaling in the presence of MARCH-7, where the cell is sensitive to cues that guide lineage differentiation: here exogenous LIF does not perturb the homeostatic control. In the null, increased levels of LIF-R result in increased endogenous LIF signaling levels, sufficient to either specifically activate Nanog gene expression or to counteract heterochromatic gene silencing to result in expression of previously masked LIF-target genes, including Nanog.

per 6 x 10^6 cells in 1.5 ml eppendorf tubes. After 15 minutes at room temperature RNA was extracted using standard methods, taking care not to carry over DNA. Quality controls confirmed measurement of non-intronic transcripts, pretreated twice with DNAase, was not due to contaminating DNA. Target gene expression was normalized to GAPDH.

Q-PCR. Transcript expression was measured by quantitative PCR (Q-PCR) following extraction of total RNA (see above) and cDNA synthesis as previously reported.[1] Probes were obtained from ABI including probes for actin, MARCH-7, c-kit, cyclin B2, Foxp3, H-1, IL-6, IL-7 receptor, LIF, Nanog, neuropilin 1, Oct-4, SIRT1, SOCS-3, STAT-3, p53, gp130, gp190, TGFbeta. No-template controls without cDNA were included for each sample and probe.

Cell culture. Spleens were collected from age-matched wild-type and MARCH-7 null BALB/c mice at 10–12 weeks old. The spleen cells at 1 x 10^7/ml were cultured in 4 ml IMDM containing 10% FCS and 5 μg/ml concanavalin-A using Corning 25 cc flasks with 0.22 μ filter-caps. The flasks were tilted at around 10° during culture to generate a local micro-environment along the bottom edge: the flasks were not disturbed during culture so as to preserve this microenvironment. Duplicate flasks were removed at 48 h, 72 h and 120 h when cells were harvested, gently pelleted and immediately snap frozen for RNA

extraction. In a second experiment where the effect of exogenous LIF was to be measured, serum-free culture medium was used and exogenous recombinant mouse LIF added at 10 μg/ml to the experimental LIF-treated cultures. T cell stimulation used immobilized anti-CD3 and soluble anti-CD28[1] and plates were kept flat.

In vivo/ex vivo transplant model. Age-matched wild-type and MARCH-7-null mice on a BALB/c background were used as heart graft recipients at 8–12 weeks of age. Using published protocols[32-35] vascularised heart allografts from full mismatch CBA donors were transplanted under cover of alternate day therapy of 11 doses of non-depleting rat monoclonal blocking antibodies against CD4 (YTS 177.9) and CD8 (YTS 105.18). This resulted in operational immune tolerance in both MARCH-7 null and wild-type recipients. After 100 d tolerant spleen cells were harvested and cultured ex vivo, using 4 x 10^7 recipient (i.e., in vivo primed tolerant) cells plus 6 x 10^7 irradiated donor-type spleen cells as antigen-specific stimulators in a total volume of 10 ml IMDM containing 10% FCS per flask. The flasks were tilted to provide 3D conditions as described earlier. At 120 h, a further aliquot of 6 x 10^7 irradiated donor-type cells was added to each flask in 1 ml growth medium to boost stimulation. Duplicate flasks were harvested at 0 h (immediately after adding the irradiated stimulators); 48 h; 120 h (immediately after the boost of stimulators); and 123 h. Previous experiments using this model established high reproducibility both within and between experiments and that the responding T cell population is donor-specific.[34]

Summary

We show for the first time that Nanog is expressed in adult T lymphocytes and that in T cells Nanog expression is linked to the E3-ligase MARCH-7 and is responsive to LIF. We also demonstrate for the first time specific patterns of miR expression linked to MARCH-7 gene dose including miR346 that targets LIF and several others were linked to development in the CNS or homeostasis of stem or percursor cell populations. We propose that common regulatory pathways exist between T cells and stem cells and that these pathways reflect core regulatory mechanisms intimately linked to MARCH-7 and the LIF-R.

Acknowledgements

We are grateful to Toby Gibson, EMBL Heidelberg for discussion on the bioinformatics of MARCH-7; and to Gary Lyons for the founding stock of the MARCH-7 null mouse.

Financial Support

Addenbrookes Hospital Trust Cambridge UK (S.M.M.); and NIHR Cambridge Biomedical Research Centre (S.M.M.); NCI P01CA76259, P01CA81534, P01CA56036 (C.C.).

Author Contributions

S.M.M. designed the experiments, interpreted the data and wrote the manuscript; H.L.T., R.A.W. and Y.R. performed the experimental work; C.C., C.C.L. and C.T. performed the comparative miR analyses.

Note

Supplementary materials can be found online at: www.landesbioscience.com/supplement/ThompsonCC9-20-Sup.pdf

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