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Autocrine GM-CSF transcription in the leukemic progenitor cell line KG1a is mediated by the transcription factor ETS1 and is negatively regulated through SECTM1 mediated ligation of CD7

Christina Bade-Döding [a], Wiebke Göttmann [a], Anja Baigger [a], Matthew Farren [b], Kelvin P. Lee [b], Rainer Blasczyk [a], Trevor Huyton [a,*]

[a] Institute for Transfusion Medicine, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany
[b] Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14226, USA

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ABSTRACT

Background: CD7 expression is found on ~30% of acute myeloblastic leukemias (AML). The leukemic progenitor cell line KG1a (CD7+) constitutively expresses GM-CSF while the parental KG1 (CD7-) cell line does not. This study focuses on the molecular basis of CD7 mediated GM-CSF regulation.
Methods: KG1a cells were treated with recombinant SECTM1-Fc protein, the PI3K kinase inhibitors wortmannin, LY292004, or PI4K activator spermine. Stable KG1-CD7+, KG1a-shCD7, KG1a-shETS1 as well as KG1a-GFP, KG1a-PKCβII-GFP cell lines were generated and the levels of CD7, GM-CSF and ETS-1 mRNA and protein were compared by real-time-PCR, western blotting, flow cytometry and ELISA.
Results: SECTM1 is expressed in Human Bone Marrow Endothelial Cells (HBMEC) and its expression can be up-regulated by both IFN-γ. KG1a cells demonstrated high expression levels of CD7 and ETS-1 allowing a constitutative signaling through the PI3K/Atk pathway to promote GM-CSF expression, while KG1 cells with low expression of CD7 and ETS-1 showed low GM-CSF expression. On KG1a cells GM-CSF expression could be negatively regulated by PI3K inhibitors or by recombinant SECTM1-Fc. Overexpression of CD7 in KG1 cells was insufficient to promote GM-CSF expression, while silencing of CD7 or ETS-1 resulted in reduced GM-CSF expression levels. Differentiation capable KG1a cells overexpressing PKCβII illustrated complete loss of CD7, but maintained normal levels of both ETS-1 and GM-CSF expression.
Conclusion: These findings add an additional layer to the previously described autocrine/paracrine signaling between leukemic progenitor cells and the bone marrow microenvironment and highlight a role for SECTM1 in both normal and malignant hematopoiesis.
General Significance: This work shows that SECTM1 secreted from bone marrow stromal cells may interact with CD7 to influence GM-CSF expression in leukemic cells.

1. Introduction

The CD7 antigen is expressed on T/NK cells, bone marrow (BM) progenitor cells and on a variety of leukemic progenitor cells including myeloid leukemic cells [1]. Despite the detailed characterization of CD7 knockout mice [2–5] the complete functions of CD7 are still unclear. Most earlier work relied heavily upon the use of activating anti-CD7 monoclonal antibodies (mAbs) to ligate the receptor and thereby mimic the effects of ligand binding [6–11]. The identification of secreted and transmembrane protein 1 (SECTM1) as a ligand for CD7 has now provided some further insights into its functions [12–14] and has highlighted its role as a co-stimulatory receptor on T/NK cells. SECTM1 is a 27 kDa type I integral membrane protein that is known to be localized to the golgi, however a soluble N-terminal cleaved form of the SECTM1 protein (~16 kDa) can be specifically secreted by cells [12] and can bind to CD7 with high affinity to stimulate both T cell proliferation and cytokine secretion [13]. SECTM1 is expressed predominantly in the peripheral blood by neutrophils and monocytes, in the bone marrow [12] and in several cancer cell lines [15]. The expression of SECTM1 gene is a characteristic of an interferon early response gene [16,17] being expressed rapidly upon IFN stimulation and it clearly plays an important role in the innate immune response to both pathogenic [18] and chemical stimuli [19].

The release of growth factors such as GM-CSF, G-CSF and IL-3 is critical for the proliferation, differentiation and survival of normal hematopoietic cells and has been suggested to influence the pathogenesis of myeloid malignancies including AML [20]. The autocrine release of GM-CSF has been observed in a number of studies and results in the ability of up to 70% of blast cells from patients with AML being able to form colonies on methyl cellulose without further addition of cytokines

Abbreviation: SECTM1, secreted and transmembrane protein 1

* Corresponding author. Tel.: +49 511 532 9710; fax: +49 511 532 9701.
E-mail address: huyton.trevor@mh-hannover.de (T. Huyton).

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[21]. A number of mechanisms for this phenomenon have been investigated including the observation that autocrine IL-1 secretion induced the production of GM-CSF by AML cells, for a detailed review see [22].

Since myeloid leukemic blasts still need pro-survival cytokines to survive, it is still of interest to look for novel molecules that might modulate the release of growth factors and therefore play a role in both normal and malignant hematopoiesis. In this study we therefore sought to investigate the role that CD7 and its ligand SECTM1 might play in the regulation of GM-CSF production by leukemic progenitor cells. The human KG1 and KG1a leukemia cell lines [23] have been widely studied as *in vitro* models to investigate the molecular events taking place during differentiation of CD34+ progenitors to dendritic cells [24–26]. It has also been shown that antibody mediated cross-linking of CD7 on these cell lines [27] or on gamma delta T cell lines [28] is able to induce GM-CSF mRNA and protein expression.

2. Materials and methods

2.1. Expression of SECTM1 in the HBMEC-33 cell line

The HBMEC-33 cell line [29] was a generous gift from Prof. C.E. van der Schoot (University of Amsterdam). HBMEC-33 was cultured on human plasma fibronectin (Milipore) coated plates as previously described [29]. Cells were detached in buffer consisting of 5 mM EDTA, 132 mM NaCl, 1 mM MgSO4, 6 mM KCl, 132 mM KH2PO4, 20 mM Hepes, 5.5 mM glucose, 0.5% w/v human serum albumin pH 7.4 for 1 h at 37 °C. The cells were then incubated briefly with FcR blocking reagent (Miltenyi Biotech) before the addition of a SECTM-1 specific antibody (Aviva Systems biology). Cells were incubated for 30 min at 4 °C, washed with PBS/0.2% BSA and incubated with a FITC-conjugated goat-anti-mouse-Ig or isotype control (AbD Serotech) for 30 min at 4 °C. The cells were then washed with PBS/0.2% BSA before Flow cytometry analysis using a FACSCanto II instrument (BD Biosciences).

For mRNA expression cells were treated with 500 U mL^{-1} recombinant IFN-γ (Peprotech) where indicated for 24 h and samples prepared as described for the KG1 and KG1a cell lines.

For western blotting cells were treated with 500 U mL^{-1} recombinant IFN-γ (Peprotech) where indicated and total cell lysates prepared in SDS loading buffer. Native SECTM1 protein was detected by using a Rabbit polyclonal antibody targeting the C-terminal region of SECTM1 (Santa Cruz Sc-139364) and mouse anti-Rabbit-HRP labeled secondary antibody (Dako).

2.2. AML cell lines

Early passage KG1 and KG1a cells were generously provided by Dr. Guenter Bernhardt of the Institute of Immunology, Hannover Medical School, Germany. Cells were cultured at 37 °C in a 5% CO2-humidified atmosphere in RPMI 1640 supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin, 2 mM L-glutamine and 20% fetal bovine serum (FBS).

2.3. Construction and expression of soluble SECTM1-Fc fusion proteins

The full-length SECTM1 cDNA (Genbank U77643.1) was obtained from the Imagenes clone collection. A fragment of the SECTM1 gene comprising the Ig-like domain (residues 29–145) was then amplified by PCR and subcloned into the pHLSEC vector system [30] to generate a construct with an N-terminal IgG-kappa leader sequence and SECTM1 residues 29–145 fused to the Fc region of human IgG1. Transfection and expression were carried out essentially as described [30] in HEK293T cells grown in DMEM media containing 1% FCS. After 4–5 days conditioned media were harvested, centrifuged at 1500 ×g to remove cell debris, diluted 3-fold with PBS, filtered through a 0.2 μM filter and the pH adjusted to 8.0 using 1 M Tris pH 8.0. Media were then loaded onto a 5 ml Hi-Trap protein A column (GE Healthcare) and washed extensively with PBS. Purified SECTM1-Fc fusion proteins were eluted from the column using 50 mM sodium citrate pH 3.0 and fractions neutralized immediately with 1 M Tris pH 8.0. The protein was then dialysed in PBS at 4 °C overnight before further purification using Ni-NTA resin (Sigma), elution fractions were dialysed into PBS at 4 °C overnight and then concentrated using a 10 kDa concentrator (Amicon) before analysis by SDS-PAGE and western blotting with a mouse anti-His mAb (Santa Cruz).

2.4. Flow cytometry

To determine the surface CD7 expression or binding of SECTM1-Fc protein to CD7 on the surface of cells, 1×10^6 cells were harvested and washed twice in PBS. The cells were then incubated briefly with FcR blocking reagent (Miltenyi Biotech) prior to incubation with anti-CD7-FITC (BD Biosciences) or 1 μg/ml of recombinant purified Fc-fusion protein in PBS for 30 min before being washed again with PBS. Bound SECTM1-Fc was detected by incubating with a mouse anti-human IgG(Fc)-FITC (AbD Serotech) secondary antibody. Data acquisition was carried using a FACSCanto II instrument (BD Biosciences) and data analysis performed with Flowjo (Treestar inc.). Each binding experiment was repeated three times, a representative of which is shown.

2.5. Evaluation of mRNA levels by RT-PCR

The indicated amounts of SECTM1-Fc fusion protein (0.1–10 μg/mL in PBS) were incubated at 50 μL per well of a 96 well plate overnight at 4 °C. Following adhesion of the Fc-fusion to the plate the wells were then rinsed with complete RPMI 1640 medium to remove any unbound protein. 2×10^5 (KG1, KG1a) cells were then added in complete RPMI 1640 medium. Where indicated the inhibitors LY2942002 and Wortmannin were added at 50 μM and 100 nM or 10 μM concentrations respectively.

Total cellular RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (1 μg) was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. RT-PCR was carried out using GM-CSF (*CSF2*), *CD7*, *ETS1*, *SECTM1*, *β-actin* and *GAPDH* specific TaqMan assay kits (Applied Biosystems, Darmstadt, Germany). Reactions were carried out on a StepOne Plus Real-Time PCR System (Applied Biosystems) with thermal cycling conditions of 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. *β-Actin* and *GAPDH* mRNA were amplified as reference standards for normalization of mRNA levels and all reactions were performed in triplicate. The relative C_T method (described in the User Bulletin No. 2, ABI PRISM 7700 Sequence Detection System, pp. 11–15) as implemented in the Steponeplus software was used to calculate the relative mRNA level of the target gene normalized to *β-actin* or *GAPDH* in each sample. All values are expressed as fold-changes relative to their appropriate untreated cell controls (set to 1.0) and are representative of multiple experiments. Statistical analyses were performed using a *t* test (GraphPad Prism, Version 4.03, GraphPad Software, San Diego, CA).

2.6. Evaluation of Akt phosphorylation

SECTM1-Fc fusion protein (5 μg/mL in PBS) were incubated at 500 μL per well of a 24 well plate overnight at 4 °C. Following adhesion of the Fc-fusion to the plate the wells were then rinsed with complete RPMI 1640 medium to remove any unbound protein. 1×10^6 KG1a cells were then added in complete RPMI 1640 medium. Where indicated the inhibitors LY2942002 and Wortmannin were added at 50 μM and 100 nM or 10 μM concentrations respectively. Cells were prepared and stained as described using a PE-labeled mouse anti-Akt(pS473) antibody (BD Biosciences). Each experiment was repeated three times, a representative of which is shown.

2.7. GM-CSF elisa

The human GM-CSF ELISA kit (eBioscience) was used according to the manufacturer's instructions and a standard curve with recombinant GM-CSF (1000–7.8 pg/mL) was run in each assay. The results are expressed as the mean ± SEM of data obtained from three or more experiments performed in duplicate. Where indicated 1 mL SECTM1-Fc fusion protein (5 μg/mL in PBS), was coated per well of a 24 well plate overnight at 4 °C. Following adhesion of the Fc-fusion to the plate the wells were then rinsed with complete RPMI 1640 medium to remove any unbound protein. 0.5×10^6 (KG1, KG1a) cells were then added in complete RPMI 1640 medium.

2.8. Design of lentiviral vectors for CD7 expression and silencing of CD7 and ETS1

The full-length CD7 cDNA was obtained from the Imagenes clone collection. A PCR product containing the full-length coding sequence was ligated into the eukaryotic expression vector pcDNA3.1V5/HisTOPO (Invitrogen, Karlsruhe, Germany). The recombinant pcDNA3.1V5/His/CD7 vector was then used as a template for the lentiviral pRRL.PPT.SFFV.mcs.pre/CD7 vector. The CD7 insert was digested with BamHI and XbaI and ligated into the same restriction sites from the pRRL.PPT.SFFV.mcs.pre lentiviral vector.

For expression of stable short-hairpin RNA (shRNA) to silence CD7 and ETS1, we used the lentiviral vector pLVTHM [31] containing enhanced green fluorescent protein (eGFP) as the reporter gene. Individual shRNA expression cassettes were generated with the oligonucleotides given in Supplementary Table 2. The 19-mer gene-specific target sequences for CD7 interference were designed using the ambion siRNA target finder program using standard rules, while ETS1 target sequences were taken from [32]. All constructs were verified by sequencing (forward and reverse direction) using an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, CA, USA).

2.9. Production of lentiviral particles in HEK293T cells

Lentivirus was produced essentially as described [33] after transfection of HEK293T cells with expression or shRNA constructs, packaging plasmid: psPAX2 and envelope plasmid: pDM2G. Concentrated lentivirus dissolved in RPMI 1640 (Invitrogen) was used for transduction of KG1 or KG1a cells.

2.10. Transduction of KG1 or KG1a cells and purification of transductants

The lentiviral transduction of the leukemic cell lines KG1 and KG1a was performed by adding the dissolved lentiviral pellet in the presence of 8 μg/mL protamine sulfate (Sigma-Aldrich) to the cells followed by incubation for 8 h. Transduced cells were then cultured in complete RPMI 1640 media. For the pRRL.PPT.SFFV.mcs.pre/CD7 lentivirus transduction using pRRL.PPT.SFFV.mcs.pre/GFP lentiviral particles was performed in parallel to monitor the transduction efficiency, which for the KG1 cells was quite low at ~10–15%. Cells transduced with CD7 were expanded before being purified using a CD7-FITC conjugated antibody (BD) and anti-FITC indirect microbead kit (Miltenyi Biotech) according to the manufacturer's protocol. Purified KG1-CD7 + cells were then expanded and CD7 expression was confirmed by RT-PCR, western blotting and flow cytometry.

For the pLVTHM-shCD7 and pLVTHM-shEST-1 lentivirus, co-expression of eGFP was used to monitor transduction efficiency which was again only ~5%. Cells were expanded and eGFP cells were sorted twice using a FacsAria cell sorter (BD Biosciences) to select the strongest GFP expressing cells. Sorted KG1a-shCD7 and KG1a-shEST-1 cells were then expanded and expression analysis was determined by RT-PCR, western blotting, flow cytometry and ELISA.

2.11. Western blotting

Typically ~10×10^6 cells were lysed in RIPA lysis buffer and protein estimation was determined using a BC Assay kit (Uptima). A total of 50 μg protein was then resolved on 4–20% SDS-PAGE gels (Invitrogen). After transfer to polyvinylidene difluoride (PVDF) the membrane was blocked for 1 h in 5% low fat Milk powder in PBST. The membrane was then incubated overnight at 4 °C with anti-CD7 antibody (Santa Cruz H-126), anti-ETS-1 (Santa Cruz C-20) and anti-GAPDH (Santa Cruz 0411) in blocking solution. The blot was then washed 3 times with PBST followed by incubation with goat-anti-mouse-HRP (DAKO) or goat-anti-rabbit-HRP (Bio-Rad) conjugated secondary antibody for 1 h at room temperature. Following 3 washes with PBST detection was performed by incubating the blot directly with Roti-Lumin substrate (Roth) and measured on a MultiImage II chemiluminescence imaging system (Alpha Innotech).

For Phosphorylation specific ETS-1 antibodies (ETS-1pT38, ETS-1pS282/S85, Invitrogen) modified RIPA buffer supplemented with 50 mM sodium fluoride and 1 mM sodium vanadate was used for cell lysis. Blocking was carried out in 5% albumin TBST and washing in TBST.

Where indicated 1 mL SECTM1-Fc fusion protein (5 μg/mL in PBS), was coated per well of a 24 well plate overnight at 4 °C. Following adhesion of the Fc-fusion to the plate the wells were then rinsed with complete RPMI 1640 medium to remove any unbound protein. 0.5×10^6 (KG1, KG1a) cells were then added in complete RPMI 1640 medium. Cells were then harvested, washed in PBS and total cell extracts resolved by SDS-PAGE before blotting with ETS1 or ETS1-pS282/85 specific antibodies.

2.12. Microarray analysis

KG1 and KG1a were grown as previously described [26]. Total cellular RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). cDNA was prepared from RNA for analysis on Human Genome U133-plus 2.0 gene chips (Affymetrix, Sunnyvale, CA) by Expression Analysis (Durham, NC), following the manufacturer's instructions. Signal intensity and expression status for each transcript on the array were determined using the statistical algorithms contained within the Affymetrix GCOS software by Expression Analysis.

3. Results

3.1. Expression of SECTM1 in the HBMEC-33 cell line

We first sought to confirm that SECTM1 is indeed expressed by cells of the bone marrow microenvironment using the immortalized human bone marrow endothelial cell line HBMEC-33. Flow cytometry was performed using a SECTM1 specific antibody and showed that the ~10% of the HBMEC-33 cells stained positive for surface expression of SECTM1 (Fig. 1c). The expression of SECTM1 mRNA could be upregulated ~100-fold by treatment with IFN-γ (Fig. 1d) correlating well with our previous observations in monocytes. We also performed western blotting using an antibody directed against the C-terminal region of SECTM1 (Fig. 1e). Two protein bands were observed corresponding to both full-length SECTM1 and the remaining "membrane tethered" form of SECTM1 left after proteolytic cleavage of the extracellular domain. The levels of both SECTM1 protein bands were also seen to be increased upon treatment with IFN-γ.

3.2. CD7 protein expression in KG1 and KG1a leukemic cell lines

The KG1 cell line was first established in 1978 from the cells of a 59 year old male patient suffering from acute myeloblastic leukemia. KG1 cells were able to form colonies in soft agar in a colony stimulating factor (CSF)-dependent manner and were able to differentiate after treatment with phorbol derivatives [34]. The KG1a variant cell line was subsequently established from the parent human AML cell line KG1 during

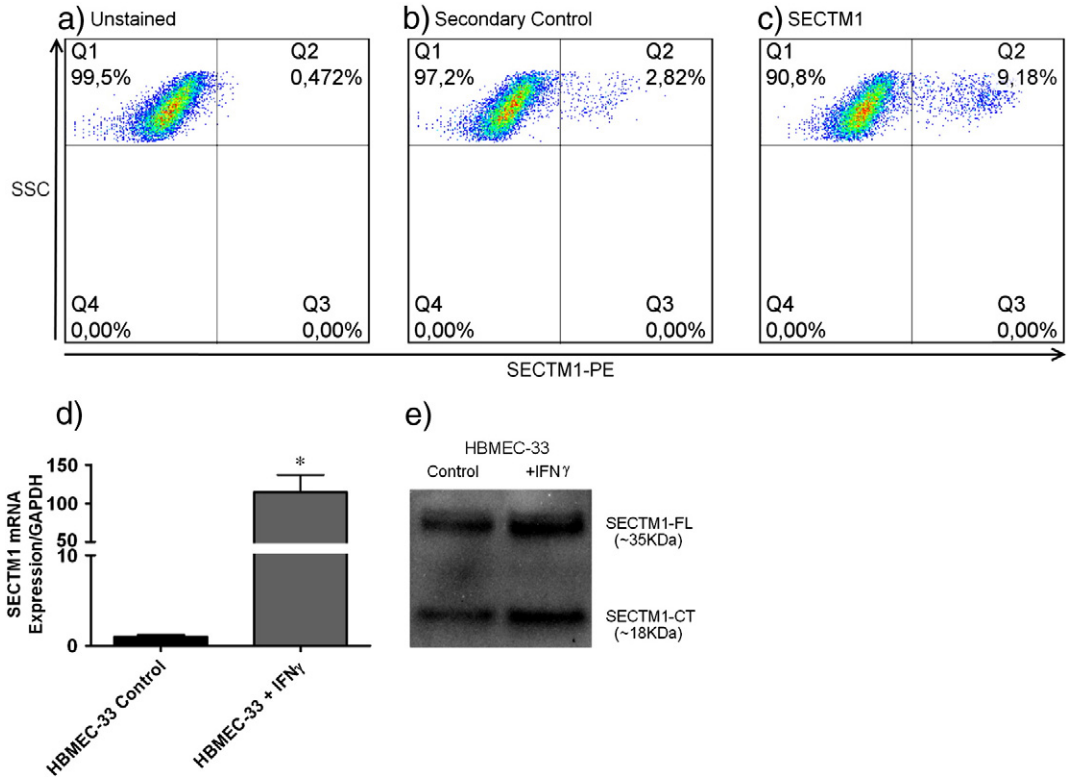

Fig. 1. Expression of SECTM1 protein and mRNA in bone marrow endothelial cells (BMEC). The immortalized human bone marrow endothelial cell line HBMEC-33 was cultured as described. Cells were detached from fibronectin coated plates and analyzed for surface staining of SECTM1 by flow cytometry (a–c). (d) RT-PCR was used to detect SECTM1 mRNA after stimulation of HBMEC-33 cells 24 h with IFN-γ. All data were normalized to a β-actin control and values are expressed as fold-changes relative to untreated cell controls $^*p < 0.05$. (e) Western blotting of HBMEC-33 total cell lysates with SECTM1 C-Terminal specific antibody.

routine subculture and although it is almost identical karyotypically [23] KG1a cells appear to be morphologically, cytochemically and functionally less mature than KG1 cells [23,35]. As a more 'immature' cell line KG1a also illustrates a different surface expression of certain differentiation markers than the parent KG1 cell line [23,35]. These differences in the immunophenotypes have been demonstrated, including the specific expression of the T cell antigen CD7 specifically on the surface of KG1a cells [23]. We confirmed CD7 cell surface expression on our KG1 and KG1a cell lines by flow cytometry using an anti-CD7-FITC antibody. While KG1 cells express very low levels ~6% (Fig. 2a) the KG1a cell line expresses high levels ~75% of CD7 surface expression (Fig. 2b).

3.3. SECTM1-Fc binding to the KG1a leukemic cell line

We expressed the secreted extracellular domain of SECTM1 as an Fc-fusion protein in HEK293T cells and purified the protein from conditioned media by affinity chromatography. Recombinant SECTM1-Fc subjected to SDS-PAGE under reducing and non-reducing conditions is resolved with apparent molecular weights of ~37 kDa and 60 kDa respectively suggesting that the protein exists as a disulfide bonded SECTM1-Fc dimer (Fig. 2c). To characterize the specific binding of SECTM1-Fc and demonstrate its functional activity, affinity-purified SECTM1-Fc binding to the KG1 and KG1a cell lines was measured by flow cytometry (Fig. 2d, e). The binding of SECTM1-Fc to cells was comparable to the staining observed with anti-CD7 antibody since SECTM1-Fc fusion protein bound weakly to the KG1 cell line (~9%) while KG1a cells stained with ~78% binding.

3.4. SECTM1-Fc inhibits autocrine production of GM-CSF by the KG1a cell line

Previously published data has demonstrated that antibody mediated ligation of the CD7 antigen on the surface of the KG1 and KG1a cell lines was able to upregulate the expression of GM-CSF [27]. We were unable to obtain this specific antibody clone however we tested two anti-CD7 antibodies, the commercially available mouse IgG2a clone 8H8.1 (Beckman Coulter) and the LAUA1/88 antibody previously described to upregulate GM-CSF on gamma delta T cell lines [28]. Since SECTM1 is the natural ligand for CD7 we also sought to determine the effect of SECTM1 mediated CD7 ligation on GM-CSF transcription. The KG1a cells incubated with 5 μg/mL of plate bound antibodies showed no significant differences compared to control cells, while to our surprise incubation with plate bound SECTM1-Fc significantly decreased the levels of GM-CSF mRNA transcripts (Fig. 3A).

Varying concentrations (0–10 μg/mL) of plate bound recombinant SECTM1-Fc protein were then incubated with KG1a cells for 24 h and GM-CSF transcription measured by RT-PCR. We observed that the levels of GM-CSF mRNA transcripts in the KG1a cell line decreased in a dose dependent manner with increasing amounts of SECTM1-Fc (Fig. 3B).

3.5. CD7 signaling inhibition by PI3K inhibitors

PI3-kinases participate in diverse cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. The CD7 receptor is known to signal in T cells through both the PI3K and PI4K pathways [36,37]. We therefore sought to determine if the constitutative signaling from CD7 leading to GM-CSF transcription could be influenced by the inhibitors wortmannin and LY294002. Wortmannin is a potent class I PI3K inhibitor at low concentrations ~100 nM while LY294002 is active at ~50 μM concentrations. Interestingly cells treated with wortmannin appeared to be unable to inhibit GM-CSF mRNA transcription at 100 nM while it was effective at 10 μM, a characteristic of the involvement of class II PI3K or PI4K since these enzymes commonly show variable responses to treatment with wortmannin (Fig. 3C). To ascertain the involvement of PI4K we also treated cells with spermine, a known PI4K activator [38] and looked

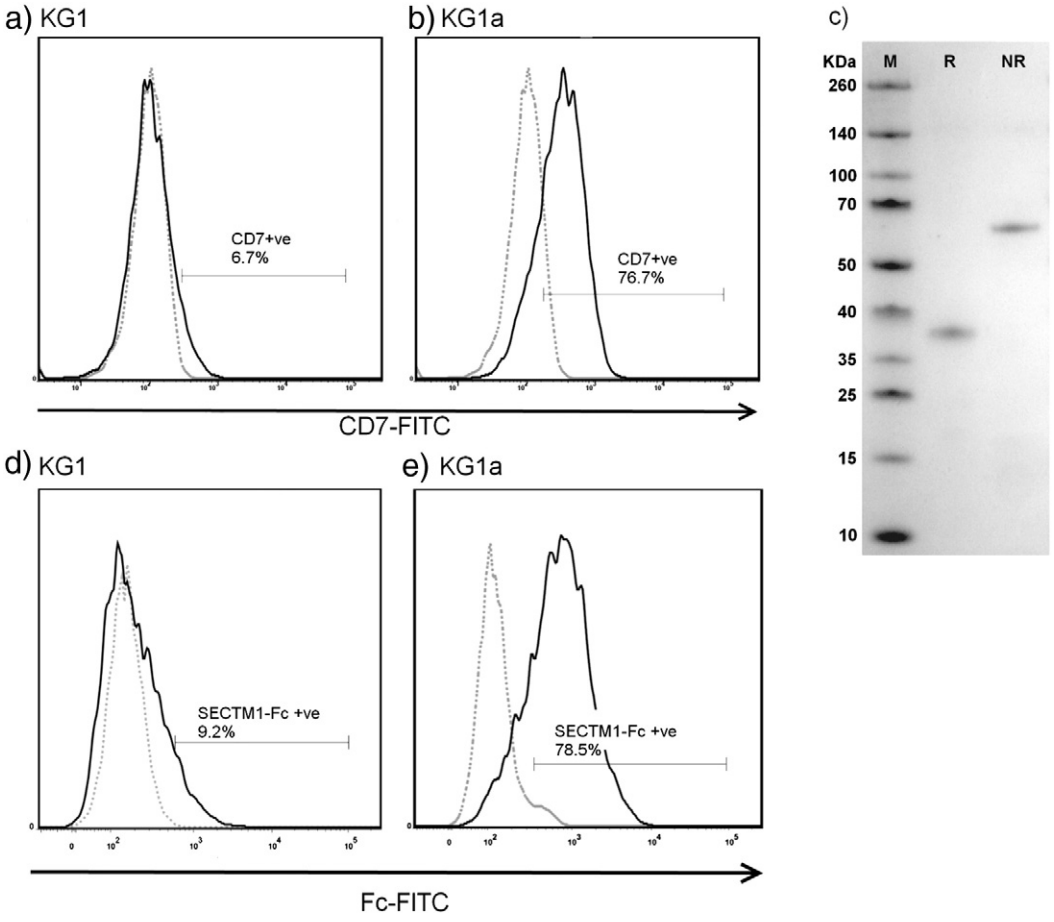

Fig. 2. CD7 surface expression and SECTM1-Fc binding in KG1/KG1a AML cell lines. Flow cytometry using CD7-FITC labeled antibody to illustrate the differential surface expression of CD7 between the parent KG1 (a) and variant KG1a (b) cell lines. c) SDS-PAGE gel stained with SimplyBlue showing markers M and purified recombinant SECTM1-Fc fusion protein under Reducing (R) and non-Reducing (NR) conditions, running with apparent molecular weights of ~37 kDa and 60 kDa respectively. d) Binding of SECTM1-Fc fusion protein to KG1 cells e) Binding of SECTM1-Fc fusion protein to KG1a cells. Gray dotted lines illustrate the isotype control and solid black line indicates SECTM1-Fc binding.

for increases in GM-CSF mRNA expression levels. Cells treated with low levels of spermine (10 μM) illustrated little increase in GM-CSF transcription however when high levels (100 μM) were used then cells upregulated GM-CSF mRNA 3 fold (Fig. 3C).

3.6. Effects on the downstream Akt signaling pathway

Many of the functions of PI3-kinases are attributed to its downstream activation of protein kinase B (PKB, Akt). To determine the activation state of Akt we performed flow cytometry analysis of KG1a cells treated with wortmannin, LY294002 and SECTM1-Fc. We observed that the treatment of KG1a cells with PI3K inhibitors or SECTM1-Fc reduced the levels of Akt phosphorylation compared to control cells (Fig. 3D) suggesting that SECTM1-Fc binding to CD7 on KG1a acts as a negative regulator of the downstream Akt signaling pathway.

3.7. Lentiviral transduction of the CD7-cell line KG1 to generate KG1-CD7 + cell line

To determine if the expression of CD7 in the KG1 cell line would generate a cell line capable of autocrine secretion of GM-CSF we transduced KG1 cells with a lentivirus encoding the full-length CD7 gene. A cell line expressing surface CD7 >98% was produced and purified from non-transduced cells by using magnetic microbeads (see Materials and methods). The KG1-CD7 + cell line shows high levels of CD7 mRNA and protein expression as determined by RT-PCR and western blotting (Fig. 4A & B). The transduced cells also demonstrated high CD7 cell surface expression as judged by flow cytometry (Supplementary Fig. 1). Although the KG1-CD7 + cell line produced 2.1 fold more GM-CSF mRNA transcript than the parent KG1 cell line (Fig. 4C), this is still significantly less than the KG1a cell line which produces 60-fold more GM-CSF mRNA transcripts (Fig. 4C).

3.8. Lentiviral silencing of CD7 in the KG1a cell line

We performed the reciprocal experiment and silenced the CD7 antigen in the KG1a cell line by lentiviral transduction of an shRNA cassette specific for CD7. The KG1a-shCD7 cell line shows reduced levels of CD7 mRNA (Fig. 4A), with a reduction from 90.1 to 50.3 fold relative to the KG1 cell line (~45% silencing at the mRNA level) and visible reduction at the protein level by western blotting (Fig. 4B). Reduced surface expression was also seen in flow cytometry (Supplementary Fig. 2). The KG1a-shCD7 cell line produced significantly less GM-CSF mRNA transcript than the parent KG1a cell line (Fig. 4C).

3.9. Microarray analysis of KG1 and KG1a cell lines

To determine the differential sets of transcripts found in KG1 and KG1a cells mRNA samples were prepared and analyzed using Affymetrix Genechips (see Materials and methods section). The CD7 antigen was seen to be significantly expressed in KG1a in comparison to KG1 cells (Supplementary Table 1). We then searched the transcript data for known transcriptional regulators of the GM-CSF promoter ETS-1, ETS-2, RUNX1, NFκB and AP-1 and found significant differences in the expression of the ETS-1 transcription factor (Supplementary Table 1).

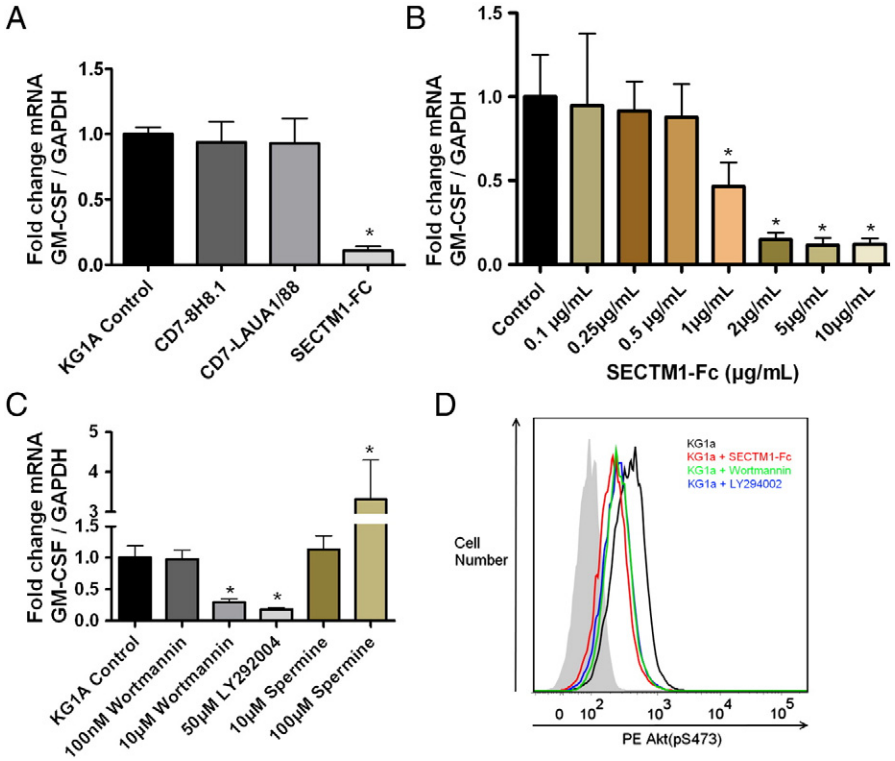

Fig. 3. CD7 mediated PI3K signaling affects GM-CSF expression in KG1a cells. A) RT-PCR showing the expression of GM-CSF after treatment of KG1a cells with plate bound anti-CD7 antibodies (5 μg/mL) or recombinant SECTM1-Fc (5 μg/mL), *$p < 0.05$. B) RT-PCR showing the dose dependent decrease in the expression of GM-CSF after treatment of cells with increasing amounts of SECTM1-Fc fusion protein *$p < 0.05$. C) Treatment of KG1a Cells with PI3K/PI4K inhibitors (wortmannin and LY292004) and the PI4K activator spermidine *$p < 0.05$. D) Flow cytometry of Akt activation using PE-Akt(pS473) antibody. Unstained KG1a cells (gray shaded histogram, MFI 89), KG1a (black histogram, MFI 411), KG1a + 5 μg/mL plate bound SECTM1-Fc (red histogram, MFI 239), KG1a + 10 μM Wortmannin (green histogram, MFI 282) and KG1a + 50 μM LY292004 (blue histogram, MFI 275).

3.10. Confirmation of microarray analysis for ETS-1 by RT-PCR and western blotting

To confirm the differential expression of ETS-1 suggested by the microarray data we performed RT-PCR using Taqman assays (Fig. 4D) and western blotting of KG1, KG1a and transduced cell lysates with an ETS-1 specific antibody (Fig. 4B). The results confirmed that there is very low levels of ETS-1 in KG1 cell lines, while KG1a derived cell lines contained significantly higher levels of both ETS-1 mRNA and protein.

3.11. Lentiviral silencing of ETS1 in the KG1a cell line

We performed lentiviral mediated silencing of ETS-1 in the KG1a cell line using previously described shRNA sequences [32]. Silencing was assessed by RT-PCR and western blotting with ETS-1 specific antibodies (Fig. 4B & D), reducing the ETS-1 expression from 5.3 to 2.7-fold relative to the KG1 cell line (~50%). As expected ETS1 silencing also significantly reduced the level of GM-CSF transcript from the parent KG1a cell line (Fig. 4C).

3.12. GM-CSF mRNA transcripts and protein secretion by AML cell lines

The autocrine production of GM-CSF by the 5 leukemic cell lines was assessed through detection of GM-CSF mRNA transcription by RT-PCR. We therefore sought to confirm differences at the protein level by ELISA measurement of the secretion of GM-CSF into the cell culture media. Cells were grown for 4 days in the presence or absence of 5 μg/mL plate bound SECTM1-Fc and supernatants used for ELISA measurement (Table 1). KG1 and KG1 transduced cells showed no detectable GM-CSF protein in their supernatants, while KG1a cell lines showed low levels of 8–11 pg/mL. KG1a cells cultured in the presence of 5 μg/mL SECTM1-Fc also showed no detectable GM-CSF secretion (Table 1).

3.13. Phosphorylation status of ETS-1

The human *ETS-1* gene produces two major protein isoforms, a 51 kDa protein (p51 Ets-1) and a 42 kDa protein (p42 Ets-1) and the activity of these Ets-1 isoforms is known to be regulated by isoform specific phosphorylation. The phosphorylation by ERK1/2 at Thr38 is activitory whereas phosphorylation by calmodulin-dependent kinase II (CaMKII) and myosin light-chain kinase (MLCK) at Ser251, Ser257, Ser282, Ser285 stabilizes the auto-inhibitory module thereby lowering the affinity of ETS-1 towards its DNA binding sequence [39]. We therefore performed western blotting of cell lysates using an antibody specific for p-S282/85 to compare the ETS-1 phosphorylation status (Fig. 5A). To determine the effects of SECTM1-Fc on the ETS-1 phosphorylation status KG1a cells were incubated with 5 μg/mL plate bound SECTM1-Fc for 24 h. As can be seen in Fig. 5B, SECTM1-Fc treated cells demonstrated an increased level of inhibitory phosphorylation.

3.14. Expression of GM-CSF, CD7 and ETS1 in differentiation capable KG1a cells

We have previously shown that KG1 and KG1a cells illustrate differences in the expression of PKC isoforms and that expression of PKC-βII allows KG1a cells to undergo DC lineage commitment in response to PMA stimuli [26]. KG1a cells transfected with a GFP vector control or with PKC-βII-GFP were assessed for their expression of CD7, GM-CSF, and ETS1 mRNA transcripts (Fig. 6A, B and C).

As can be seen from Fig. 6A PKC-βII-GFP expressing cells showed complete loss of CD7 mRNA expression levels compared to the GFP vector control. Surface staining of cells and analysis by flow cytometry confirmed that PKC-βII-GFP overexpression generated a CD7-ve phenotype (Fig. 6D) suggesting that PKC-βII activity is able to negatively regulate CD7 expression in KG1a cells. The mRNA expression level of GM-CSF in PKC-βII-GFP overexpressing cells decreased dramatically compared

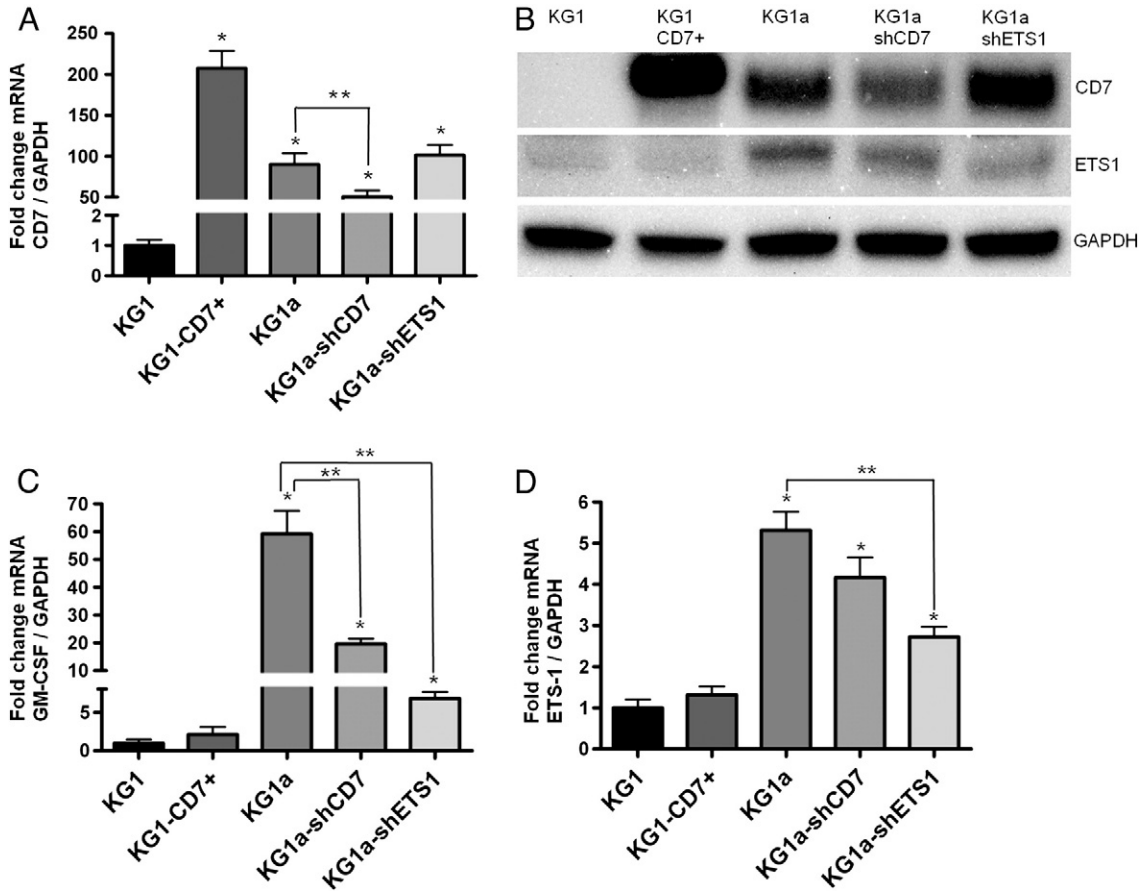

Fig. 4. Comparison of the KG1, KG1a and transduced cell lines. RT-PCR showing the mRNA expression of A) CD7, C) GM-CSF D) ETS-1 in the 5 cell lines studied *$p < 0.05$ compared to KG1, **$p < 0.05$ compared to KG1a. A) Western blotting of cell line lysates illustrating the levels of CD7 and ETS1 in the 5 cell lines.

to the GFP control vector, with almost no expression observed (Fig. 6B). A small decrease in ETS-1 mRNA for PKC-βII-GFP overexpressing cells was also observed relative to the GFP control cells (Fig. 6C), although significant differences in the ETS-1 protein level were not observed in western blotting of cell lysates (Fig. 6E).

4. Discussion

The CD7 antigen has been well studied as a T/NK cell co-receptor and has roles in cellular activation, proliferation, cytokine secretion and adhesion. CD7 is considered as one of the earliest T cell specific markers and is found on early hematopoietic progenitors and is a common leukemic marker, however the true function of CD7 in hematopoietic development still remains unclear.

The tissue expression of the CD7 ligand SECTM1 has been shown to include both the bone marrow and thymus, suggesting that SECTM1 expression in these microenvironments could play a distinct role in both hematopoietic and T cell development. Indeed expression data shows SECTM1 to be specifically expressed in the bone marrow during granulocytic differentiation [40]. Although SECTM1 has predominantly been described as a secreted molecule surface expression was also detected in transfected cell lines [13]. Our observations confirmed that SECTM1 is also expressed on the surface of bone marrow endothelial cells (BMEC), albeit at very low levels (Fig. 1c). SECTM1 mRNA and protein were both inducible upon treatment of cells with IFN-γ and the secreted domain appears to be proteolytically processed since western blotting using an antibody directed against the C-terminal domain of SECTM1 illustrates two bands that correspond to the molecular weights of the full-length protein and remaining "membrane tethered" form left over after proteolytic processing.

A role for CD7 in regulating the expression of GM-CSF in the KG1 and KG1a leukemic progenitor cell lines has been inferred as previous work and illustrated that antibody mediated ligation of CD7 was able to upregulate the expression of GM-CSF mRNA transcripts [27]. We were unable to obtain the anti-CD7 antibody (clone MoAb69) used in this study, however in our hands two other plate bound anti-CD7 antibodies illustrated no effect on GM-CSF mRNA levels (Fig. 3A). One of the antibodies we tested (LAU-A1/88) was previously shown to upregulate GM-CSF mRNA transcription in the gamma delta T cell line MOLT-13 [28], but only in the presence of Phorbol myristate acetate (PMA). In contrast to antibody treatment, stimulation using recombinant SECTM1-Fc protein demonstrated a significant downregulation of the constitutative GM-CSF mRNA (Fig. 3A, B) and protein expression (Table 1) in KG1a cells. The negative regulation of GM-CSF in KG1a leukemic progenitors by SECTM1-Fc was somewhat unexpected, we therefore checked to see if SECTM1-Fc was able to regulate GM-CSF transcription when incubated with human Peripheral Blood Mononuclear Cells (PBMCs).

Table 1
The GM-CSF ELISA assay was performed in triplicate, the means and standard deviations are presented.

Cell line	pg/mL GM-CSF	Standard deviation
KG1	<1	ND
KG1 CD7+	<1	ND
KG1a	10.83	±1.11
KG1a shCD7	10.53	±0.61
KG1a shETS1	8.71	±0.61
KG1a + 5 μg/mL SECTM1-Fc	<1	ND

Abbreviation: ND, not determined.

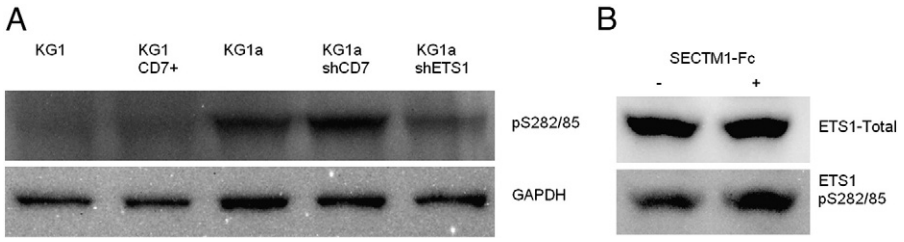

Fig. 5. ETS-1 phosphorylation in the KG1, KG1a and transduced cell lines. A) Western blot illustrating the ETS1 inhibitory phosphorylation at pS282/85 in the 5 cell lines. B) Western blot of KG1a cells cultured in the absence or presence of SECTM1-Fc, illustrating an increase of inhibitory phosphorylation at pS282/85 in treated cells.

However the incubation of SECTM1-Fc with PBMCs increased the level of GM-CSF mRNA (Supplementary Fig. 3) highlighting that there are functional differences between the different CD7 expressing cell types. We suspect that these functional differences may relate to the local environment surrounding CD7 on the surface of different cell types. Indeed CD7 has been shown to be associated with both CD3 and CD45 on T-cells [41], however these molecules are unlikely to be associated on NK cells or on leukemic cells, which could lead to different signaling capabilities and regulation.

The transduction of KG1 cells which lack CD7 with lentivirus encoding full-length CD7 was not sufficient to generate the same constitutative transcription of GM-CSF mRNA we observed in KG1a cells (Fig. 4C), highlighting that an additional signaling pathway/transcriptional differences between the KG1 and KG1a cell lines are also required. The regulation of the GM-CSF promoter is complex [42] but is known to be activated by a variety of transcription factors including ETS-1 [43], ETS-2 [44], RUNX1 [45], NFκB and AP-1 [46]. Subsequent microarray analysis highlighted that the transcription factor ETS-1

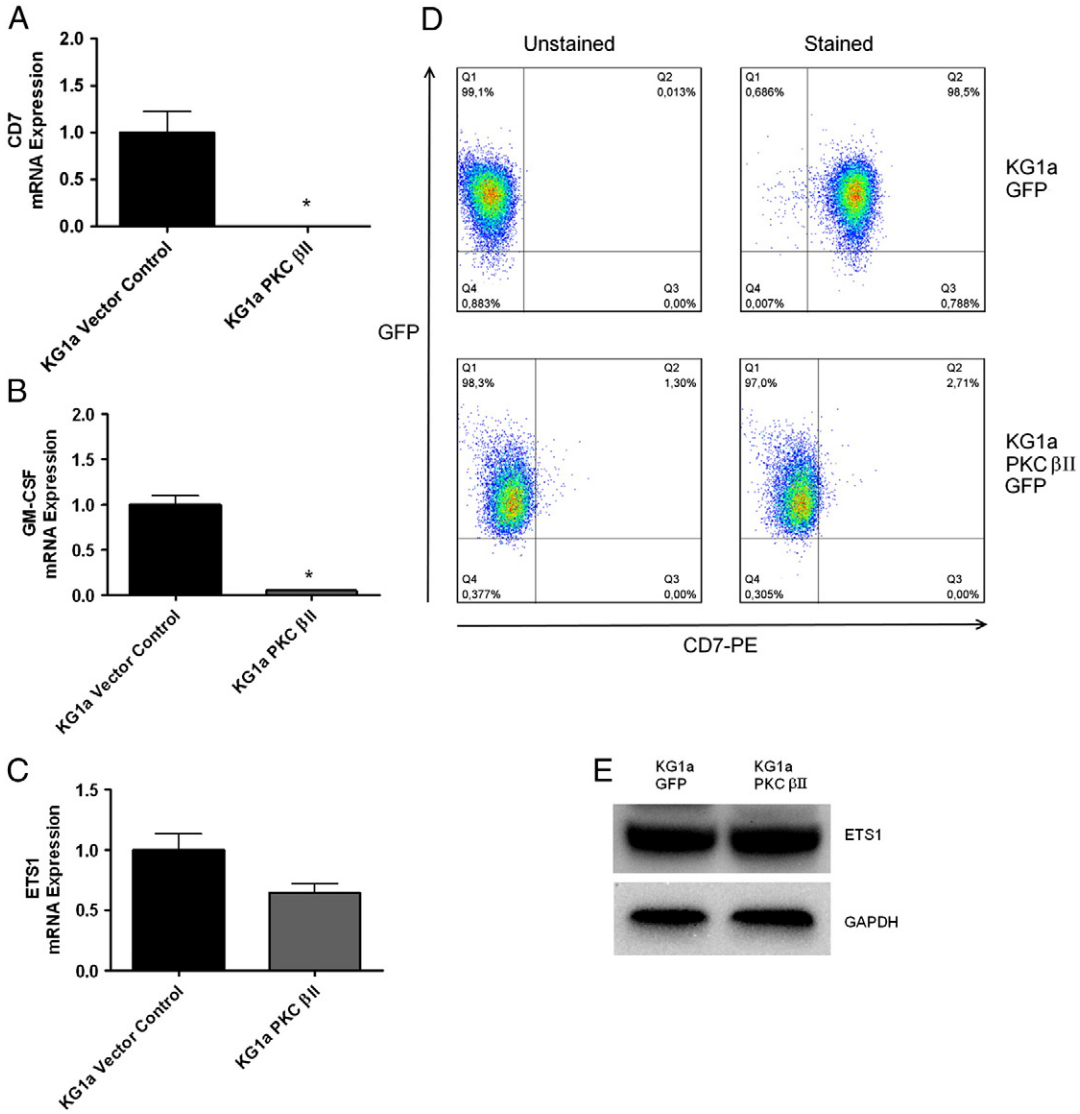

Fig. 6. Comparison of differentiation capable and incapable KG1a cell lines. RT-PCR showing the mRNA expression of A) CD7, B) ETS1, C) GM-CSF, D) Surface staining of CD7, E) ETS-1 protein expression levels in the KG1a cell lines stably transfected with GFP (control vector) and PKCβII-GFP. *$p < 0.05$.

might be a requirement to mediate the effects of CD7 signaling on transcription since we observed significantly higher expression levels in KG1a cells compared to KG1 (Supplementary Table 1).

ETS-1 is a member of the ETS family of transcription factors and binds to conserved DNA sequences to influence the expression of specific genes involved in hematopoietic development, angiogenesis, and tumor progression, for a detailed review see [39]. ETS-1 is not known to be a direct mediator of CD7 signaling, however it has been demonstrated that ETS1 transcription can be upregulated through a PI3K/Akt dependent pathway in certain cell types [47]. We observed in KG1a cells that constitutative CD7 mediated PI3K/Akt activation leads to expression of GM-CSF mRNA and protein. These low levels of GM-CSF could however be inhibited by treatment of cells with either the PI3K inhibitors (wortmannin and LY294002) or incubation of cells with recombinant SECTM1-Fc protein as illustrated in Fig. 3C and depicted schematically in Supplementary Fig. 4. The high CD7 expression in KG1a also appears to correlate with high expression levels of ETS-1 mRNA and protein, while in KG1a-shCD7 or KG1 cells loss of CD7 corresponds to lower levels of ETS-1 (Fig. 4B, D) consistent with possible regulation of ETS-1 expression by CD7 mediated signaling.

Recently ETS-1 over-expression has been characterized in CD34+ hematopoietic progenitor cells derived from patients with acute myeloid leukemia (AML) and decreased expression of ETS-1 was shown to be associated with differentiation of leukemic cells [48]. Unfortunately in this study the CD7 positivity of the patient cells was not measured however it would be interesting to investigate if a correlation between the levels of CD7 and ETS-1 and the differentiation potential of certain leukemic cells exists.

It is well known that the KG1a cell line is resistant to differentiation, while KG1 cells are able to undergo DC differentiation mediated by either cytokines [49] or direct activation of Protein kinase C (PKC) βII [26]. The activation of PKC isoforms (α, β and γ) appears to influence how haematopoietic cells respond to inducers of cell growth and differentiation and can show variability between leukemic cell types. Extensive clinical data suggests that atypical AML blasts expressing T cell antigens such as CD2 and CD7 have significantly lower PKC activities compared to typical AML blasts consistent with the theory that such leukemic cells are derived from transformation of early progenitors and that PKC activity is maturation linked [50]. Interestingly we observed that KG1a cells stably transfected with PKC βII completely lost their CD7 expression (Fig. 6A, D) directly indicating a role for PKC βII activity in the negative regulation of CD7 expression. The expression of CD7 has been linked to CCAAT/enhancer binding factor alpha (CEBPA) regulation in conditional knockout experiments in mice, where expression of CD7 is upregulated in the absence of CEBPA [51]. Recently Rohrs et al. confirmed that the inverse correlation between CEBPA and CD7 expression reported for many primary AML cells also occurs in a majority of AML cell lines, although the primary factor responsible for repression of CD7 was promoter methylation [52]. How overexpression of PKC βII in KG1a is able to regulate the CD7 promoter is presently unclear and requires further investigation.

The various timepoints in hematopoiesis can be represented by the many leukemic cell lines that have been established over the past few decades and although we must remember that these cells are genetically abnormal the snapshots that they provide have given us a wealth of information. Here we have studied only one such timepoint in the KG1/KG1a cell lines and future work confirming the effects of SECTM1 on patient leukemic blasts is needed to confirm what we have observed in KG1a cells. The importance of SECTM1 in the bone marrow microenvironment and its effects on normal hematopoietic and leukemic progenitors is still poorly understood. That SECTM1 has been described as a detectable biomarker in blood and urine samples [53] may allow correlation of the expression level of SECTM1 with the maintenance of leukemic cells or determine if SECTM1 expression might be a factor that is detrimental to successful bone marrow transplantation.

5. Conclusion

These findings add an additional layer to the previously described autocrine/paracrine signaling between leukemic progenitor cells and the bone marrow microenvironment and highlight a role for SECTM1 in both normal and malignant hematopoiesis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.10.043.

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