The TAL1/SCL Transcription Factor Regulates Cell Cycle Progression and Proliferation in Differentiating Murine Bone Marrow Monocyte Precursors

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Monocytopoiesis involves the stepwise differentiation in the bone marrow (BM) of common myeloid precursors (CMPs) to monocytes. The basic helix-loop-helix transcription factor TAL1/SCL plays a critical role in other hematopoietic lineages, and while it had been reported to be expressed by BM-derived macrophages, its role in monocytopoiesis had not been elucidated. Using cell explant models of monocyte/macrophage (MM) differentiation, one originating with CMPs and the other from more committed precursors, we characterized the phenotypic and molecular consequences of inactivation of TAL1 expression ex vivo. While TAL1 knockout had minimal effects on cell survival and slightly accelerated terminal differentiation, it profoundly inhibited cell proliferation and decreased entry into and traversal of the G1 and S phases. In conjunction, steady-state levels of p16(Ink4a) mRNA were increased and those of Gata2 mRNA decreased. Chromatin immunoprecipitation analysis demonstrated the association of TAL1 and E47, one of its E protein DNA-binding partners, with an E box-GATA sequence element in intron 4 of the Gata2 gene and with three E boxes upstream of p16(Ink4a). Finally, wild-type TAL1, but not a DNA binding-defective mutant, rescued the proliferative defect in TAL1-null MM precursors. These results document the importance of this transcription factor in cell cycle progression and proliferation during monocytopoiesis and the requirement for direct DNA binding in these processes.

Mononuclear phagocytes serve as critical effectors of both innate and adaptive immunity and have important functions in the maintenance of tissue homeostasis. Macrophages derive from common myeloid precursors (CMPs) that differentiate in the bone marrow (BM). Monocytopoiesis is driven by specific growth factors that include interleukin-3 (IL-3) and macrophage colony-stimulating factor (M-CSF) and by transcription factors, including PU.1 and C/EBPα (10). This generates monoblasts, promonocytes, and finally monocytes, which are released into circulation (10, 12). While monocytes are capable of phagocytosis and cytokine secretion, their functional capabilities are enhanced with differentiation to macrophages in tissues.

The T-cell acute lymphoblastic leukemia 1 (TAL1) gene, also known as stem cell leukemia (SCL), encodes a class II basic helix-loop-helix (bHLH) transcription factor that regulates both embryonic and adult hematopoiesis (25, 28, 39, 42, 43, 45). In the adult, TAL1 has a recognized role in the differentiation of erythroid, megakaryocytic, and mast cell progenitors (1, 3, 13, 14, 31, 41, 54) and is expressed in vascular and lymphatic endothelial cells under both physiological and pathological conditions (49). Heterodimers of TAL1 and ubiquitous bHLH proteins known as E proteins regulate transcription by binding to specific regulatory regions in target genes in association with the zinc finger transcription factor Gata1 or Gata2. The sequence element recognized by those complexes includes an E box (CANNTG) that binds a bHLH heterodimer and a WGATAR motif recognized by a GATA protein (34, 35, 56, 58, 60). TAL1/E protein heterodimers can also bind E boxes to effect transcriptional repression (8, 11, 19, 20, 55). In murine erythroleukemia and T-cell acute lymphoblastic leukemia cell lines, TAL1 had been shown to interact with a number of nuclear corepressors, including mSin3A and HDAC1 (21, 23), BrgL and HDAC2 (59), and Mtv16/Eto-2 and Mgr1 (6, 44).

Although an important role for TAL1 has been demonstrated in other hematopoietic lineages, a function in mononuclear phagocyte production, while suggested by a number of observations, had not been established. First, TAL1 had been found to contribute to specific E box DNA-binding complexes in the M1 monocytopoiesis cell line (55). While studies with M1 cells and the human bipotent cell line TF-1 suggested that it was downregulated during monocytic differentiation (18, 50, 55), TAL1 mRNA or protein was detected in mouse peripheral blood mononuclear cells and BM macrophages (25) and zebrafish (62) and human (41) macrophages. In addition, higher and earlier expression of IL-6 receptor (Il6r) and colony-stimulating factor 1 receptor (Csf1r) mRNA (our unpublished data) was observed in TAL1-transduced primary mouse BM monocyte/macrophage (MM) precursors and M1 cells induced to differentiate with mouse M-CSF (mM-CSF) and IL-6 (mIL-6), respectively. In contrast, knockdown of TAL1 in human cord blood CD34+ cells by short hairpin RNA reduced the numbers of both myeloid and erythroid cell progenitors (5). Finally, while deletion of TAL1 in adult hematopoietic stem cells (HSCs) did not affect granulocyte-macrophage or macrophage progenitor numbers (16, 30), 5-fluorouracil (5-FU)

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treatment of Tal1−/− mutant mice resulted in a transient but significant delay in the recovery of circulating monocytes (David Curtis, unpublished data).

In this study, we establish that Tal1 is expressed at all stages of monocyte differentiation from CMP to macrophage and report a previously unrecognized function of this hematopoietic transcription factor in cells of this lineage. Using an ex vivo method of Tal1 gene deletion, knockout of Tal1 in BM mono-nuclear cells was found to suppress cellular proliferation at a relatively late stage of monocytopoiesis and delay entrance into and progression through S phase. Finally, we identify two genomic targets of Tal1 in differentiating MM precursors whose altered expression likely contributed to the abnormal phenotype of knockout cells.

MATERIALS AND METHODS

Cell lines and culture reagents. The mouse myeloid leukemia cell line M1, connective tissue cell line L-929, and retroviral packaging cell line BOSC23 were purchased from the American Type Culture Collection (Manassas, VA). M1 cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin-streptomycin. L-929 cells were cultured in Eagle’s minimum essential medium (Invitrogen)–15% FBS–2 mM l-glutamine (Invitrogen)–1 mM sodium pyruvate (Fisher Scientific, Pittsburgh, PA)–1% penicillin-streptomycin. The BOSC23 cell line was maintained in Dulbecco’s minimum essential medium (DMEM; Invitrogen)–10% FBS–1% penicillin-streptomycin. The BOSC23 cell line was maintained in Dulbecco’s minimum essential medium (DMEM; Invitrogen)–15% FBS–2 mM l-glutamine (Invitrogen)–1 mM sodium pyruvate (Fisher Scientific, Pittsburgh, PA)–1% penicillin-streptomycin and G418 selection medium (Millipore, Billerica, MA).

For preparation of L-cell conditioned medium, 1.25 × 105 L-929 cells were seeded into 30 ml of medium. Medium was harvested 7 to 10 days later, centrifuged at 1,500 × g for 10 min, and then filtered through a 0.2-μm filter to remove cellular debris. This conditioned medium served as a source of M-CSF in mouse MM progenitor cell cultures.

Plasmids and cDNAs. The murine stem cell virus (MSCV)-based bicistronic vectors MSCV-ires-GFP, MSCV-ires-GFP-Tal1, and MSCV-ires-GFP-Tal1-IRES-YFP have been previously described (22). A Gata2 cDNA was obtained from J. Douglas Engel (University of Michigan, Ann Arbor), a Cre expression plasmid from Lishan Su (University of North Carolina, Chapel Hill), and the MSCV-ires-YFP vector from Derek Persons (St. Jude Children’s Research Hospital, Memphis, TN).

Mouse genotyping and breeding. Three-week-old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice with a fost1-targeted Tal1 allele (fost1Cre; LoxP-loxP) and SCL-loxP mice (20) have been previously described (9, 16). Mice were housed in an AAALAC-accredited facility at Vanderbilt University Medical Center according to approved guidelines. SCL-loxP-loxP and SCL-loxP/WT mice were intercrossed to generate SCL-loxP/loxP mice, with genotyping carried out by PCR analysis of DNA extracted from tail biopsy samples. The forward primer for the floxed and wild-type Tal1 alleles was TCCCAAGCCAAGATTTCCCCTAGC; and for the floxed allele the excised allele was GCCAGCTCTGATGACACAGGGAATTGCC. A common reverse primer with the sequence CAAAGCTGTAGATTACATGAGCT was used for all three alleles. For detection of the LacZ knock-in allele, the forward and reverse primer sequences were, respectively, GAGATGCACGCTGCAGACTCTGC and TCGCGGTGGGATGAGCAGAGT.

Gene targeting. BM cells from SCL-loxP/loxP mice were transduced with MSCV-GFP-Cre (where GFP is green fluorescent protein) or its parental vector using the methods described below. Cre expression resulted in excision of the single floxed Tal1 locus, generating SCL-loxP/loxP cells, which were effectively nullizygous, while compound heterozygotes (SCL-loxP/WT) genotype that were transduced with the parental vector served as controls. GFP-expressing cells were isolated in a fluorescence-activated cell sorter and returned to culture for subsequent studies.

Fluorescence-activated cell sorting (FACS) and flow cytometry analysis. FACS analysis and flow cytometry analysis were carried out in FacsAria and BD FACsCanto II instruments, respectively, running FACSdiva software (Becton Dickinson, Franklin Lakes, NJ). Data analysis was performed using WinList software, and PKH26 dye dilution data were modeled with ModFit LT software (Verity Software House, Topsham, ME).
periods, including 12, 24, 36, and 48 h. These cells were processed for flow cytometry analysis identically to the others.

Chromatin immunoprecipitation (ChIP) analysis. ChIP analysis was carried out using a commercial kit (Millipore) with certain modifications (59). Formaldehyde was added at a final concentration of 1% to 2 \times 10^7 cells for 20 min at 37°C. For M1 cells, cross-linking was carried out with 1 \times 10^8 cells cultured in the presence or absence of recombinant mIL-6 for 48 h. All subsequent steps were carried out according to the manufacturer’s instructions. Antibodies used in immunoprecipitation of chromatin fragments included rabbit polyclonal anti-Tal1 (25), anti-E47 (sc-763X; Santa Cruz), and anti-E2A (Yae) (sc-416X; Santa Cruz), in addition to normal rabbit IgG (sc-2027; Santa Cruz). Immunoprecipitated DNA was analyzed by real-time PCR with iQ SYBR green Supermix (Bio-Rad), and occupancy was quantified for all antibodies and IgG and expressed as a percentage of the input. The sequences of the primers used were as follows: p16\textsuperscript{(Ink4a)}13 region forward, CCACTGGTCACACGACTG; p16\textsuperscript{(Ink4a)}13 region reverse, GCACGTCATGCACACG; p16\textsuperscript{(Ink4a)}2592 region forward, CCCTAGCCAAATCTGACAT; p16\textsuperscript{(Ink4a)}2592 region reverse, TCCAGGTAGTACATGCTTTCA; p16\textsuperscript{(Ink4a)}4638 region forward, CCTCTCCAGGGTAGTCATGG; p16\textsuperscript{(Ink4a)}4638 region reverse, GATGAAAGAATCACACAAAGG; p16\textsuperscript{(Ink4a)}5106 region forward, CACTTTGCTTATACTGGATGG; p16\textsuperscript{(Ink4a)}5106 region reverse, CCAGTGAAACCCTGTGTATCTT; p16\textsuperscript{(Ink4a)}6932 region forward, GCTTTCACAGGACACAGAAGG; p16\textsuperscript{(Ink4a)}6932 region reverse, TTACTCTTTCCACCATGACC; p16\textsuperscript{(Ink4a)}3’ UTR (untranslated region) forward, GGAGGGAGCAGAAGGAGG; p16\textsuperscript{(Ink4a)}3’ UTR reverse, ACAATCCAGCCATTATTC; Gata2 intron 4 forward, CCTGCGGAGTTCCTATCC; Gata2 intron 4 reverse, ACTAGTCCGGGTGCTCTGA; Gata2 3’ UTR forward, TCGATGCTGTGTTGTGGTT; Gata2 3’ UTR reverse, ACTCCGGTTAGGGGTCTCT.

Western blot analysis. Whole-cell lysates of MM precursors were prepared at days 1, 4, 6, and 8 of culture. Western blot analysis was performed according to previously described procedures (21) with antibodies to Tal1 (sc-12982; Santa Cruz) and β-actin (ab 8226; Abcam, Cambridge, MA).

RESULTS

Tal1 is expressed continuously during murine monocytopoiesis. To characterize Tal1 expression in monocyte progenitors, we made use of a previously developed method for isolating CMPs from mouse BM and inducing their differentiation (48). Using quantitative reverse transcription (RT)-PCR analysis, Tal1 mRNA was detected at all stages from the common myeloid progenitor to the postmitotic macrophage (Fig. 1A). Although Tal1 message fluctuated in abundance, it was detectable throughout, as well as in LPS- and IFN-γ-activated macrophages, albeit at lower levels than in unactivated cells (Fig. 1A). Macrophage differentiation was verified by morphological analysis of Wright-Giemsa-stained cytospin preparations (Fig. 1B), quantitative RT-PCR analysis of well-
established macrophage gene markers (data not shown) and flow cytometry analysis of the cell surface antigens CD31 and Ly6C. As previously described, CMPs (CD31+Ly6C−) differentiated sequentially to CD31+Ly6C+, CD31+Ly6C−, and CD31−Ly6C− (data not shown).

Expression studies were also carried out with MM precursors explanted at a later stage of differentiation. Using methods developed by Stanley and colleagues (15, 47, 53), terminal differentiation of mouse BM monoblasts and promonocytes was induced in vitro with recombinant murine M-CSF. Western blot analysis of cellular extracts prepared after 1, 4, 6, and 8 days of culture revealed only modest changes in Tal1 expression over this period (Fig. 1C and D).

**Loss of Tal1 impairs proliferation of mouse MM precursors.** For subsequent studies, monoblasts and promonocytes were isolated from mouse BM using established procedures (15, 47, 53) and Tal1 expression was manipulated in them in culture. In the absence of a suitable promoter to direct Cre expression to floxed delete the gene in MM precursors isolated from mice with a 53) and isolated from mouse BM using established procedures (15, 47, 53), terminal differentiation of mouse BM monoblasts and promonocytes was induced in vivo with recombinant murine M-CSF. Western blot analysis of cell extracts prepared after 1, 4, 6, and 8 days of culture revealed only modest changes in Tal1 expression over this period (Fig. 1C and D).

**Tall knockout MM precursors show reduced viability and accelerated differentiation.** To determine whether the reduced accumulation of SCLloxP cells resulted from decreased viability, apoptosis was analyzed using annexin V and 7-AAD staining, with nonapoptotic cells characterized by the ability to exclude both stains (Fig. 3A). The percentage of viable cells in the SCLloxP group was very slightly decreased relative to that of SCLloxP cells at days 4 and 6 but was not different at days 1 and 8 of culture. These results show that Tal1 gene loss had minimal effects on cell survival and that apoptosis could not account for the marked reduction in cell number observed.

Cellular differentiation was then assessed by flow cytometry analysis of cell surface antigens CD31 and Ly6C (Fig. 3B). A higher percent of the most mature CD31−Ly6C− cells was noted in the SCLloxP population (59.2% at day 2 and 91.2% at day 4) than in the SCLloxP group (52.2% at day 2 and 82.6% at day 4) at early times in culture. In contrast, less differentiated CD31+Ly6C+ cells made up a smaller proportion of SCLloxP cultures (26.9% at day 2 and 8.5% at day 4) than of SCLloxP cultures (36.1% at day 2 and 16.4% at day 4). Expression of another widely used macrophage marker, F4/80, did not reliably discriminate between different stages of monocytopenesis in our studies, with approximately 90% of the cells in both groups becoming F4/80 positive after only 24 hr in culture (data not shown). In sum, these data indicate that SCLloxP cells lacking a functional Tal1 gene differentiated slightly more rapidly than SCLloxP cells.

**SCLloxP cells are defective in cell cycle progression.** To better characterize the proliferative defect in Tall knockout cells, a BrdU pulse-chase analysis was carried out. To that end, GFP-expressing cells were cultured for 3 days, labeled with BrdU for 45 min, and followed through at least one cell cycle by flow cytometry (Fig. 4A). Analyzed immediately after pulsing, the majority of the BrdU-labeled cells from both the SCLloxP population and SCLloxP groups were in S phase (94.9% and 87.3%, respectively), with smaller percentages in early S phase or late G2/M population (5.1% and 12.7%, respectively; shaded dark in the 0-h population, Fig. 4A, i and v). At later times, SCLloxP cells progressed rapidly to G2/M and then to G0/M, with a concomitant decrease in the percentage in S phase (Fig. 4A, ii, iii, and v). In contrast, while some SCLloxP cells advanced to last S phase 1 hr after pulsing, as evidenced by the two peaks of labeling (Fig. 4A, vi), these cultures had a larger proportion of cells in G2/M after 2 and 4 hr (18.1% and 35.2%, respectively), lacked a distinct G0/M population (0% and 18.8%, respectively), and contained a higher percentage of cells still in S phase (81.9% and 46.0%) than did the control cells (Fig. 4A, vii and viii).
Tal1 knockout cells, with delayed entry into and traversal of S phase. As only cells able to incorporate the BrdU label can be analyzed by the pulse-chase approach, MM precursors were also cultured with BrdU for longer times (12, 24, 36, and 48 h) before analysis by flow cytometry (Fig. 4B). As could have been predicted, the SCLΔlacZ population contained a higher proportion (27.5% versus 7.6% for SCLloxP/lacZ cells, Fig. 4B, iv).
and viii) of cells that never incorporated BrdU during a 48-h labeling period. Of note, the number of BrdU-positive SCL\(^+/\)^/H9004/LacZ cells decreased slightly over time in culture (Fig. 4B, vii and viii), consistent with their increased tendency toward apoptosis (Fig. 3A). Finally, and most directly, double staining with Hoechst 33342 and pyronin Y showed a threefold higher percentage of cells in G0 in SCL\(^+/\)^/H9004/LacZ than in SCL\(^+/\)^/LacZ cultures (data not shown). Together, these studies suggest a role for TAL1 in cell cycle progression in MM precursors that can largely account for the proliferative defect associated with homozygous Tal1 gene loss.

Tal1 knockout MM precursor cells contain lower Gata2 and higher p16\(^{Ink4a}\) mRNA levels. Tal1 gene loss would be expected to impact the transcription of its target genes, especially direct targets like those coregulated with E proteins. Accordingly, real-time PCR analysis was used to evaluate expression of genes known to have a role in MM differentiation, including putative TAL1 targets, with comparisons made among SCL\(^WT/WT\), Tal1-overexpressing, and SCL\(^DLacZ\) cells. The same bicistronic retroviral vector used to deliver Cre to MM precursors for inactivation of the floxed Tal1 allele was also employed in enforcing Tal1 expression (Fig. 5A). These studies revealed that Gata2 mRNA was reduced seven- and fourfold, respectively, at days 1 and 4 in SCL\(^DLacZ\) relative to SCL\(^WT/WT\) cells (Fig. 5B). Although the level of Gata2 expression decreases physiologically with differentiation of MM precursors (Fig. S5B and reference 48), Tal1-null (SCL\(^DLacZ\)) cells showed even lower Gata2 expression than wild-type controls at days 6

FIG. 3. Viability and differentiation of SCL\(^loxP/LacZ\) and SCL\(^DLacZ\) MM precursor cells in culture. (A) Viability of cells was determined by flow cytometry analysis. Viable cells were characterized by absent APC-annexin V binding and 7-AAD staining. Each bar represents the mean percentage ± the standard deviation of cells negative for both markers (from triplicate plates). (B) Flow cytometry analysis of APC-CD31 (y axis) and PE-Ly6C (x axis) staining of SCL\(^loxP/LacZ\) and SCL\(^DLacZ\) cells at days 1, 2, and 4 of culture. A representative profile from three independent experiments is shown.
FIG. 4. Cell cycle analysis of SCLloxP/LacZ and SCLΔM/LacZ MM precursor cells. (A) Flow cytometry analysis of BrdU incorporation versus DNA content (7-AAD staining) for SCLloxP/LacZ and SCLΔM/LacZ cells at 0, 1, 2, and 4 h after pulsing. G0/G1, S, and G2/M populations were modeled computationally. A representative profile from three independent experiments is shown. (B) Flow cytometry analysis of SCLloxP/LacZ and SCLΔM/LacZ cells cultured with BrdU for 12, 24, 36, and 48 h. DNA content (x axis) is plotted versus BrdU incorporation (y axis). Values inside the upper and lower quadrants denote the percentages of BrdU-positive and -negative cells, respectively.
and 8. In contrast, enforced Tal1 expression did not increase the abundance of Gata2 mRNA at any time point examined (Fig. 5B).

Because of the delay in cell cycle progression observed with Tal1 loss (Fig. 4A and B) and the previously published evidence of their regulation by E proteins, expression of the genes for several cyclin-dependent kinase (CDK) inhibitors was analyzed. p16(Ink4a), which was shown to be a target of repression by Tal1 (17, 33, 37), was upregulated approximately three- to fourfold in SCLΔLacZ cells and reduced, albeit to a lesser extent, in Tal1-overexpressing cells (Fig. 5C). In contrast, expression of p21(Cip1), another putative target of Tal1 and E proteins (29, 37, 40), was only slightly increased in SCLΔLacZ cells compared to SCLWT cells (data not shown). While elevation of p16(Ink4a) and p21(Cip1) could relate to the cell cycle delay in Tal1 knockout cells, especially given the phase of the cell cycle affected, the only slight reduction in these mRNAs appears to be attributable to the cell cycle delay itself and not a reduction in gene expression over this same time period. Primer sequences designed for the Gata2 3′ UTR did not support any amplification (data not shown), indicating specific occupancy of this intronic region in MM precursor cells.

Scanning a 12-kb region upstream of the p16(Ink4a) translational start site identified five preferred E box sequences (CATCTG or CAGATG) for TAL1/E protein binding (bases −13, −2592, −4638, −5106, and −6932). PCR primers complementary to each of these regions and to the gene’s 3′ UTR were then designed, and a quantitative ChIP analysis was carried out on sonicated chromatin from day 4 and day 7 wild-type (SCLWT) cells with polyclonal antibodies to Tal1 and E47. The amount of DNA immunoprecipitated was then quantified in a real-time PCR analysis using region-specific primers and expressed as a percentage of the input (Fig. 6A and B).

Antibodies to Tal1 and E47, but not normal rabbit IgG, were able to precipitate this intronic fragment from day 4 cells (Fig. 6A) but not day 7 cells (Fig. 6B), concordant with the decline in Gata2 expression over this same time period. Primer sequences designed for the Gata2 3′ UTR did not support any amplification (data not shown), indicating specific occupancy of this intronic region in MM precursor cells.

Gata2 intron 4 and p16(Ink4a) upstream regions are occupied by a Tal1-E protein complex in MM precursors. The above-described studies revealed that knockout, and to a lesser extent overexpression, of Tal1 affected the expression of Gata2 and p16(Ink4a). Previous studies defined an enhancer in intron 4 of the Gata2 gene (kb +9.5) with a consensus E box-GATA DNA-binding motif that is occupied by Tal1, E proteins, and GATA proteins in endothelial and fetal liver cells (27, 57). Moreover, this DNA sequence element appeared to be essential for the proper expression of Gata2 in endothelial cells (27).

To ascertain whether Tal1 occupies this region in MM precursors, a ChIP analysis was carried out on sonicated chromatin from day 4 and day 7 wild-type (SCLWT) cells with polyclonal antibodies to Tal1 and E47. The amount of DNA immunoprecipitated was then quantified in a real-time PCR analysis using region-specific primers and expressed as a percentage of the input (Fig. 6A and B).

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Antibodies to Tal1 and E47, but not normal rabbit IgG, were able to precipitate this intronic fragment from day 4 cells (Fig. 6A) but not day 7 cells (Fig. 6B), concordant with the decline in Gata2 expression over this same time period. Primer sequences designed for the Gata2 3′ UTR did not support any amplification (data not shown), indicating specific occupancy of this intronic region in MM precursor cells.
Ink4a) expression increases with IL-6-induced differentiation (2) of the murine myeloid leukemia cell line M1 in parallel with a decrease in Tal1 expression and Tal1/E protein DNA-binding activity (55). As this phenocopies the results of Tal1 gene knockout, a ChIP analysis was also carried out on chromatin from untreated and IL-6-treated M1 cells. As predicted from its drastic decline in expression (55), Tal1 occupancy was detected only in uninduced cells (Fig. 6C). In contrast, E2a association with these same E box elements was detected in both IL-6-treated and untreated cells, indicating that its E12 and/or E47 protein products acted to augment the expression of this gene unopposed by TAL1 when these cells were induced to differentiate (Fig. 6D).

Tal1 function in MM precursor cell proliferation requires direct DNA binding. TAL1 has been shown to regulate transcription through direct binding to DNA and as a non-DNA-binding cofactor (34, 56, 58). To determine whether the proliferative defect in SCL<sup>Loxp</sup>/LacZ cells could be rescued by the wild-type Tal1 cDNA, with the growth curve of Tal1-transduced SCL<sup>Loxp</sup>/LacZ cells virtually identical to that of control cells (Fig. 7). Since Tal1 deletion resulted in the downregulation of Gata2 expression, a Gata2 cDNA was also tested in rescue experiments. However, cells transduced with the Gata2 cDNA were nonviable after only 1 day in culture, demonstrating an adverse effect of Gata2 overexpression in a Tal1-null background (data not shown). Finally, to investigate whether DNA-binding activity is required for TAL1 function in MM proliferation, SCL<sup>Loxp</sup>/LacZ cells were transduced with a cDNA for a well-characterized DNA binding-defective mutant form of Tal1, Tal1 T192P (21). Significantly, cells transduced with this protein behaved identically to SCL<sup>Loxp</sup>/LacZ cells and showed no recovery of proliferative capacity (Fig. 7). This result indicates that, similar to its actions in erythroid model systems (26), Tal1 DNA-binding activity is required for outgrowth of murine MM precursors in explant cultures.

**FIG. 6.** ChIP analysis of Tal1 and E47 occupancy of the p16(Ink4a) and Gata2 genes in wild-type MM precursors cultured for 4 (A) or 7 (B) days. DNA derived from chromatin fragments immunoprecipitated with antibodies to Tal1 or E47 or normal rabbit IgG was quantified by real-time PCR analysis using region-specific primers. Each bar represents the mean abundance ± the standard deviation of each fragment relative to the input as determined from triplicate PCR measurements. ChIP analysis of Tal1 (C) and E2a (D) occupancy of the p16(Ink4a) and Gata2 genes in M1 cells cultured with or without IL-6 for 48 h. DNA derived from chromatin fragments immunoprecipitated with antibodies to Tal1 or E2a or normal rabbit IgG was quantified by real-time PCR analysis using region-specific primers. Each bar represents the mean abundance ± the standard deviation of each fragment relative to the input as determined from triplicate PCR measurements.

*p16(Ink4a)* expression increases with IL-6-induced differentiation (2) of the murine myeloid leukemia cell line M1 in parallel with a decrease in Tal1 expression and Tal1/E protein DNA-binding activity (55). As this phenocopies the results of Tal1 gene knockout, a ChIP analysis was also carried out on chromatin from untreated and IL-6-treated M1 cells. As predicted from its drastic decline in expression (55), Tal1 occupancy was detected only in uninduced cells (Fig. 6C). In contrast, E2a association with these same E box elements was detected in both IL-6-treated and untreated cells, indicating that its E12 and/or E47 protein products acted to augment the expression of this gene unopposed by TAL1 when these cells were induced to differentiate (Fig. 6D).
gene loss or because growth in culture simulates stress mono-
out an abnormal phenotype, either because of the acuteness of

(16), loss of the granulocyte-macrophage progenitor number significantly
isolated by dual-color FACS. The Tal1T192P cDNA encodes a DNA
binding-defective protein. Parental vector-transduced cells were
used as controls, and the total number of cells per plate for each day is
plotted. Each data point represents the mean ± the standard deviation
from three plates of cells.

FIG. 7. Rescue analysis in Tal1 knockout cells. Cell counts in MM
precursors from SCLLacZ/LacZ mice simultaneously transduced with
MSCV-GFP-Cre and either a Tal1 or a Tal1T192P cDNA in the retro-
viral vector MSCV-IRE-YFP. GFP- and YFP-expressing cells were
isolated by dual-color FACS. The Tal1T192P cDNA encodes a DNA
binding-defective protein. Parental vector-transduced cells were
used as controls, and the total number of cells per plate for each day is
plotted. Each data point represents the mean ± the standard deviation
from three plates of cells.

DISCUSSION

Previous studies have established the importance of Tal1 in
erythroid, megakaryocytic, and mast cell differentiation. Al-
though derived from the same progenitor cell as these, the
CMP, there is conflicting evidence for Tal1 function in MM
production. First, work carried out with the TF-1 (18) and M1
(55) cell lines showed that Tal1 expression declined with
macrophage differentiation and that enforced expression of
this transcription factor inhibited this process (51), although
monocytic differentiation of TF-1 cells was assessed with the
CD45 and FcyRII (CD32) markers, neither of which is mac-
rophage specific. Regardless, Tal1 expression has been de-
tected in primary mouse macrophages by several groups and in
mouse peripheral blood mononuclear cells and BM macro-
phages (25) and in zebrafish (62) and human (41) macro-
phages. In addition, the murine Tal1 cDNA was originally
cloned from a BM macrophage cDNA library (4).

We investigated here whether the abundance of Tal1 mRNA
was altered during in the differentiation of CMPs to monocytes
and whether Tal1 knockout affected the survival, proliferation,
or differentiation of mouse MM precursors. While deletion of
Tal1 in HSCs in IFN-inducible Mxi-Cre mice did not change
the granulocyte-macrophage progenitor number significantly
(16), loss of Tal1 in cells also nullizygous for the related Lyl1
gene did reduce myeloid colony-forming activity, raising the
possibility of genetic redundancy (46). Deletion of the Tal1
gene ex vivo, as done in these studies, may also have brought
out an abnormal phenotype, either because of the acuteness of
gene loss or because growth in culture simulates stress mono-
cytopoiesis. In support of the latter notion, SCLΔLacZ mice
showed a small but significant delay in the recovery of circu-
lating monocytes after 5-FU treatment (Curtis, unpublished).

The complete loss of Tal1 caused proliferative and cell cycle
defects in macrophage precursors, and wild-type and heterozy-
gous knockout cells also differed in their growth rates. This was
most apparent at later times in culture (Fig. 2D), however, suggesting that more differentiated cells such as monoblasts or
promonocytes are more sensitive to Tal1 gene dosage. In con-
trast, overexpression of Tal1 in MM progenitors resulted in a
substantial increase in cell number (Fig. 2D), similar to what
was reported for the 32D and HL-60 cell lines (7). As enforce-
ment of Tal1 expression in mouse MM precursors was associ-
ated with Il6r and Csf1r upregulation (data not shown), the
increased proliferation of these cells could reflect a heightened
sensitivity to cytokines.

The genes for several CDK inhibitors, including p16(Ink4a)
and p21(Cip1), have been shown to be positively regulated by
E proteins, while Tal1/E protein heterodimers were suggested
to repress their transcription (8, 17, 19, 33, 37, 40). Further,
restoration of E2A activity in the Jurkat cell line, in which
Tall-E2A function is low, profoundly depressed growth and
increased apoptosis (36). In Tall knockout MM precursor
cells, abrogation of Tall inhibition of E protein action was
likely a major contributor to both the upregulation of p16
(Ink4a) and p21(Cip1) expression and the impairment of cell
cycle progression and proliferation observed. The gradual in-
crease in p16(Ink4a) mRNA abundance (Fig. 5C) in the face of
nearly invariant Tall expression during late-stage monocyo-
poiesis (Fig. 1A and C) suggests that while Tall may be re-
quired for initial suppression of p16(Ink4a) transcription, it is
less important for maintenance of gene repression. This is also
supported by the decreased Tal1/E protein occupancy of E
boxes upstream of the p16(Ink4a) gene at day 7 of culture
compared to day 4 (Fig. 6A and B). The further upregulation of
p16(Ink4a) expression in Tall-null Lyl1-/- CMPs (Curtis,
unpublished) suggests that there is, in addition, genetic redu-
dancy in these bHLH proteins, at least for this target gene. In
contrast, p16(Ink4a) mRNA abundance was only slightly de-
creased in Tall-overexpressing cells (Fig. 5C), making it un-
likely to account for their increased proliferative rate (Fig.
2D). Regardless of the timing of its actions, the direct associ-
dation of Tall with three preferred E box sites in the p16(Ink4a)
upstream region in both primary MM precursors and the M1
cell line provides the best evidence that this gene is a target of
this transcription factor in cells.

While p16(Ink4a) transcription was inhibited by Tall in dif-
ferentiating cells of this lineage, the Gata2 gene appeared to be
transactivated by Tall. A region in intron 4 of Gata2 was
shown previously to have strong enhancer activity in endothel-
ial and mouse fetal liver cells (27, 57), and the present data are
compatible with its also being active in mononuclear phago-
cytes. Indeed, the slight acceleration of differentiation in Tall
knockout cells could be explained by their reduced levels of
Gata2, as a similar finding was reported for Gata2-deficient
juvenile myelomonocytic leukemia cells (61) and brown adipo-
cytes (32, 52). Furthermore, Gata2 knockdown reduced G1-S
transition while increasing myeloid gene expression in G1ME
cells (24), and the further decrease in Gata2 expression in
SCLΔLacZ cells could also have contributed to their abnormal
cell cycle progression. In contrast to p16(Ink4a) transcription,
Gata2 transcription was not affected by Tall overexpression
and was reduced only with Tall nullizygosity, reflecting a dif-
ference in the quantitative requirement for Tall in genes ac-
tivated and repressed by this transcription factor.

Finally, the proliferative defect in SCLΔLacZ cells was fully
rescued by the reintroduction of a Tall cDNA, which confirms
that this phenotype is attributable to Tall loss and is not an
indirect effect of retroviral transduction or Cre expression.
The failure of a DNA binding-defective mutant to rescue prolifer-
ation in these cells, in contrast, shows that DNA-binding activity is as important for Tal1 function in this lineage as in erythroid progenitors (26).

In summary, these studies demonstrate that the bHLH transcription factor Tal1 is expressed throughout monocytopenesis and that loss of Tal1 impairs the proliferation of late-stage MM precursors. This was associated with decreased exit from G0, delayed entrance into S from G1, and slowed traversal of S phase, resulting in part from the altered expression of a direct Tal1 target, the CDK inhibitor gene p16(Ink4a).

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