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Enforced expression of E47 has differential effects on Lmo2-induced T-cell leukemias

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Abstract

LIM domain Only-2 (LMO2) overexpression in T cells induces leukemia but the molecular mechanism remains to be elucidated. In hematopoietic stem and progenitor cells, Lmo2 is part of a protein complex comprised of class II basic helix loop helix proteins, Tal1 and Lyl1. The latter transcription factors heterodimerize with E2A proteins like E47 and Heb to bind E boxes. LMO2 and TAL1 or LYL1 cooperate to induce T-ALL in mouse models, and are concordantly expressed in human T-ALL. Furthermore, LMO2 cooperates with the loss of E2A suggesting that LMO2 functions by creating a deficiency of E2A. In this study, we tested this hypothesis in Lmo2-induced T-ALL cell lines. We transduced these lines with an E47/estrogen receptor fusion construct that could be forced to homodimerize with 4-hydroxytamoxifen. We discovered that forced homodimerization induced growth arrest in 2 of the 4 lines tested. The lines sensitive to E47 homodimerization accumulated in G1 and had reduced S phase entry. We analyzed the transcriptome of a resistant and a sensitive line to discern the E47 targets responsible for the cellular effects. Our results suggest that E47 has diverse effects in T-ALL but that functional deficiency of E47 is not a universal feature of Lmo2-induced T-ALL.

Keywords

LMO2; E2A; T-ALL; T-cell leukemia

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Introduction

The LIM domain Only-2 (LMO2) gene is frequently deregulated in human acute T-cell lymphoblastic leukemias (T-ALL). LMO2 was first cloned from T-ALLs with chromosomal translocation breakpoints at 11p13 (1) but more recently, LMO2 and its paralog, LMO1, have been found to be deregulated by diverse mechanisms. LMO2, the best characterized member of the gene family, encodes an 18 kilodalton (kDa) protein that has two Zinc-binding domains called LIM domains that serve as interfaces for interactions with class II basic helix-loop-helix (bHLH) proteins, GATA proteins (1-3), and the scaffolding protein, LIM domain binding 1 (Ldb1)(2, 3). Lmo2's protein function has been best described in erythroid progenitor cells where it has been shown to be part of a macromolecular complex at E box-GATA sites within enhancers and promoters of target genes. In T-ALL, the LMO2-associated complex may differ in content from the complex described in erythroid cells and may occupy sites other than E box-GATA motifs (4-6). Nevertheless, gene expression studies of human and murine T-ALL show remarkable correlation between gene expression of LMO2 (and LMO1) and class II bHLH genes, TAL1, LYL1, or OLIG2, implying that LMO2 and the bHLH proteins cooperate in T-ALL pathogenesis (7, 8). Indeed, Tal1/Scl transgenes are weak tumor initiators but the co-expression of LMO genes can accelerate T-ALL onset and increase penetrance in these mouse models (9-11).

The class II bHLH proteins heterodimerize with the more ubiquitously expressed class I bHLH E proteins, E47, E12, E2-2, and Heb (TCF12) (12). E47 and E12 are encoded by the E2A gene (i.e. TCF3) and are identical except for residues at the carboxyl-terminus that result from alternative splicing (13). Heb is encoded by the TCF12 gene and also has multiple isoforms from alternative splicing and promoter usage (14). Mice with knockout of the E2A gene show profound defects in the generation and maintenance of multipotent progenitor cells and in the development of T- and B-cell lineages(15). Heb^{-/-} mice have pronounced defects in T-cell development whereas E2-2^{-/-} (i.e. TCF4) mice have normal numbers of mature T- and B-lymphocytes (16-18). Most strikingly, E2A^{-/-} mice spontaneously develop T-ALL. E2A^{-/-} and Heb^{-/-} thymocytes have a differentiation arrest at a similar developmental stage as Lmo2-overexpressing thymocytes, the double negative stage 2-3 prior to expression of CD4 and CD8 co-receptors (19, 20). This has led to a paradigm in understanding Lmo2 oncogene function. Since Lmo2 appears to require class II bHLH genes to induce T-ALL (21), the Lmo2/Tal1(or Lyl1)-associated complex may bind and redirect E2A proteins away from their normal targets. Alternatively, an Lmo2-associated complex may recruit cofactors different from those recruited by E2A homodimers at the same target genes, such as co-repressors in place of co-activators; or, Lmo2 and E2A deficiency may cooperate and separate parallel pathways (22-24). Whether by sequestration or by recruitment of alternate co-factors, current hypotheses suggest that Lmo2 causes functional E2A deficiency (25). There is compelling evidence from mouse models supporting this idea. As mentioned, Lmo2 and class II bHLH genes are concordantly expressed in mice where Lmo2 expression is enforced by retroviral insertional mutation or transgenesis (5, 26). Lmo2 transgenic mice that are heterozygous for E2A or Heb deletion, show accelerated T-ALL onset in comparison with mice with wild type E2A and Heb alleles (20, 27). There is equally compelling evidence that Lmo2 transcriptionally regulates a

cohort of factors responsible for hematopoietic stem cell-like features; and this function for Lmo2 may not be mutually exclusive with its effects on E2A (5, 28, 29).

In this study, we analyzed the effects of enforced expression of E47 in T-ALL cell lines derived from CD2-Lmo2 transgenic mice (5). Since wild type E47 is not tolerated by T-cell leukemia lines, we enforced expression of an E47/estrogen receptor fusion protein that could be forced to homodimerize with 4-hydroxytamoxifen (4-HT) (30). Prior studies show that E47 replacement into T-ALLs derived from E47^{-/-} induced cell death (30). We expected similar findings in Lmo2-induced T-ALL if functional deficiency of E47 was the mechanism of transformation, but in striking contrast to this model, we observed diverse effects of E47 on Lmo2-induced T-ALL lines. Our results suggest that E47 functional deficiency is not a universal mechanism by which Lmo2 induces leukemia.

Materials and Methods

Cell culture and transductions

Four B6 T-ALL cell lines; 007,020, 027, and 080 were maintained in IMDM medium containing 10% fetal bovine serum and 1% penicillin and streptomycin. For E47-ER stably expressing lines, 007,020, 027, and 080 cells were transduced with E47-ER using spinfection and then selected with hCD25. The E47/estrogen receptor (E47-ER) (31, 32) plasmid was graciously provided by Dr. Cornelis Murre (UC San Diego, California). To produce virus, the E47-ER and pCL-Eco plasmids were co-transfected into the Phoenix packaging cell line (ATCC) using calcium phosphate precipitation as described (33, 34). Viral supernatant was collected 48 hours after transfection from transfected Phoenix cells and titered on 3T3 cells by FACS and detection hCD25. The T-ALL cell lines were transduced by spinfection; cells were pelleted at 2000 rpm with 8 µg/mL of polybrene for 1 hour at 4°C. On average, 50-90% of virally infected cells expressed hCD25 at the cell surface. CD25-expressing cells were sorted 48 hours after transduction and maintained in culture for experiments. THE Protein 4.2 luciferase construct was kindly provided by Dr. Stephen Brandt. It was transfected at 1.5 µg per 10⁶ U2OS cells along with 50 ng of pcDNA3.1-E47 or MSCV-E47/ER-ires-hCD25 and 1 ng of pCMV-Renilla. 48 hours after transfection, the cells were lysed in passive lysis buffer (Promega) and aliquotted to be read with a dual luciferase luminometer. All transfections were done in triplicate and luciferase values were normalized to Renilla luciferase. Statistics were done using GraphPad Prism 6.0.

Antibodies, cell cycle, and apoptosis analysis

We analyzed growth by counting cells directly by hemavet and by CyQuant Cell Proliferation Assay kit (Life Technologies, Grand Island, New York). Western blot analysis was done with anti-E47 (sc-763, Santa Cruz Biotechnology), anti-Lmo2 (monoclonal antibody provided by Dr. Ron Levy, Stanford), and anti-tubulin (sc-55529, Santa Cruz Biotechnology) antibodies. FACS analyses were done using anti-CD4 (FITC-conjugated Rat anti-mouse, 553650, BD Pharmingen) and anti-CD8 (PE Rat anti-mouse, 55032, BD Pharmingen). Sorting was done using FITC-conjugated anti-hCD25 (347643, BD Biosciences). Quantification of proteins by Western blotting was done using 680nm and

800nm infrared dye-conjugated secondary antibodies (LI-COR) on the Odyssey machine. Cleaved caspase 3 was analyzed by FACS using antibody 9661 per manufacturer's instructions (Cell Signaling Technology). FACS data was imported into Flojo for further analysis. Bromodeoxyuridine (BrdU) incorporation was analyzed per manufacturer's instructions (BD Biosciences, San Jose, California) as previously described (29). Intracellular cleaved caspase 3 staining was performed using the BD Cytoperm/ Cytofix kit (BD Biosciences, San Jose, California). Statistical analyses were done using GraphPad Prism 6.0.

Gene expression analysis

Total RNA was purified by TRIzol (Life Technologies, Grand Island, New York) per manufacturer's instructions. For standard qRT-PCR, first strand cDNA was synthesized using oligo-dT primers, random hexamers and reverse transcriptase enzyme (Omniscript, Qiagen, Valencia, California) followed by quantitative PCR on cDNA using Sybr green (Bio-Rad, Hercules, California), primers shown in Table S6, and the MyIQ (Biorad).

Results

Enforced homodimerization of E47-ER causes growth arrest of some but not all Lmo2-induced T-cell leukemias

To directly test whether E47 is required in Lmo2-induced T-cell leukemia, we enforced expression of a chimeric E47 protein, E47-ER, fused with the ligand binding domain of the estrogen receptor and expressed in a retroviral vector (32, 35). This chimeric protein construct contains amino acids 1-651 of human E47 fused to amino acid 251-599 of the murine estrogen receptor. This retroviral vector has been extensively used to identify E47 transcriptional targets in hematopoietic cells (Figure 1A) (31, 36-38). The E47-ER vector also encodes the human CD25 (ires-hCD25) which allows for rapid selection of transduced cells. E47-ER is inactive until 4-hydroxytamoxifen (4-HT) releases the fusion protein from chaperones and induces homodimerization(39). We co-transfected wild type E47 or E47-ER expression constructs along with the P4.2 luciferase reporter which has 2 E boxes within the promoter sequence (40). E47 was able to activate transcription (see lane 2, Figure 1B) of this construct whereas E47-ER was inactive until 300 nM 4-HT was added to the media (compare lanes 4 and 5, Figure 1B, Student t-test, $P=0.001$). 4-HT had no effect on E47-induced transcription (lane 3, Figure 1B).

We transduced 4 Lmo2-induced T-cell leukemia cell lines (03007, 03020, 03027, 32080, abbreviated 007, 020, 027, and 080) with a gammaretroviral vector expressing E47-ER-ires-hCD25 and sorted hCD25⁺ cells. The cell lines tested had constitutive intracellular Notch1 by Western blot (data not shown) and all lines except for 007 had mutations in exon 34 in the carboxyl-terminal PEST domain (020: S2398stop, 027: L2276Q, 080: 68 base pair duplication)(41). All of the lines stably expressed hCD25 allowing them to be flow sorted (Figure 1C). Stable cell lines expressing E47-ER (designated 007-E47, 020-E47, 027-E47, 080-E47) showed no change in their growth rates compared to the parental lines and they expressed E47-ER at comparable levels as analyzed by Western blot (see lanes 2, 6, 10, and 14 in Figure 1D). We treated the cells with 300 nM of 4-HT which increased E47-ER

protein (see lanes 4, 8, 12, 16 in Figure 1D). We analyzed the growth of the parental lines and stable lines expressing E47-ER with or without 4-HT. Cell lines 007 and 027 showed the same growth pattern as stable 007-E47 and 027-E47 (Figure 2). 4-HT treatment did not affect growth of the parental lines (red lines in Figure 2) or growth of the stable lines expressing E47-ER (orange lines in Figure 2). In contrast, stable cell lines 020-E47 and 080-E47 had markedly attenuated growth with 4-HT treatment (day 7 cell counts compared with 4-HT-treated parental cells by 2-way ANOVA, $P < .001$). Hence, forced dimerization of E47 affected some but not all Lmo2-induced T-cell leukemias.

Lmo2-induced T-cell leukemias sensitive to E47-ER homodimerization undergo G1 growth arrest

To understand the mechanistic basis for the attenuated growth observed in 4-HT-treated 020-E7 and 080-E47, we pulsed all the lines with bromodeoxyuridine (BrdU) and analyzed cell cycle profiles by FACS with anti-BrdU and 7-AAD. As shown in Figure 3A, a 45 minute pulse of BrdU revealed extensive proliferative activity in all the cell lines. Lines 007-E47, 020-E47, 027-E47, and 080-E47 showed comparable number of BrdU⁺ cells as the parental lines. 4-HT treatment showed a marked decrease in BrdU⁺ cells for 020-E47 and 080-E47 and a reciprocal increase in G0/G1 cell proportions (Figure 3B). We also analyzed the proportion of cells undergoing apoptosis as a result of forced homodimerization of E47-ER by intracellular staining for cleaved caspase 3. Cell line 007 actually showed decreased apoptosis with stable expression of E47-ER (see 007-E47 in Figure 4) and cell line 020-E47 showed decreased apoptosis with 4-HT treatment (Figure 4). There was no change for cell line 027 but line 080-E47 showed increased apoptosis with 4-HT treatment. Thus, cell lines sensitive to forced E47 homodimerization underwent G1 arrest and decreased S-phase entry but did not show a consistent pattern of apoptosis as a result of E47-ER homodimerization.

Forced E47-ER homodimerization activates expression of CD4

The Lmo2-induced T-cell leukemias established lines bear a resemblance to various stages in the T-cell developmental program based on their expression of CD4 and CD8 (42). Line 007 expressed CD8 only and was Intermediate Single Positive (ISP)-like. Line 020 expressed both CD4 and CD8 and was double positive (DP)-like. Line 027 resembled double negative (DN) cells which express neither CD4 nor CD8 and 080 cells had a mixture of cells that appeared like normal ISP and DP cells. The stable expression of E47-ER increased CD4 in 007 which was markedly increased with treatment of 4-HT (Figure 5). Line 020-E47 showed no change in CD4 or CD8 expression even after 4-HT treatment but expression of both antigens was high at baseline. Line 027-E47 showed increased expression of both CD4 and CD8 which did not change with 4-HT treatment. Line 080 showed no change with E47-ER expression but a marked increase in the proportion of CD4⁺ cells with 4-HT. The CD4 protein expression analyzed by FACS correlated with increased *CD4* mRNA abundance (qRT-PCR, Figure 5B) consistent with a transcriptional effect of E47-ER homodimerization. E47-ER activated expression of *CD4* in lines that were both sensitive (i.e. 080) and resistant (i.e. 007 and 027) to E47-ER-induced growth arrest. Interestingly, transcriptional profiling showed genes that were repressed with the addition of 4-HT such as *Il2ra* (-2 fold compared to 027-E47, -6.2 fold compared to 080-E47), *Cd24a* (-1.2 fold in 027-E47 and 080-E47), or activated by 4-HT such as *Cd8a* (1.93-fold in 080-E47-4HT v.

080-E47), suggesting that forced E47 dimerization promoted differentiation in Lmo2-induced T-cell leukemias, a process distinguishable from that of growth arrest.

Differential gene regulation by E47-ER in 080 and 027 cell lines

To further analyze the transcriptional effects of nuclear E47-ER, we performed RNA-seq on 027, 027-E47, and 027-E47-4HT and compared gene expression to 080, 080-E47, and 080-E47-4HT in order to identify E47 targets that may correlate with sensitivity to E47 dimerization. Our analysis showed 830 genes were differentially expressed between 027-E47 and 027-E47-4HT and 820 genes between 080-E47 and 080-E47-4HT at the corrected P-value less than .05. As shown in Table 1, previously described E47 targets were not differentially expressed unless the cells were treated with 4-HT confirming the tight regulation of the E47-ER fusion protein in the stable lines. In 027-E47 cells, 486 genes were downregulated and 334 were upregulated with 4-HT treatment but this distribution was significantly different in 080-E47 cells which showed 377 genes that were downregulated and 453 genes that were upregulated with 4-HT treatment (Figure 6, Chi square test, $P < .001$). A comparison of these two gene lists showed that 268 genes were in common (Figure 6). We hypothesized that among the 551 genes in 080 cells that were differentially expressed due to 4-HT treatment and not similarly regulated in 027-E47, some may account for the growth arrest phenotype. To pare down our gene lists, we performed pathway analysis on the differentially expressed genes between 027-E47 and 027-E47-4HT and 080-E47 and 080-E47-4HT by Ingenuity Pathway Analysis (<http://www.ingenuity.com>). E47 (i.e. TCF3) was identified as the upstream driver of the gene expression datasets for both cell lines, confirming the accuracy of the overall pathway analysis (43). Other top upstream regulators in common between 027 and 080 were Tp53, IL2, and Myc. Interestingly, upstream analysis of 027 showed consistent activation of genes downstream of Hras, E2F, and E2F1.

Discussion

Several lines of evidence from human T-ALL gene expression and mouse models of T-ALL suggest that LMO2 and its paralogs induce leukemia by causing a functional deficiency of E2A transcription factors, E12 and E47. E47^{-/-} mice spontaneously develop T-ALL which undergo cell death if E47 is transduced (30, 44). In this study, we followed this same logic, hypothesizing that if Lmo2 induces a functional deficiency of E47, then its enforced expression should cause similar cellular effects as observed in E47^{-/-} T-ALL. We enforced expression of an E47-ER fusion protein allowing inducible dimerization with 4-HT and established stable cell lines which grew like their parental counterparts. However, with the application of 4-HT, two of the cell lines underwent growth arrest and two of the cell lines continued to grow, oblivious of E47 expression. By BrdU labeling, we determined that the growth arrest was in the G1-S transition of the cell cycle. We observed activation of the *Cd4* gene in all of the lines independent of E47's growth inhibitory effects. Interestingly, apoptosis was induced by E47-ER only in the 080 line but this line showed higher levels of apoptosis in the CD4⁺ subset of cells without 4-HT treatment (42). Our results suggests that apoptosis is not a prominent cellular pathway employed by E47 in Lmo2-induced T-ALLs.

Our analysis of the global gene expression pattern showed remarkable similarity between a line that was resistant (i.e. 027) to E47-induced growth inhibition and a line (i.e. 080) that was sensitive. The induction and repression of genes involved in G1-S phase transition followed a similar pattern in both 027 and 080 cells and the G1/S checkpoint regulation pathway was a top canonical pathway regulated by E47 in both cell lines. Nevertheless, the end result of G1 growth arrest was only observed in 080 and 020. Indeed, variant analysis of the RNA-seq data revealed a Kras G12D mutation in 027 cells that was not present in 080 which could account for the activation of these pathways and resistance to E47's growth inhibitory effects.

The top canonical pathways in 027 cells were Molecular Mechanisms of Cancer ($P=1.19E-9$) and GADD45 signaling ($P=1.93E-8$) among others (Table S1). In 080 cells, the top canonical pathways were Glioblastoma multiforme signaling ($P=3.01E-6$), Granzyme A signaling ($P=3.68E-6$), and PTEN signaling ($P=8.68E-6$) among others (Table S2). The top pathway, Glioblastoma multiforme signaling, contained many genes in the PI3K/AKT pathway including *Pik3cg* and *Pik3cd* (Table S3) which were also represented in the PTEN signaling pathway enrichment (Table S5). These two paralogous genes had not been previously described as E47 targets but both mRNAs were repressed in 080-E47-4HT (1.53- and 1.56-fold, respectively) and did not change in 027-E47-4HT. Interestingly, the *Pik3cg* and *Pik3cd* enzymes are the targets of novel kinase inhibitors that induce apoptosis of human T-ALL cells(45). The Granzyme A pathway was significantly enriched in 080 cells due to the downregulation of the Histone 1 cluster (4 of the 20 genes in this pathway) in this cell line (Table S4). Linker histones are part of this pathway because they are directly digested by granzyme A. The Histone 1 cluster is transcriptionally upregulated with cell cycle entry and the whole cluster may be downregulated as a result of the G1 arrest in 080 cells (46). Alternatively the genes may be direct targets of E2A transcription factors (47).

Cell cycle G1/S checkpoint regulation was a top canonical pathway in both 027 ($P=1.56E-7$) and 080 ($P=6.01E-6$) cells (Table S1 and S2). Among those genes playing a role in G1-S phase transition, *Ccne1* was identified as a target in prior studies that was activated 1.8 fold by E47. Consistent with these findings, the 027-E47-4HT showed 2.4 fold increased *Ccne1* mRNA but 080-E47-4HT showed a decrease in *Ccne1* mRNA by 1.3 fold. Similarly, *Cdkn1b* mRNA was decreased in 027-E47-4HT 1.3 fold but was increased 1.6 fold in 080-E47-4HT. *Cdkn1a* (2 fold) and *E2F4* (1.5 fold) had comparable increases with 4-HT treatment (columns 8 and 9 in Table 1) as observed by Schwartz et al (37). Schwartz et al also generated a list of repressed genes such as *Cdk6* (-2.6 fold), *Myc* (-1.4 fold), and *Caspase 3* (-1.3 fold) which were downregulated to the same degree by 4-HT in 027-E47 and 080-E47. There were some genes that did not correlate with prior findings, like *Rb1*, which was induced 1.5 fold in E47-knockout lymphomas, but was repressed in 027-E47 (-1.75 fold) and 080-E47 (-2.5 fold). E47 replacement in *E47^{-/-}* lymphomas induced *Gfi1b* mRNA which in turn repressed *Gata3* (38). This same pattern was observed in both 027-E47-4HT and 080-E47-4HT (Table 1) but did not correlate with E47-induced growth arrest. *Trib2*, an oncogene identified recently by TAL1 ChIP-seq studies, was downregulated to the same degree in 020-E47-4HT and 080-E47-4HT (24).

The difference between 027 and 080 cells could be explained if E47's growth inhibitory effects in 080 were post-transcriptional. For example, *Cdkn2a* mRNA has no mutations and is highly expressed in the 080 line. It is possible that E47-homodimerization may affect expression of p16Ink4a protein, a potent inhibitor at the restriction point in G1 phase (48). We made multiple attempts to prove this by Western blotting but we could not detect p16 protein in 080 cells treated with 4-HT. An alternative explanation would be that 080 cells are more sensitive than 027 to the transcriptional effects of E47 homodimerization. Thus, the pattern of gene expression could be the same in 027 and 080 but the cellular effects would be markedly different. One prominent example is the *Cd4* mRNA which is upregulated in both lines (Table 1) but to different degrees which is revealed by protein expression analysis in 080 than 027 (see FACS in Figure 4). A regulator of the G1-S phase transition may be subject to similar transcriptional effects, like Rb1, which was downregulated by E47-ER homodimerization in both 027 and 080 cells but the repression was more pronounced in 080 cells (Table 1). E47 targets may have different chromatin states depending on their differentiation stage. It is interesting that the two Lmo2-induced T-ALLs sensitive to E47-induced growth arrest are more mature than their resistant counterparts. Both 080 and 020 cells resemble the double positive stage of T-cell development. Another possibility is that Kras (G12D) gain of function conferred resistance to E47's growth inhibitory effects. The experiments in this paper are focused on E47 homodimerization and it remains a possibility that the cell lines resistant to E47's growth inhibitory effects may be more dependent on Heb (18, 49). Heb mRNA was downregulated in both 027 and 080 cells with 4-HT (see last row in Table 1, P=NS).

Although the similarity of the E47-induced gene expression program between 027 and 080 cells was very striking, the global distribution of genes, upregulated versus repressed, was markedly different between the two lines. The 080 line had fewer genes that were downregulated compared to the 027 line, a statistically significant difference. This may be explained by the absence of key co-repressors in the 080 cell line. For example, Mtg16, a component of the Lmo2-associated complex, is expressed in 027 cells but not in 080 cells. Since it can bind E2A proteins and Heb, it could perturb the balance of these proteins either in macromolecular complexes or as homo- or heterodimers, which may ultimately affect the transcription of targets. Mtg16 is a relatively weak interactor with E47 compared with Heb and it appears to be dispensable for the development of Lmo2-induced T-cell leukemia since it is absent in the 080 line (50). Even so, it is required for normal T-cell development and it is part of the Lmo2-associated complex in erythroid progenitor cells (51). We were unable to isolate 080 lines that co-expressed E47-ER and Mtg16 which may imply that these two cooperate to induce growth arrest. Nevertheless, bona fide targets repressed by E47 identified in prior studies were effectively downregulated in 080-E47-4HT cells, suggesting that Mtg16 was not an essential co-repressor for these specific targets. Mtg8 was not present in the two lines analyzed but Mtgr1 was present; Mtgr1's expression did not change with E47-ER homodimerization but it may functionally compensate for the loss of Mtg16 in the 080 line. Hence, although the molecular basis for E47-induced growth arrest in Lmo2-induced T-ALL is still not clear, our results show that E47 deficiency is not a universal feature of Lmo2-induced T-cell leukemia. This finding supports an important role for Lmo2

in transcriptional regulation and argues for further analysis of its chromatin occupancy and its binding partners in T-ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We address the paradigm that E47 deficiency is the mechanism by which Lmo2 induces T-ALL.
- Enforced E47 homodimerization causes G1 arrest in some but not all Lmo2-induced T-ALL cells.
- E47 functional deficiency is not a universal feature of Lmo2-induced T-ALL.

Figure 1. Enforced expression of E47-ER in Lmo2-induced T-cell leukemias is stable

A, schematic shows the construct used in the experiments. An E47-ER fusion encoding cDNA followed by an internal ribosomal entry site followed by the human CD25 cDNA was driven by the LTR of a gammaretrovirus. B, bar graph shows the fold activation (mean $\pm$ SD) of P4.2-luciferase reporter above reporter alone in U2OS cells. The P4.2-luciferase reporter was transfected on its own (lane 1) or along with pcDNA3.1-E47 or the MSCV-E47-ER construct shown in panel A; lanes 3 and 5 show cells that were transfected and then treated with 300 nM 4-hydroxytamoxifen (4-HT) for 24 hours. The bar graphs show the mean of triplicate transfections. All luciferase readings were normalized to pCMV-Renilla to control for transfection efficiency and then expressed as fold over reporter alone (lane 1); statistical analysis comparing lanes 4 and 5 were by Student t test generating the P value shown. C, shows FACS plots of 4 Lmo2-induced T-ALL cell lines, 007, 020, 027, and 080 that were transduced with the retrovirus shown in A; x-axis showed expression of hCD25

and y-axis shows side scatter. D, whole cell lysates from the parental cell lines were subjected to SDS-PAGE and analyzed by Western for E47, tubulin, and Lmo2. The E47-ER fusion protein had slower migration than endogenous E47. Molecular weight markers in kilodalton (kDa) are shown to the left of the blot.

Figure 2. Forced dimerization of E47-ER with 4-hydroxytamoxifen induces attenuated growth in some but not all Lmo2-induced leukemias

The 4 Lmo2-induced T-ALL cell lines and those stably expressing E47-ER were analyzed for growth with or without 4-hydroxytamoxifen (4-HT). The growth curves and error bars represent three independent experiments. The y-axis shows growth in arbitrary fluorescence units. Each point of the growth curve was compared by 2-way ANOVA. Cell lines 007 and 027 showed no statistically significant differences at day 7 whereas 020-E47 and 080-E47 showed marked attenuation of growth with 4-HT treatment compared to 020 and 080, respectively ($P < 0.0001$).

Figure 3. Attenuated growth due to E47-ER dimerization is due to G1 arrest

A, Cell lines, either parental or stably expressing E47-ER, were treated with 4-hydroxytamoxifen (4-HT) for 24 h and then pulsed for 45 min with bromodeoxyuridine. Representative FACS plots for BrdU (x-axis) and for 7-AAD (y-axis) are shown. Conditions and cell lines are labeled with the percentage of cells in S phase in parentheses. B, bar graph shows the mean and standard error of the mean for three independent cell cycle analyses. Pairwise comparisons for the proportions of cells in G1 and S phases were done by Student t-test. Asterisks denote statistically significant comparisons: $P=.002$ for G1 phase and $P=.002$ for cells in G1 and S phase comparisons of 080-E47-4HT versus 080-4HT; $P=.0007$ for G1 phase and $P=.003$ for S phase comparisons of 020-E47-4HT versus 020-4HT.

Figure 4. E47-ER homodimerization has differential effects on apoptosis

Lmo2-induced T-ALL lines and their counterparts stably expressing E47-ER were treated with 4-hydroxytamoxifen for 24 h and then analyzed for intracellular cleaved caspase 3 by FACS. The bar graphs represent 3 independent experiments on the lines and conditions shown with mean and standard error shown. The P values were generated by pairwise comparison by Student t-test.

Figure 5. E47-ER homodimerization activates CD4 expression

A, Parental cell lines and those stably expressing E47-ER were analyzed for CD4 expression with and without the addition of 4-hydroxytamoxifen (4-HT). FACS plots show the staining of cells for anti-CD4 (x-axis) or anti-CD8 (y-axis) 24 h after 4-HT treatment. Conditions and lines are labeled under each individual contour plot and the percentage of CD4+CD8+ cells (in upper right quadrant) is shown in parentheses. B, shows quantitative RT-PCR analyses for CD4 mRNA for the same conditions and lines as in A; y-axis represents fold change from baseline which is the mRNA present in the parental line. The mean and standard error of mean are shown for triplicate analyses.

Figure 6. Global gene expression changes in Lmo2-induced T-ALLs resistant and sensitive to E47-ER's effects

020-E47 and 080-E47 were treated with 4-hydroxytamoxifen (4-HT) for 24 h and then harvested for whole RNA; libraries were constructed for Illumina cDNA sequencing. Gene expression was compared in 020-E47 before and after 4-HT treatment and in 080-E47 before and after 4-HT treatment. Venn diagram shows the total number of genes significantly (corrected $P < .05$) upregulated and downregulated in 020-E47 after 4-HT and in 080-E47 after 4-HT. The distribution of genes upregulated versus downregulated was compared between 020 and 080 by Chi square test generating $P < .0001$. The Venn diagram shows a comparison of the two datasets and the number of genes similarly regulated in 020 and 080 lines.

RNA-seq analysis of cell lines sensitive (080) and resistant (027) to E47-ER dimerization**Table 1**

Select genes that were E47 transcriptional targets in previously published studies and their fold change from those studies are shown in column 2 (pub result, fold) (36, 37). Columns 3-8 show RPKM (reads per kilobase of gene per million reads) for each mRNA. Columns 9-10 show the fold changes in E47+4HT divided by RPKM in the parental lines (i.e 027 and 080). The RPKMs of E47+4HT were compared between 080 and 027 cell lines (column 5 v. 8) generating P values which were corrected for multiple hypothesis testing; ns, not significant or corrected P>.05.

<i>Gene</i>	Pub result, fold	027	027 +E47	027 +E47 +4HT	080	080 +E47	080 +E47 +4HT	027 Fold	080 Fold	<i>P</i>
<i>Rorc</i>	4.3	8.83	10.47	46.83	67.96	137.47	296.75	5.3	4.37	0
<i>Gpr56</i>		4	2.34	19.17	46.41	59.1	232.03	4.79	5	0
<i>Dhrs3</i>		0.17	0.16	0.34	6.93	10.6	33.41	1.98	4.82	0
<i>Pipre</i>		3.69	2.97	15.21	1.94	2.07	8.72	4.13	4.49	ns
<i>Socs3</i>		3.6	6.8	14.13	5.91	10.85	28.05	3.93	4.75	ns
<i>Hes1</i>		68.13	84.25	89.6	86.98	79.8	99.06	1.32	1.14	ns
<i>Xbp1</i>	3.1	63.19	51.87	114.16	20.14	23.59	89.72	1.81	4.45	ns
<i>Sell</i>		146.66	58.62	23.07	37.84	8.61	1.71	0.16	0.05	3.17E-05
<i>Cdk6</i>	-2.6	8.3	10.39	4.83	40.91	34.75	20.25	0.58	0.47	0.000202
<i>Jund1</i>		5.48	9.77	12.48	4.92	4.5	5.45	2.28	1.11	ns
<i>E2F4</i>	1.5	106.34	106.85	169.67	66.65	76.6	142.67	1.6	2.14	ns
<i>Myc</i>	-1.4	93.28	113.12	68.88	141.58	142.05	104.76	0.74	0.74	ns
<i>Axin2</i>	-3.7	12.51	12.82	8.75	22.58	22.03	15.04	0.7	0.67	ns
<i>Casp3</i>	1.3	49.44	48.61	46.91	75.02	42.59	33.55	0.95	0.45	ns
<i>Casp6</i>	-1.4	22.14	20.14	21.17	18.13	18.71	24.72	0.96	1.36	ns
<i>Bid</i>		25.21	36.76	38.04	63.15	64.15	75.51	1.51	1.2	0.022

<i>Gene</i>	Pub result, fold	027	027 +E47	027 +E47 +4HT	080	080 +E47	080 +E47 +4HT	027 Fold	080 Fold	<i>P</i>
<i>Gadd45a</i>	2.4	47.14	50.36	98.81	23.31	14.65	11.85	2.1	0.51	5.10E-11
<i>Gadd45b</i>	2.2	0.86	0.97	2.8	0.16	0.12	0.18	3.27	1.13	0.0003
<i>Cdkn1a</i>	2	6.36	14.27	25.25	25.42	9.27	34.82	3.97	1.37	ns
<i>Ccn1</i>	1.8	20.2	21.76	48.01	12.47	3.26	9.39	2.38	0.75	1.38E-05
<i>Rb1</i>	1.5	8.4	8.37	4.77	14.06	10.32	5.42	0.57	0.39	0.057
<i>Cyp11a</i>	0	0.05	0.36	0.36	0.05	0	0.43	n/c	8.17	ns
<i>Ptgs2</i>	9.03	9.93	10.69	10.69	0.04	0.04	0.03	1.18	0.68	0
<i>Dgke</i>	10.69	10.55	10.43	10.43	11.65	11.54	10.29	0.98	0.88	ns
<i>Cerk</i>	8.47	10.34	12.98	12.98	10.22	20.97	36.83	1.53	3.6	1.40E-05
<i>Ets2</i>	42.79	48.63	65.48	65.48	11.08	12.53	32.64	1.53	2.95	0.021
<i>Cd3e</i>	-1.6	196.58	181.08	210.6	158.37	170.44	243.32	1.07	1.54	ns
<i>Gfi1b</i>	2.7	0.8	1.03	16.05	0.03	0.06	3.01	19.96	107.53	0.001965
<i>Gfi1</i>	46.91	46.1	59.53	59.53	41.81	43.8	69.94	1.27	1.67	ns
<i>Gata3</i>	-2	60.01	62.01	33.15	147.97	124.04	43.43	0.55	0.29	ns
<i>Foxo1</i>	-2.9	31.54	33.88	29.69	11.6	13.1	17.68	0.94	1.52	ns
<i>Cd1d1</i>	14.51	14.54	9.78	9.78	14.37	11.59	9.4	0.67	0.65	ns
<i>Id2</i>	83.67	85.46	492.49	492.49	19.57	27.32	248.41	5.89	12.69	ns
<i>Rag2</i>	1.7	27.48	22.95	21.51	27.35	33.94	57.96	0.78	2.12	0.027
<i>Rag1</i>	1.9	91.24	83.49	95.22	89.96	96.59	250.49	1.04	2.78	0.005
<i>Id1</i>	30.47	36.62	171.36	171.36	10.8	25.35	284.4	5.62	26.33	0
<i>Cd4</i>	1.68	8.37	30.05	30.05	27.49	67.63	257.12	17.91	9.35	4.43E-12

<i>Gene</i>	Pub result, fold	027	027 +E47	027 +E47 +4HT	080	080 +E47	080 +E47 +4HT	027 Fold	080 Fold	<i>P</i>
<i>Tcf12</i>		166.66	154.60	95.93	89.05	83.30	51.33			