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Adapting *in vitro* embryonic stem cell differentiation to the study of Locus Control Regions

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Abstract

Numerous Locus Control Region (LCR) activities have been discovered in gene loci important to immune cell development and function. LCRs are a distinct class of cis-acting gene regulatory elements that appear to contain all the DNA sequence information required to establish an independently and predictably regulated gene expression program at any genomic site in native chromatin of a whole animal. As such, LCR-regulated transgenic reporter systems provide invaluable opportunities to investigate the mechanisms of gene regulatory DNA action during development. Furthermore the qualities of LCR-driven gene expression, including spatiotemporal specificity and “integration site-independence” would be highly desirable to incorporate into vectors used in therapeutic genetic engineering. Thus, advancement in the methods used to investigate LCRs is of considerable basic and translational significance. We study the LCR present in the mouse T cell receptor (TCR)- α gene locus. Until recently, transgenic mice provided the only experimental model capable of supporting the entire spectrum of LCR activities. We have recently reported complete manifestation of TCR α LCR function in T cells derived *in vitro* from mouse embryonic stem cells (ESC), thus validating a complete cell culture model for the full range of LCR activities seen in transgenic mice. Here we discuss the critical parameters involved in studying LCR-regulated gene expression during *in vitro* hematopoietic differentiation from ESCs. This advance provides an approach to speed progress in the LCR field, and facilitate the clinical application of its findings, particularly to the genetic engineering of T cells.

1. Introduction

Locus Control Regions (LCRs) are cis-acting gene regulatory elements known to confer a high degree of integration site-independence to the expression of a linked transgene *in vivo*

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[reviewed in (Li et al., 2002)]. This rare property yields copy number-dependent transgene mRNA production levels with predictable spatiotemporal characteristics paralleling those of the specific LCR's gene locus of origin. Many of the identified LCRs regulate genes expressed in cell types of the hematopoietic system (Li et al., 2002). LCRs usually consist of multiple DNase I hypersensitive sites (HS), each of which supports a distinct set of properties contributing to overall LCR function. The functional interactions of these HS regions can be complex and challenging to characterize. But, they ultimately synergize to produce the unique properties that distinguish LCR activity from that of other types of *cis*-acting DNA elements.

Until recently, the only model proven to support all aspects of LCR activity has been transgenic mice. While this gold standard approach has significantly advanced the field, investigating the complex structure-function relationships that culminate in LCR activity in this model is resource intensive and involves protracted experimental timetables. It has therefore been a goal of the field to obtain a cell culture model that can support all characteristics of an LCR. Such methodology could serve as a more rapid and cost effective approach to the investigation of these complex and powerful gene regulatory elements.

Previous attempts to use cultured cell lines to study LCRs have only been partially successful. For instance, β -globin LCR activity is incomplete in erythroid cell lines that are directly transfected with a transgene linked to the LCR (Skarpidi et al., 1998). A follow-up study reported an important clue to explaining this unexpected result (Vassilopoulos et al., 1999). In this work, mouse embryonic fibroblasts, transfected with a reporter gene linked to the β -globin LCR, were subsequently fused with an erythroid cell line. The fusion resulted in restoration of the copy number dependent reporter transgene expression indicative of full LCR activity. These studies suggested the need for the β -globin LCR DNA to be present, initially, in the genome of an uncommitted cell type prior to the establishment of its complete activity upon later exposure to a differentiated nuclear environment. Inspired by this work, we sought to design an *in vitro* cell culture model of complete LCR activity that would meet these apparent requirements.

Technology is now readily available for differentiating mouse embryonic stem cells (ESCs) to cells of the hematopoietic lineage, including T cells, *in vitro* (Holmes and Zuniga-Pflucker, 2009). Briefly, ESCs can be differentiated to hematopoietic stem cells (HSCs) when co-cultured with a bone marrow derived cell line (OP9) (Nakano et al., 1994). The addition of fms-like tyrosine kinase 3 ligand (Flt3-L) and interleukin 7 (IL-7) supports differentiation of HSCs to erythroid, monocytic, and B cell types (Cho et al., 1999). Further inclusion of a Notch ligand DLL1 or DLL4 in the OP9 cells signals differentiation of HSCs and ESCs into T lineage cells (Schmitt and Zuniga-Pflucker, 2002; Schmitt et al., 2004). Virtually the entire course of T cell development in the thymus can be modeled in this co-culture system, with each developmental stage readily distinguishable by multi-parameter flow cytometry. Thus, we believed this system offered the opportunity to model the activity of LCRs that function in T lineage cells after their *in vitro* differentiation from reporter gene transfected ESCs. LCRs have been discovered in several gene loci expressed at varying stages of T cell development and function, making the study of LCR activity in T cells of heightened significance.

We study the LCR derived from the mouse T cell receptor- α (TCR α) gene. It was originally identified as a cluster of nine HS spread over 13-kb in the intervening DNA between the C α exons and Dad1 gene (Diaz et al., 1994). These HS confer copy number-dependent mRNA expression levels to a transgene with a similar profile of tissue specificity, and developmental timing to that observed for the endogenous TCR α gene (Ortiz et al., 1997). It has been shown that at least four of these HS regions are indispensable for complete LCR activity. Two of the four required HS (HS1 and HS1') confer TCR α gene-like spatiotemporal specificity on linked transgene expression (Ortiz et al., 1999). The other two, HS4 and HS6, are considered to contain transcriptional "insulator-like" activities that protect against integration site-dependent position effects on transgene expression (Gomos-Klein et al., 2007).

Analogous to the results seen for the β -globin LCR, we have reported that the TCR α LCR cannot drive transgene expression in a copy number-dependent manner after its direct introduction into differentiated T cells (Lahiji et al., 2013). This is in stark contrast to the activity of identical TCR α LCR-driven reporter constructs in transgenic mice, within which all the hallmarks of TCR α LCR activity are consistently observed (Harrow and Ortiz, 2005; Knirr et al., 2010). These results led us to use the TCR α LCR to test the ability of the aforementioned *in vitro* ESC differentiation model to support full LCR activity. Indeed, we found that this system enables full recapitulation of all aspects of TCR α LCR activity seen *in vivo* (Lahiji et al., 2013). This work has validated *in vitro* ESC differentiation, as a much needed alternative approach to transgenic mice for the study of LCRs. Here we describe in detail how we adapted and optimized this technology to examine the multi-faceted function of an LCR.

2. Materials and Methods

2.1. Reporter gene constructs

The hCD2 T transgene (Melton et al., 1996) was excised from the pBluescript SK vector using *Sal* I and *Xba* I. This transgene fragment was co-transfected with an SV40 promoter-driven Neomycin-G418 resistance cassette that was excised from the pEYFP-C1 vector (Clontech) using *Ssp* I and *Eco*O109 I. The V α -YFP-LCR reporter construct contained a YFP cDNA (from pEYFP-C1) driven by a 324-bp fragment of the V α 17 promoter (Kouskoff et al., 1995) and linked to the 7.4-kb *Xho* I – *Sac* I fragment containing the fully active TCR α LCR (Ortiz et al., 1999).

2.2. TCR α /Dad1 bacterial artificial chromosome (BAC) dual-reporter construct

The BAC utilized in this study is based on a TCR α /Dad1 genomic region-containing sub-fragment (Knirr et al., 2010) of clone RP23-94I14 (BACPAC Resources, Oakland, CA). Our BAC fragment spanned from the extreme 3'-Ja region to approximately 38-kb downstream of the Dad1 exons. BAC modifications were done using Red/ET recombination technology (Gene Bridges) following the manufacturers instructions. The genomic human CD2 reporter gene (approximately 5.4-kb) (Melton et al., 1996), driven by a 428-bp V α 17 promoter sequence, was inserted approximately 3.7-kb upstream of exon 1 of the TCR α constant region in the transcriptional orientation of the TCR α gene. The second reporter gene was a

703-bp cDNA of the rat CD2 gene (Hozumi et al., 2000) linked to an SV40 poly-adenylation signal (from pEYFP-C1). This reporter was recombined in frame to the ATG in exon 1 of the Dad 1 gene. Thus, the Dad1 promoter drives transcription of the rat CD2 reporter gene. Prior to transfection, the dual-reporter BAC construct fragment was released from the pBACe3.6 vector backbone using *Not* I and *Fse* I restriction enzymes and separated by the Field-Inversion Gel Electrophoreses (FIGE). The 76.2-kb band was isolated from the gel by electro-elution into TE buffer followed by standard phenol/chloroform extraction and ethanol precipitation (Ausubel et al., 1987).

2.3. T cell line culture and transfection

T cell line VL3-3M2 (Groves et al., 1995) was cultured in RPMI 1640 with 5% FBS, supplemented with 1% penicillin streptomycin (Corning), 1% Glutagro (Corning) and 54 μ M β -mercaptoethanol (Sigma). Cells were transfected using nucleofection Amaxa (Lonza) program G13 according to the manufacturers protocol. Approximately 5.0×10^6 cells were re-suspended in 0.1 ml Nucleofector solution with 5 μ g of the Va-YFP-LCR reporter gene fragment, and an equimolar amount of neomycin-G418 resistance cassette. Twenty-four hours after co-transfection, Neomycin-G418 was added at a concentration of 0.4 mg/ml. Individual clones were obtained by serial dilution.

2.4. ESC culture and transfection

The mouse ESR1 cell line was cocultured with mitomycin C arrested mouse embryonic fibroblasts (Millipore) in DMEM (Corning) supplemented with 20% FBS (Gemini), 1% Glutagro (Corning), 1% penicillin streptomycin (Corning), 1% HEPES (Millipore), 1% nonessential amino acids (Millipore), 0.1% gentamicin (Life Technologies), 55 μ M β -mercaptoethanol (Life Technologies), and 10 ng/ml LIF (Millipore). Cells were transfected with a Bio-Rad Gene Pulser (0.24 kV and 500 μ F). Approximately 1.0×10^7 ESCs were re-suspended in 0.5 ml electroporation buffer (Millipore) with 10 μ g hCD2 T gene fragment or 12 μ g of the dual-reporter BAC fragment. An equimolar amount of a neomycin-G418 resistance cassette was co-transfected with each of the reporter transgenes. 24 hours after transfection, G418 selection was begun (concentration range of 0.15 to 0.25 mg/ml – see section 3.2). Selection media was changed daily. Individual colonies were picked after 10 days and clonally propagated.

ESC transfectant clones were initially screened for stable transgene integration by PCR using primers complimentary to the 5' region of the transgenes: hCD2 T-forward, 5'-GAGGAAACCAACCCCTAAGATGAG-3'; Va17-forward (for BAC detection), 5'-ATCCTGTCACTTCAGCTAGCC 3'; hCD2 T-reverse, 5'-CGTAATCTCTTTGGAGA CTGCACC-3'. The presence of intact transgene copies in PCR positive clones was subsequently confirmed by Southern blot.

2.5. In vitro ESC differentiation

The protocol for *in vitro* derivation of T cells, and other hematopoietic cell types, from mouse ESC was carried out as described previously (Holmes and Zuniga-Pflucker, 2009; Lahiji et al., 2013). Emerging hematopoietic stem cells from day eight co-cultures were harvested and transferred onto OP9-DL1 cell monolayers (to derive T cells) (Schmitt et al.,

2004) or OP9 cell monolayers (to derive monocytic and erythroid cells) (Nakano et al., 1994). In a typical experiment, multiple, independent, transfected ESC clones were differentiated in parallel with a non-transfected ESR1 control co-culture. The ESR1-derived progeny were used as negative controls for the corresponding differentiation products of the multiple transfected ESC clones assessed in parallel. Cells were analyzed by flow cytometry on day 12 of co-culture (to detect monocytic, erythroid, or early-stage developing T cells), day 16 (to detect mid-stage developing T cells), and day 18–20 (to detect later stage developing T cells).

2.6. Flow cytometry

FACScan, FACSCalibur and FACSVantage devices were used. Antibodies used were obtained from BD Biosciences or Life Technologies and included anti-human CD2 (clone S5.2), anti-rat CD2 (clone OX-34) and anti-mouse CD45 (Clone 30-F11), CD44 (Clone IM7), CD25 (Clones 3C7 or PC61), CD8 (Clone 53-6.7), CD16/32 (Clone 2.4G2), CD4 (Clones GK1.5 or RM4-5), CD11b (clone M1/70.15), Ter119 (clone TER119). Dead cell discriminator (DCD) or DAPI (Life Technologies) was used to label nonviable cells. Before staining, cells were pretreated with anti-CD16/32 (to block Fc receptors). Cells were stained with fluorochrome-conjugated antibodies for 20 min and washed three times with plain staining medium. For analyses, live cells were gated based on forward and side scatter and lack of DAPI or DCD signal. CD45 was then used to gate on the white blood cell types derived in co-culture. FlowJo (Tree Star) software was used for data analyses. Fluorescence produced by the YFP reporter gene, when present, was analyzed in the FITC channel.

3. Results & Discussion

Several critical parameters emerged during our efforts to apply *in vitro* ESC differentiation to TCR α LCR study. These included issues as basic as the selection of the reporter gene, as well as the method by which it is transfected into ESC. Furthermore, the differences between cell transfection and the pronuclear microinjection method used to make transgenic mice (the latter being more direct and gentle on the DNA) made it especially critical to characterize the integrations in each transfected ESC clone for transgene intactness. Finally, transfected ESC clones endure several procedures that could impact their fitness in the *in vitro* differentiation experiments. Therefore, we made minor but important adjustments to the established *in vitro* ESC to T cell differentiation protocol to support the proliferation of transfected ESC during their differentiation in this system.

3.1. Selection of Reporter Gene

In crafting a reporter gene construct, our first instinct was to minimize its size in order to facilitate its efficient transfection and intact integration. Thus, we initially selected a reporter cDNA encoding a yellow fluorescent protein (YFP), from *Aequorea victoria*. Fluorescent proteins are widely used as reporter genes in transfected cell lines and have, in some instances, been used successfully in transgenic mice (Yu et al., 1999a; Yu et al., 1999b). We linked the YFP cDNA to a minimal TCR V α 17 promoter fragment and further linked this transcription unit to a fully active TCR α LCR (Fig. 1A) (Ortiz et al., 1999). This reporter construct worked very well in stable-transfected T cell lines, producing robust YFP signal in

flow cytometry experiments (Fig. 1B). Unfortunately, ESC clones stably transfected with this same construct did not produce progeny with detectable YFP levels after *in vitro* differentiation (Fig. 1C).

Since the estimated number of integrated transgene copies in these clones (3 and 5) are within a range that yields copy number-dependent expression (Lahiji et al., 2013), this apparent silencing is not due to excessive transgene copy number. Thus, it seems that transfected ESCs will be less hospitable to the function to YFP, and perhaps any non-mammalian reporter cDNA, during differentiation than are standard cell lines. In this way, the ESC differentiation system more closely resembles transgenic mice, within which non-mammalian cDNAs are often observed to experience silencing, in some transgene contexts (Palmiter and Brinster, 1986).

Studies of the TCR α LCR in transgenic mice have employed both cognate (TCR α) (Diaz et al., 1994) and heterologous mammalian reporter gene fragments in their genomic configuration [human β -globin (Ortiz et al., 1997), HLAB7 (Knirr et al., 2010), and human CD2 (Harrow and Ortiz, 2005)]. All of these reporter gene fragments are susceptible to position effects in transgenic mice in the absence of an LCR (Magram et al., 1985; Townes et al., 1985; Lang et al., 1991; Kushida et al., 1997). However, when these reporter genes are linked to the TCR α LCR, their expression pattern closely resembles that of the endogenous TCR α gene in time and space, and displays copy number dependent mRNA levels irrespective of the site of integration. An ideal reporter gene for testing LCR activity in the progeny of *in vitro* differentiated ESCs would be one that produces a protein that is independently expressed on the cell surface and, thus, easily detectable by flow cytometry. Of the aforementioned reporters, the hCD2 reporter protein is consistently detected on the cell surface (Melton et al., 1996; Harrow and Ortiz, 2005). Therefore, we selected this gene for use as a reporter of the TCR α LCR activity in the *in vitro* differentiated ESCs.

3.2. Transfection of ESCs

An effective way of introducing a transgene into ESCs is electroporation. A common variation on this technique is nucleofection, which requires lower cell numbers, and less DNA, and results in higher transfection efficiency. However, upon characterization of transgene integration sites by Southern blot, severe truncations of the transgenes were prevalent in most stable-transfected ESC clones produced by nucleofection (data not shown). Truncations within the reporter transgene can lead to skewed results, due to the potential for deletion of coding and/or regulatory components in some or all integrated transgene copies. The hCD2:1-8 transgene is approximately 21-kb in length (Harrow and Ortiz, 2005). The observation that this large construct would be susceptible to severe shearing upon nucleofection has been corroborated by a recent report (Rostovskaya et al., 2012). We thus switched to exponential decay pulsing for electroporation as the method for transfection. Although this approach did require more cells and more DNA, and resulted in a smaller number of stable transfected clones, the frequency of truncated transgene integrants was much lower in the resultant clones.

The inclusion of bacterial DNA in reporter transgenes can lead to the silencing of the reporter gene after it is stably integrated into the genome of mice (Palmiter and Brinster,

1986). Therefore, in order to minimize the amount of vector DNA that will be introduced into the genome of ESCs we liberated the transgene construct from its cloning vector and co-transfected it with an SV40 promoter-driven neomycin resistance cDNA that had been similarly isolated from its prokaryotic vector. 24 hours after ESC co-transfection, Neomycin-G418 antibiotic was added at a concentration of 0.25 mg/ml for two days to initiate strong selection for stable-transfected clones. The G418 concentration was then reduced to 0.18 mg/ml to allow surviving clones to proliferate. This concentration was maintained for the duration of the selection period, if the live, remaining neomycin resistant colonies were not adequately expanding after seven days of selection, the concentration of G418 was reduced to 0.15 mg/ml, to encourage colony proliferation. After ten days of selection, each of the expanding colonies were picked using a dissecting microscope and propagated in 0.18 mg/ml of G418.

3.3. Transgene integration site analyses

We took multiple steps to characterize the transgene integration sites of the isolated clones (Lahiji et al., 2013). The first step was to confirm the presence of the transcription start site of the construct by PCR analysis. We designed primers to detect the non-endogenous hCD2 promoter region. The clones that contained this region of the transgene were then analyzed by Southern blot with a probe detecting the 3'-most, HS6 region of the LCR. Cells that incorporate multiple copies of a transgene into their genome tend to integrate these in tandem arrays in a "head-to-tail" arrangement. Therefore, the Southern blot strategy was designed to detect these head-to-tail junctions and any truncations thereof. To further distinguish *bona fide* intact head-to-tail junction fragment from similarly sized transgene truncation products, a PCR scheme was designed to detect the intact junction fragment, using a forward primer located at the 3'-end of the construct, and a reverse primer located in the 5' sequence flanking the hCD2 promoter.

One of the properties of the TCR α LCR is its ability to confer an mRNA expression level that bears a linear relationship to the number of integrated transgene copies. To test if the ESC differentiation model can support the copy number dependence property of the TCR α LCR one must carefully estimate the number of integrated transgene copies. We derive the copy number in each clone by multiple Southern blots. A probe detecting both the endogenous and transgene region of HS6 was identified. To distinguish between the endogenous and transgene regions, a restriction enzyme digestion strategy that would generate two different sizes for each was determined. The probe hybridization signal intensities of transgene and endogenous (i.e. two-copy control) bands were then quantified using a Phosphor Imager. We calculate relative copy number estimates for each clone from an average of at least three independent Southern blots. It is best to analyze DNA samples from all ESC clones to be directly compared in further quantitative analyses of LCR function on the same Southern blots. This optimizes the accuracy of relative transgene copy number estimation among the various clones.

3.4. Differentiation of ESC Clones to Cell Types of the Hematopoietic System

We use OP9 co-culture technology to differentiate ESCs *in vitro* into cells of the hematopoietic system. This ESC *in vitro* differentiation system supports the derivation of

various blood cell types, including erythroid, monocytic and T cells representative of all stages of thymic development to the CD8⁺, TCRβ^{hi} stage (Schmitt et al., 2004). This technology has been reported to work with various ESC lines (de Pooter et al., 2006b) as well as with high-G418 “double-selected” homologous recombinant ESC clones used to make chimeric mice (de Pooter et al., 2006a; Arsov et al., 2011). Developing ESC progeny can be tracked by cell surface immunophenotyping using flow cytometry. This system enables the characterization of the developmental timing and cell type restriction of LCR activity.

Briefly, the original ESC differentiation protocol (Holmes and Zuniga-Pflucker, 2009) calls for a co-culture of 5×10⁴ ESCs with an 80% confluent layer of OP9 bone marrow stromal cells. After five days of co-culture, the ESCs should display quantitative differentiation to mesoderm like structures. On this day, 5×10⁵ cells from the co-culture are re-plated on to a fresh layer of stromal cells, in the presence of Flt-3L, for each subsequent time point to be analyzed (see Materials and Methods). Eight days after the start of the co-culture, the mesoderm should give rise to hematopoietic progenitor cells (HPC). These progenitors are now gently harvested and placed, with addition of Flt-3L and IL7, onto either an OP9 cell layer to generate monocytic, erythroid, and B cells, or onto an OP9-DL1 cell layer to generate T cells. Natural lot-to-lot variability in fetal bovine serum can profoundly affect the robustness of proliferation and differentiation in this assay. Thus, it is absolutely critical to test multiple serum lots to determine those that will work best in this protocol.

To optimize this model for studies of the TCRα LCR, we made small but important adjustments to this published protocol. We had observed that some transfected ESC clones tend to proliferate less robustly than non-transfected ESCs. Therefore, to increase the yield of differentiating cells, we introduce up to two extra days of co-culture at two key time-points. The 5th and 8th days are crucial junctures in this co-culture. On day 5, if less than 80% of the ESC colonies have visually differentiated to mesoderm-like colonies (Holmes and Zuniga-Pflucker, 2009), then we allow for an extra 1–2 days of co-culture at this point prior to cell passage. We have observed that this delay improves the robustness of subsequent co-culture. On day 8, a large amount of HPCs should be visible as small round bright cells (Holmes and Zuniga-Pflucker, 2009). If the apparent number of HPCs is low, and the day 5 passage was delayed only one day, then an extra day of co-culture may be introduced at this point to increase these numbers prior to HPC harvest and transfer. These adjustments improved the yield of differentiation products from transfected ESCs. We confirmed that after this point, a similar population distribution of developing T cells is generated from both transfected and non-transfected ESC clones (data not shown).

3.5. Qualitative assessment of TCRα LCR Activity in T cells derived in vitro

Flow cytometry analyses are used to determine the qualitative aspects of LCR driven reporter gene activity in the differentiation progeny of transfected ESC clones. These aspects include gross integration site-independence, developmental timing and cell type specificity. Analyses of erythroid progeny are done on the live (i.e. DAPI negative) Ter119 positive, CD45 negative population. Otherwise, these analyses are done on the live, CD45 positive gated fraction of the co-cultures. The CD45 positive gating is indispensable in these

analyses. It eliminates the possible inclusion of OP9 stromal cells in the collected data, and ensures that only hematopoietic lineage cells are being analyzed. Within the CD45 gate, monocytic cells are detected using CD11b antibody. It is also possible to assess transgene expression in the CD19 positive B cell fraction of an OP9-ESC co-culture. But, in our hands, B cells accumulate less reliably than do the other non-T cell types in these co-cultures. It is unclear why this is the case, but it may be linked to a special sensitivity to culture conditions and/or fetal bovine serum lot variability. In contrast, T cell generation in OP9DL1-ESC co-culture is remarkably robust and consistent. Combinations of antibodies to CD44, CD25, CD4 and CD8 are used to trace reporter gene expression through the stages of T cell development.

Determining the integration site-independence of TCR α LCR activity involves examination of T cells derived from multiple, independent ESC clones bearing intact integrations of the reporter transgene. Under the control of an LCR, virtually all integration sites will be permissive for transgene activity. We previously demonstrated this property for the TCR α LCR using a linked hCD2 reporter gene (Lahiji et al., 2013). Figure 2 shows similar analyses of a dual-reporter bacterial artificial chromosome (BAC) carrying a portion of the mouse TCR α /Dad1 gene locus. Upstream of the J α -C α intron and LCR sequences, a TCR V α promoter driven genomic hCD2 reporter gene fragment has been integrated. A rat CD2 (rCD2) cDNA has also been integrated in frame with the ATG translation start site in Dad1 exon 1. Thus, the Dad1 promoter drives the rCD2 reporter (Fig 2A). Flow cytometry experiments show that ESC clones bearing this dual-reporter BAC construct generate T cells with high expression of the hCD2 reporter gene, and low, but consistently detectable, levels of the rCD2 reporter gene (Fig 2B).

Figure 3 shows flow cytometry analyses of the cell type distribution of reporter gene activity in this system. In the left column, a representative ESC clone bearing the dual-reporter BAC described above shows low to absent expression of the TCR α “representative” hCD2 reporter gene in erythroid (Ter119⁺), monocytic (CD11b^{hi}) or very early stage CD44⁺, CD25⁻, CD4⁻, CD8⁻ (DN1) T-cell progeny, as expected (Lahiji et al., 2013). In contrast, the rCD2 reporter for the Dad1 gene is consistently active at low, but detectable, levels in all these progeny cell types. Since the Dad1 gene is expressed ubiquitously, and beginning early in development, this result is also expected.

Figure 4 depicts the developmental timing of reporter gene activity during T cell development *in vitro*. In the context of the BAC reporter construct, the TCR V α -promoter-driven hCD2 gene's activity is significantly upregulated during the DN3 stage, as was previously reported with a simpler hCD2-TCR α LCR reporter construct (Lahiji et al., 2013). In the absence of an LCR, this hCD2 reporter gene fragment will only very rarely be expressed in a normal manner when integrated into the genome (Lang et al., 1991). Accordingly, the unlinked hCD2 reporter transgene (hCD2 T) was completely silenced in the differentiation progeny of 13 of 14 hCD2 T-bearing ESC clones that we examined [(Lahiji et al., 2013) and unpublished data]. In contrast, we obtained one of the rare ESC clones (1 of 14) in which the unlinked hCD2 T transgene apparently integrated into a site in the genome permissive for LCR-independent gene expression. We include analyses of this rare clone (X10) in Figure 4 (right column) for comparison purposes. As would be expected

for an hCD2 transgene (de Boer et al., 2003), its expression is upregulated early in T cell development (at the DN1 stage) and remains high through subsequent maturation stages. The data from this clone demonstrates that the hCD2 reporter gene is capable of functioning as early as the DN1 stage, and exemplifies the ability of this system to detect different programs (in terms of developmental timing) of reporter gene expression.

3.6. Controls for quantitative assessments of reporter gene mRNA levels

Assessing the quantitative aspects of LCR activity, such as copy number dependence of reporter gene expression, calls for a different approach from that described above. We caution against relying on flow cytometry data to assess this feature of LCR function, as cell surface protein levels may not always strictly correlate with reporter gene mRNA levels. As LCRs are active at the transcriptional level, an assay that directly measures mRNA levels is required. Real time, quantitative, reverse transcriptase-mediated PCR (qRT-PCR) was employed in our previous studies to undertake these analyses (Lahiji et al., 2013).

In general, the qRT-PCR protocols and procedures we used were standard, carried out and analyzed according to the manufacturers recommendations (Lahiji et al., 2013). Nevertheless, it is worthwhile to discuss here the additional controls needed for these assays of transfected LCR-driven reporter genes. First and foremost, corresponding differentiation progeny of untransfected ESCs must be included in all assays to control for background signal in the absence of the reporter transgene. One must also carefully select an appropriate control mRNA signal to use in normalizing the reporter mRNA signals. When examining mRNA levels in a single cell lineage in these assays, sources of potential sample-to-sample variability include loading error, target cell content of the differentiating co-cultures, and developmental stage of the target cells. The simplest way to control for these variables would be to normalize using the mRNA of the endogenous gene locus from which the LCR under study is derived. This is because this mRNA would be expected to parallel that of the LCR-driven reporter gene through development. We used endogenous TCR α mRNA for this purpose, which enabled us to normalize reporter gene signals for all of the above potential sources of experimental variability (Lahiji et al., 2013). If, however, it is desired to perform an experiment that requires a normalizing control gene other than the endogenous counterpart of the LCR, then two additional steps are advisable. First, one can purify the target cells of choice from the differentiating co-cultures prior to harvesting RNA. In addition, it would be prudent to select a normalizing control gene that is not expressed in the OP9 feeder cells (e.g. CD45) as RNA preparations from these co-cultures are likely to contain OP9-derived transcripts.

4. Concluding remarks

The ability to assess the full range of LCR activity in the differentiation progeny of OP9-ESC co-culture creates new opportunities to more rapidly advance the basic structure-function analyses of the various LCR activities discovered in genes expressed during hematopoietic development. In addition, the integration site-independence and spatiotemporal control properties of an LCR would be highly desirable to incorporate into the viral vector constructs used in gene therapy. But the viral vectors used to transduce therapeutically relevant cell types have limited space for exogenous DNA sequences

(Thomas et al., 2003). Thus, in addition to facilitating the understanding of the molecular bases of LCR function, isolation of the key functional DNA sequence elements of a given LCR will, in many cases, be necessary for its eventual translation to clinical applications.

The potential for LCRs to be employed in the creation of gene therapy vectors that will be useful in stem cell transplantation is very high. During genetically engineered stem cell differentiation in a transplant recipient, vector components derived from LCRs should be able to provide predictable expression patterns and levels to a gene encoding a therapeutic protein. This approach has already been taken in the design of vectors containing β -globin LCR components (May et al., 2000). In addition, the advent of chimeric antigen receptors (CARs) has made the prospect of genetically engineering T cells to target specific diseases very promising (Davila et al., 2012). CAR-expressing T cells have recently proven successful in treating B cell leukemia patients (Porter et al., 2011). Our adaptation of *in vitro* T cell differentiation from ESCs to the study of LCR activity should provide an efficient system for troubleshooting and testing the gene expression properties of viral vector constructs incorporating compilations of LCR sub-elements.

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Fig. 1.

A YFP reporter cDNA is silenced during ESC differentiation. (A) Diagram of the TCR V α promoter (V α 17p) driven YFP driven cDNA linked to the TCR α LCR. (B) Two representative independent VL3-3M2 T cell clones directly transfected with this reporter construct display robust expression of YFP (dark curves). In contrast, (C) transfecting this construct into ESCs does not yield YFP positive CD4/CD8 double positive (DP) T cell progeny (dark curves) after ESC differentiation *in vitro*. Two representative, independent clones are shown. The light curves represent the signals from control, non-transfected cells assayed in parallel.

Fig. 2.

A TCR α /Dad1 gene locus-derived dual reporter BAC construct is expressed independently of genomic integration site. (A) Diagram (not to scale) of the dual reporter BAC construct for detecting the products of a $V\alpha$ promoter driven human CD2 reporter gene ($V\alpha 17$ ghCD2), and a Dad1 promoter-driven rat CD2 (rCD2) reporter cDNA (Dad1rCD2). The numbered, dark boxes indicate the exons of the hCD2 reporter gene, TCR α constant region (C α) and Dad1 gene. The asterisk in Exon 5 of the hCD2 gene indicates a premature stop codon that results in production of a non-signaling hCD2 protein (Melton et al., 1996). The light box indicates the TCR α LCR sequences. The numbered, vertical arrows indicate the DNase I hypersensitive sites in the region of the LCR. Ea refers to the classical transcriptional enhancer element of the TCR α gene. The positions of the *Fse* I and *Not* I restriction sites used to excise the BAC reporter fragment for transfection are shown. (B) Flow cytometry detection of TCR α reporter (hCD2) and Dad1 reporter (rCD2) activity in CD4/8 DP T cells (dark curves) derived *in vitro* from three independent, BAC-transfected ESC clones. The light curves represent the signals from control, non-transfected ESR-1 cell-derived DP T cells assayed in parallel.

Fig. 3. Cell type distribution of TCRα/Dad1 BAC reporter gene activity. Flow cytometry detection of TCRα reporter (hCD2) and Dad1 reporter (rCD2) in the indicated *in vitro* differentiation progeny of a representative dual-reporter BAC transfected ESC clone (WT68). Representative gating (shown at left) of day 12 co-cultures (for erythroid, monocytic and DN1 T cells) and day 20 co-cultures (DP T cells). Signals from the progeny of transfected cells are shown by the dark curves. The light curves represent the signals from control, non-transfected ESR-1 cell-derived progeny assayed in parallel.

Fig. 4.

Flow cytometry detection of the developmental timing TCR α reporter (hCD2) expression. Analyses of differentiating T cell progeny of a representative dual-reporter BAC transfected ESC clone (WT15). Representative gating of day 16–20 ESC-OP9DL1 co-cultures is shown at left. Reporter hCD2 gene signals from the indicated T cell progeny of transfected cells are shown by the dark curves. The light curves represent the signals from control, non-transfected ESR-1 cell-derived progeny assayed in parallel.