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MYC regulates the HIF-2 α stemness pathway via Nanog and Sox2 to maintain self-renewal in cancer stem cells versus non-stem cancer cells

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Abstract

Cancer stem cells (CSC) maintain both undifferentiated self-renewing CSCs and differentiated, non-self-renewing non-CSCs through cellular division. However, molecular mechanisms that maintain self-renewal in CSCs versus non-CSCs are not yet clear. Here, we report that in a transgenic mouse model of MYC-induced T cell leukemia, MYC maintains self-renewal in Sca1+ CSCs versus Sca-1- non-CSCs. MYC preferentially bound to the promoter and activated HIF-2 α in Sca-1+ cells only. Further, the reprogramming factors Nanog and Sox2 facilitated MYC regulation of HIF-2 α in Sca-1+ versus Sca-1- cells. Reduced expression of HIF-2 α inhibited the self-renewal of Sca-1+ cells; this effect was blocked through suppression of reactive oxygen species (ROS) by N-acetyl cysteine (NAC) or the knock down of p53, Nanog or Sox2. Similar results were seen in ABCG2+ CSCs versus ABCG2- non-CSCs from primary human T cell

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lymphoma. Thus, MYC maintains self-renewal exclusively in CSCs by selectively binding to the promoter and activating the HIF-2 α stemness pathway. Identification of this stemness pathway as a unique CSC determinant may have significant therapeutic implications.

Introduction:

A hallmark of many tumors is the capacity to maintain a stable population of cancer stem cells (CSCs) during multiple generations (1). This is attributed to CSC's ability to undergo asymmetric cellular division where one daughter cell retains self-renewal ability while the other daughter cell differentiates into non-CSCs, composing the bulk of the tumor (2). Numerous studies demonstrate that CSCs retain this ability of selective or exclusive self-renewal through asymmetric cellular division even after numerous serial transplantations and maintain a stable proportion of CSCs (3, 4). Hence, this maintenance of a stable proportion of CSCs via asymmetric division suggests a revision in the notion of the clonal evolution in cancer (2, 3, 5, 6).

Various mechanisms have been proposed by which CSCs maintain asymmetric self-renewal, including cell polarity, fate determinants, microenvironment modulation (4, 7, 8), phenotypic equilibrium (9) and activation of developmental pathways such as Notch and Wnt (1, 3, 4, 10). Additionally, gene products that can confer self-renewal in cancer have been identified including the iPS gene products MYC, Nanog, Sox2, Oct-4, as well as hypoxia-inducible factors (HIFs) (11–23). However, it is not clear how MYC and other iPS genes cooperate with HIFs to maintain self-renewal in CSCs versus non-CSCs.

The MYC oncogene plays an important role in the self-renewal of normal stem cells and CSCs (22, 24, 25). MYC is a transcription factor that regulates gene expression. When overexpressed, MYC commonly contributes to human cancer (11, 14). MYC induces an embryonic stem cell signature in CSCs (26). While in cooperation with other iPS genes such as Sox2, Nanog and Oct-4, MYC elicits reprogramming of differentiated cells enabling self-renewal (27) and thereby modulating the iPS genes (19, 28). MYC cooperates with hypoxia-inducible transcription factor-2 α (HIF-2 α) (29, 30), a stemness associated transcription factor that increases self-renewal of embryonic stem cells through coordinated upregulation of Oct-4, Nanog (31, 32) and the negative regulation of p53 (33). Hence, MYC through interaction with HIF-2 α and iPS genes could regulate exclusive self-renewal of CSCs.

We investigated self-renewal of CSCs in a transgenic mouse model of MYC-induced T-cell acute lymphocytic lymphoma (T-ALL) (34, 35) and human lymphoma. In MYC-induced T-ALL, we identified Sca-1⁺ CSCs that exhibit dependency on HIF-2 α for self-renewal. In CSCs but not non-CSCs, MYC preferentially binds to the promoter and activates transcription of HIF-2 α that is facilitated by Nanog and Sox-2. Finally, MYC mediated activation of HIF-2 α in ABCG2⁺ but not ABCG2⁻ human lymphoma CSCs. Our observations thereby suggest that MYC maintains exclusive self-renewal of CSCs by preferential activation of HIF-2 α in CSC versus non-CSCs.

Materials and Methods

Details of methods are provided in the Supplementary method section.

Sca-1 cell sorting of MYC-induced transgenic lymphoma:

All the necessary experimental procedures were approved and undertaken in accordance with guidelines of Stanford University, Forsyth Institute, Gauhati University and Kavi Krishna laboratory institutional animal ethics committee. Seven such transgenic mice were selected for the study and genotype confirmed (Supplementary table 1). The generation and genotyping of Eu-tTA/tetO-MYC system transgenic lines for conditional MYC-driven lymphoma has been used as described (34). The thymus obtained from moribund animals were dissociated to flow cytometry or immunomagnetic sort Sca-1+ cells (36) and these cells were expanded in serum free media containing IL-7 and SCF, and then subjected to phenotypic analysis. Multi-color flow cytometry for HIF-2 α (#NB100–132, Novus Biologicals, CO) and Nanog (#ab184609, Abcam, MA) was done as described(33).

Measurement of intracellular GSH, ROS, apoptosis and proliferation:

GSH and ROS levels were measured as previously described (37), whereas apoptosis was measured by calorimetric assay as per manufacturer instructions. Analysis of the relative cell number was performed by using Alamar blue assay as described (37).

Measurement of Senescence Associated Beta-galactosidase (SA-beta-gal) activity:

The fluorescence method using 5-dodecanoylaminofluorescein di- β -d-galactopyranoside (C₁₂FDG) (Thermo Fisher Scientific, IL, #D2893) was used as previously described (38). MYC inactivated lymphoma cells served as positive control for senescence (39). Details are given in supplementary methods.

Clonogenic assay:

It was performed in methylcellulose medium (Methocult M3134, Stem Cell Technologies, BC) as described (15, 37).

Immunohistochemistry and Western Blot (WB):

Immunohistochemistry was done using a Vector Mouse on Mouse (M.O.M.) Elite Peroxidase Immunodetection Kit (Vector Laboratories, Inc., CA) according to manufacturer's instructions (40). Mouse HIF-2 α (Novus Biologicals, CO) antibody was used in 1:500 dilution. WB was done as previously described by using mouse HIF-2 α (#NB-132; Novus Biologicals, CO); mouse β -actin (Cell Signaling technology, MA) (40).

Real time PCR (qPCR) and specific inhibition of genes:

The real time PCR was performed using Taqman gene expression assay as described (33). The *in vitro* inhibition of mouse HIF-2 α , HIF-1 α , p53, Sox2 and Nanog were achieved by Accell siRNA obtained from GE Healthcare Dharmacon Inc., IL as described (33). The *in vivo* inhibitions of HIFs and p53 were achieved by FM19G11 (Millipore, Billerica, MA; 1.0 mg/kg/i.p) and pifithrin α (Sigma-Aldrich, MO; 4 mg/kg/i.p) after dissolving in 1.5 %

DMSO, and given thrice weekly for two weeks (41). Inhibition of ROS was accomplished by N-acetylcysteine (NAC) (100mg/kg i.p.) 30 minutes before the injection of FM19G11.

Chromatin Immunoprecipitation (ChIP) and Re-ChIP assays:

ChIP was performed using the Magnify chromatin IP kit (#1626969, Invitrogen, CA) according to manufacturer instructions. Briefly, Sca-1+ cells were fixed with 1% formaldehyde, sonicated to produce DNA fragments of approximately 100–600bp and then subjected to immunoprecipitation (19) with c-MYC (2 ug, N-262, # sc-764, Santa Cruz, TX) as well as isotype matched control for rabbit IgG (NB810–56910; Novus Biologicals, CO) as previously described (42). The mouse and human DNA products were subjected to SYBR green PCR using EpiTect ChIP qPCR primers (SABioscience, Qiagen, Valencia, CA). Results were analyzed using percent input method, where 1% of starting chromatin was used as input (42). For re-ChIP analysis, chromatin products from first IP were treated with 10mM DTT for 30 minutes at 37°C to prevent the majority of the first antibody from participating in the second IP reaction. Eluates were diluted in dilution buffer and then used for second IP by Nanog (#5232, Cell Signaling, MA) rabbit antibody, as described (42).

Collection of primary T-LBL leukemia cells, ABCG2+ cell sorting, and xenotransplantation in NOD/SCID mice:

Peripheral blood and lymph node samples from leukemia patients (Supplementary table 2) were obtained after written informed consent and with the approval from the Institutional Research Ethics Committee of respective institutions and Declaration of Helsinki. The approved institutions are Stanford University School of Medicine, KaviKrishna Telemedicine Care, a branch of KaviKrishna laboratory, Dr. B. Borooah Cancer Institute and Gauhati University. The collection and expansion of T-cell lymphoblastic lymphoma (T-LBL) samples (n=6, Supplementary table 2), immunomagnetic sorting of ABCG2+ and *in vivo* transplantation in non-obese diabetic/severe combined immune deficient (NOD/SCID) mice were performed as described (20, 43, 44). Mice were prior treated with 22 mg/kg intraperitoneal Busulfan (6mg/ml injection, Taj Pharma, Mumbai) daily over three days (20). The human cell engraftment was confirmed by flow cytometry staining with a human CD45 antibody (Biolegend, CA).

Mutant HIF-2 α transfection assay:

ABCG2+ cells were transfected using the JetPEI reagent (Polyplus transfection) with plasmids encoding constitutively active HIF-2 α mutant, the HA-HIF-2 α -P405A/P531A (45) (gift from Professor William Kaelin (addgene plasmid #18955 and #18956) and confirmed by Western Blot. The control circular plasmid encoding HA with no HIF α insert was generated as described (46).

Statistical analysis:

The statistical calculations were performed with GraphPad Prism 4.0 (Hearne Scientific Software, Chicago, IL, USA) using Student's t-test and One-Way ANOVA with Dunnett's post-hoc test. The data on the *in vivo* limiting dilution assay was analyzed by the extreme

limiting dilution analysis software, available online (<http://bioinf.wehi.edu.au/software/elda/>). Statistical comparison of Kaplan-Meier curves was based on the log-rank test.

Results:

MYC-induced lymphoma contains rare Sca-1+ CSCs.

In a transgenic mouse model of Tet system regulated MYC-induced T-ALL, we have examined whether there was an identifiable CSC population (34) that activates HIF-2 α pathway (Figure 1A). We used multiple transplanted T-ALL tumors (D9476, D9482, E0366, E2824, E2825, E6550, D9479). We confirmed that all tumors exhibited clonal expression of the T cell receptor V β 2 or 4 (Supplementary table 1), as previously demonstrated (34).

To identify potential CSC markers, we first examined the expression of multiple thymic lymphocyte lineage markers. T-cell development in the thymus involves migration of bone marrow cells that differentiate into early thymocyte progenitors (ETPs) and express Sca-1, c-kit, and CD44 (36). We found that a small number of tumor cells expressed Sca-1 (Figure 1B, left panel), c-kit, and CD44 (Supplementary Figure 1A, supplementary table 3 A–B). The Sca-1+ cells were positive for both CD4 & CD8 but did not express B cell marker CD19 (Figure 1B, right panel, supplementary table 3 A) (34). Hence, a small number of tumor cells expressing Sca-1 could be identified as potential CSC population.

Next, we examined whether Sca-1+ versus Sca-1- cells differentially expressed other stem cell genes including: HIF-2 α , and the pluripotency factors Nanog, Sox2 and Oct4 (33). Sca-1+ cells exhibited 6–10-fold higher expression of all of these genes except Oct4, which was not significantly expressed (Figure 1C, supplementary figure 1A–B, supplementary table 3B). HIF-1 α and p53 were downregulated by 4–6 -fold, whereas the level of endogenous MYC remained consistent in Sca-1+ versus Sca-1- cells (Figure 1C, supplementary figure 1C). Flow cytometry confirmed the qPCR results (Supplementary Figure 1A–B, and Supplementary table 3 A–B). Thus, Sca-1+ versus Sca-1- cells exhibited differential expression of stemness associated gene products.

Next, we evaluated the self-renewal capacity of Sca-1+ cells by performing *in vivo* limiting dilution assay. Flow cytometry sorted Sca-1+ and Sca-1- cell population from parental tumors were transplanted into immune-competent syngeneic mice. Intravenous injection of 10 Sca-1+ cells but 1 $\times$ 10⁶ Sca-1- cells resulted in engraftment of lymphoma in recipient syngeneic mice (Figure 1D, left panel, Supplementary Figure 2A–C and Supplementary table 4). The maximum likelihood analysis revealed that Sca-1+ cells exhibit 5,724-fold higher tumorigenic capacity in comparison to Sca-1- cells ($p < 0.00001$; Supplementary table 4). These Sca-1+ cells derived primary tumors re-established parental heterogeneity. The expression of ETPs and stemness markers CD44, C-kit, HIF-2 α , Nanog, and Sox2 were consistently high in Sca-1+ versus Sca-1- cells. Whereas the expression of p53 and HIF-1 α remained low, similar to parental tumors (Supplementary table 3B). Thus, we have identified Sca-1+ cell population enriched with CSCs in MYC induced lymphoma.

Sca-1+ cells maintain exclusive self-renewal during serial transplantation

To evaluate whether Sca-1+ cells maintain stable frequency and self-renewal state during serial transplantation, a monoclonal transplantation assay was performed where tumors were derived from a single CSC (47). For this purpose, Sca-1+ cells were isolated from multiple primary tumors: D9476, E6550, D9479 and E0366 (Figure 1D left panel, and Supplementary Figure 2A–C). A single Sca-1+ cell from these primary tumors was then injected to syngeneic mice to obtain secondary tumors or further re-injected to obtain tertiary tumors. We found secondary and tertiary tumors derived from each of the four parental tumors maintained stable proportion of Sca-1+ cells, and their phenotype including the high HIF-2 α protein expression in comparison to Sca-1- cells (Figure 1D–E, supplementary figure 2D, Supplementary table 3 A&B). Additionally, Sca-1- cells from these tumors expressed TCR V β expression as parental Sca-1+ cells (Supplementary Table 1 and 3C). Then, Sca-1+ and Sca-1- cells from these primary, secondary and tertiary tumors were subjected to *in vivo* limiting dilution assay to obtain CSCs proportions. We found that the ratio of CSC frequency between Sca-1+ versus Sca-1- cells remained stable. (Figure 1F, Supplementary table 4). Our results suggest that Sca-1+ CSCs maintain stable proportion during serial transplantation.

A stable proportion of CSC may be maintained either via asymmetric self-renewal or by conversion of non-CSC to CSCs by the process of phenotypic equilibrium (7, 9). To address this possibility, we subjected Sca-1- cells from primary, secondary and tertiary tumors (Figure 1D–F) to *in vivo* limiting dilution assay. Interestingly, CSC frequency did not become elevated during serial transplantation (Supplementary table 4), suggesting the unlikely contribution of Sca-1- cells to CSC frequency.

HIF-2 α pathway is essential to maintain the stemness of Sca-1+ cells

Inactivation of MYC by doxycycline treatment downregulates HIF-2 α without significantly changing HIF-1 α in Sca-1+ cells (Supplementary Figure 3A), suggesting that HIF-2 α pathway may be required to maintain the stemness state of Sca-1+ cells. By Flow cytometry, the expression of HIF-2 α , Nanog and Sox2 were found to be consistently higher in Sca-1+ cells of serially transplanted tumors. While, the expression of HIF-1 α and p53 remained low in the Sca-1+ cells (Figure 2A, Supplementary table 3B). Thus, Sca-1+ cells maintained high HIF-2 α during serial transplantation. In contrast, the Sca-1- cells maintained low HIF-2 α expression (Supplementary Figure 2D). Thus, serially transplanted Sca-1+ cells but not Sca-1- cells maintain high HIF-2 α , suggesting the potential role of HIF-2 α in the exclusive self-renewal of Sca-1+ versus Sca-1- cells.

Hence, we evaluated the role of HIF-2 α in the self-renewal of Sca-1+ cells. Suppression of HIF-2 α but not HIF-1 α expression by siRNA resulted in decreased Nanog and Sox2, but increased p53 expression (Figure 2B and Supplementary Figure 3A–B). These changes were associated with decreased HIF-2 α protein levels, *in vitro* cell growth, but no change in cell death (Figure 2B–C and Supplementary Figure 3C–D). Suppression of HIF-2 α but not HIF-1 α was associated with multiple senescence and differentiation markers including reduced BrdU incorporation and increased beta-galactosidase staining (Figure 2D) p21, and p16 (39) and decreased expression of CD44 and C-kit (Supplementary Figure 3E).

Apoptosis measured by caspase-3 activity was not altered (Figure 2D). Therefore, HIF-2 α is required to maintain the stemness state of Sca-1+ cells.

HIF-2 α maintains the self-renewal of Sca-1+ cells by suppressing p53 and ROS.

In ES cells, HIF-2 α negatively regulated p53 and ROS to maintain an undifferentiated state of high glutathione (GSH) redox state (33). We explored whether HIF-2 α employs similar mechanisms of maintaining low p53 and ROS levels in Sca-1+ cells. Flow cytometry-based evaluation of p53, ROS and GSH were performed in Sca-1+ cells subjected to HIF-2 α silencing. Interestingly, suppression of HIF-2 α indicated decreased GSH while increased p53 and ROS levels in Sca-1+ cells. Further, these changes were accompanied by loss of Sca-1 expression (Figure 2E–F, supplementary table 5A). Therefore, HIF-2 α appears to negatively regulate p53 and ROS for maintaining the stemness state of Sca-1+ cells. Indeed, siRNA p53 gene silencing blocked HIF-2 α silencing from inducing proliferative arrest or senescence in Sca-1+ cells (Figure 2G–H, Supplementary Figure 3F). Similarly, inhibition of ROS by co-treatment with N-acetyl cysteine (NAC) blocked HIF-2 α silencing from inducing p53 (Supplementary Figure 3F) and proliferative arrest or senescence (Figure 2H). Hence, HIF-2 α suppresses both p53 and ROS to maintain proliferation of Sca-1+ cells.

Next, we examined if HIF-2 α was required for the self-renewal of Sca-1+ cells by injecting these cells to immunocompetent mice. We measured *in vivo* tumorigenic growth and self-renewal of HIF-2 α silenced Sca-1+ cells with or without p53 silencing or NAC treatment. HIF-2 α silencing in Sca1+ cells decreased tumorigenic growth by 115-fold (Figure 3A–B, Supplementary table 5B) and self-renewal by 11-fold (Figure 3C–E, Supplementary table 5C–D). However, concomitant NAC treatment or p53 silencing restored both tumorigenic growth and self-renewal (Figure 3B–E, Supplementary table 5C–D). Importantly, HIF-2 α silencing did not prevent Sca-1+ cells from entering the thymus (Supplementary Figure 4 A–C). Thus, HIF-2 α appears to maintain the self-renewal of Sca-1+ cells by negative regulation of ROS and p53.

We examined if the *in vivo* inactivation of HIF-2 α through the small molecule FM19G11 influenced tumor growth. Tumors in mice treated with FM19G11 showed increased survival comparable to vehicle treated group (Figure 4A). Importantly, Sca-1+ cells from FM19G11 treated tumors indicated increased p53 but decreased HIF-2 α as well as self-renewal capacity of Sca-1+ cells (Figure 4B–C and Supplementary table 6A–B). FM19G11 combined with NAC reduced the p53 and ROS levels, increased GSH and rescued the self-renewal capacity of Sca-1+ cells (Figure 4 D–F, and Supplementary table 6A–B). This further supports the conclusion that HIF-2 α is required for self-renewal of Sca-1+ cells.

However, we noted that 4 out of 10 tumor bearing mice treated with FM19G11 exhibited morbidity within 20–30 weeks (Figure 4A) suggesting continuing growth of thymic tumors despite treatment. Sca-1+ versus Sca-1- cells recovered from these tumors maintained the expression of high HIF-2 α and low p53, whereas MYC level remained equal (Figure 4G). Furthermore, Sca-1+ versus Sca-1- cells of these post-FM19G11 treated tumors maintained high tumorigenic capacity (Supplementary table 6C). This suggests that Sca-1+ cells that escape sensitivity to FM19G11 did not evolve to a different phenotype but maintained the high HIF-2 α phenotype.

MYC through Nanog and Sox2 regulates HIF-2 α in Sca-1+ versus Sca-1- cells

We examined MYC's role in the exclusive regulation of HIF-2 α in Sca-1+ versus Sca-1- cells. MYC was found to be equally expressed in Sca-1+ and Sca-1- cells, obtained from a single Sca-1+ cell derived tumor #D9476 (Figure 1D, and Figure 5A) and expressing TCR V β 4 (Supplementary table 3C). We infer that other transcription factors may be required for differential regulation of HIF-2 α in Sca-1+ versus Sca-1- cells. One possibility was that the iPS genes Nanog and Sox2 might be involved in this MYC mediated regulation of HIF-2 α , since they both were found to be highly expressed in Sca-1+ but not Sca-1- cells (Figure 1C, Supplementary Figure 1A–C and Supplementary table 3B). Indeed, we found that MYC extensively binds to the promoters of HIF-2 α , Nanog and Sox2 but not Oct4 in Sca-1+ versus Sca-1- cells (Figure 5B). Notably, MYC binds preferentially to the HIF-1 α promoters in Sca-1- versus Sca-1+ cells (Figure 5B). Suppression of MYC expression decreased HIF-2 α , Nanog and Sox-2, but upregulated HIF-1 α expressions in Sca-1+ versus Sca-1- cells (Figure 5C). Thus, MYC preferentially bound and regulated the expression of HIF-2 α , Sox-2 and Nanog in Sca-1+ versus Sca-1- cells.

Next, we examined if MYC regulation of HIF-2 α expression was mediated by Nanog and/or Sox-2. We found that co-siRNA silencing of Nanog and Sox2 decreased MYC binding to HIF-2 α , reduced HIF-2 α protein levels by 4-fold and decreased the self-renewal capacity without altering MYC protein level (Figure 5D–G, Supplementary Figure 5A–C). Thus, MYC cooperates with Nanog and Sox2 to increase HIF-2 α expression and regulate self-renewal in Sca-1+ versus Sca-1- cells.

MYC through HIF-2 α maintains self-renewal in human lymphoma

To confirm our findings from transgenic mouse model system in a human equivalent tumor, we first identified the equivalent ABCG2+/HIF-2 α CSC subpopulation in relapsed cases of human adult Acute Lymphoblastic Lymphoma (T-LBL). ABCG2, a stem cell marker regulated by HIF-2 α (33), was highly expressed in T-LBL patients treated with chemotherapy/radiation (48). Our results indicated that T- cells recovered from cervical lymph nodes and peripheral blood of the subjects (n=6, Supplementary table 2) were highly enriched in ABCG2+/HIF-2 α + cells (Supplementary Figure 6A–C). These ABCG2+ cells were CD7 positive but CD8 and CD1a negative (Figure 6A and Supplementary Figure 6D), a phenotype associated with T-ALL CSCs (49).

Serial transplantation assay in NOD/SCID mice showed a 2000-fold higher self-renewal capacity of ABCG2+ versus ABCG2- cells (Figure 6B–C, Supplementary Figure 7A–D). Phenotypically, ABCG2+ cells represented undifferentiated lymphoma gene signature with high expression of MYC, HIF-2 α , Nanog, Sox2, and CD44. p53 Expression was lower in ABCG2+ versus ABCG2- cells (Figure 6D) thereby indicating similar CSC property and stemness phenotype to Sca-1+ cells. Importantly, silencing of HIF-2 α but not HIF-1 α in ABCG2+ cells exhibited similar results that we observed in Sca-1+ CSCs (Figure 6E–G, and Supplementary Figure 8A–B). The results suggest that HIF2 α maintains the self-renewal of ABCG2+ cells in human equivalent cancer.

We furthered our findings and revealed that MYC differentially binds to promoter of HIF-2 α in ABCG2⁺ versus ABCG2⁻ cells while directly regulates the Nanog and Sox2 binding. Inhibition of MYC led to marked decrease of HIF-2 α , Nanog and Sox2 expression in ABCG2⁺ cells (Figure 7A–B, and Supplementary figure 8C), indicating that MYC and HIF-2 α may mediate self-renewal of ABCG2⁺ cells.

To confirm the role of Nanog and Sox2 in the MYC mediated regulation of HIF-2 α in ABCG2⁺ versus ABCG2⁻ cells, we performed ChIP assay. Inhibition of Nanog but not Sox2 led to marked decrease of MYC binding to HIF-2 α promoter (Figure 7C, and Supplementary figure 8C). Importantly, Nanog binding to the HIF-2 α promoter was observed in re-ChIP samples of MYC (Figure 7D), suggesting that MYC and Nanog may cooperate to regulate HIF-2 α expression. Furthermore, in Nanog silenced ABCG2⁺ cells, while MYC protein levels remained unaltered, the HIF-2 α protein and GSH levels were markedly reduced, p53 and its target genes p21, MDM2 and BAX were induced, and the self-renewal was negatively affected as measured by serial transplantation assay (Figure 7E–G and Supplementary Figure 8D). Interestingly, Nanog's role in ABCG2⁺ cell self-renewal was not independent of HIF-2 α , since Nanog knockdown ABCG2⁺ cells could be rescued by the constitutive expression of a HIF-2 α transgene (45) (Figure 7F–G; Supplementary Figure 8E). These results therefore suggest that Nanog participated in MYC regulation of HIF-2 α in ABCG2⁺ versus ABCG2⁻ cells to confer exclusive self-renewal capacity and tumor stemness.

Discussion:

Cancer stem cells contribute to chemotherapy failure and tumor relapse (2, 3). How CSCs maintain exclusive self-renewal is not clear. Here, we report that Sca-1⁺ CSCs in transgenic mouse model of MYC driven lymphoma and ABCG2⁺ CSCs from lymphoma subjects demonstrate exclusive self-renewal via specific MYC binding to HIF2 α promoter regions specifically in CSCs versus non-CSCs. Further, stemness factors Nanog and Sox2 cooperates with MYC to regulate HIF-2 α that in turn decreases p53 expression and reduces ROS levels in CSCs. Thus, MYC-HIF-2 α stemness pathway (Figure 7H) may contribute to the exclusive self-renewal mechanism of CSCs and regulate their frequency during multiple generations.

HIFs signaling have a complex role in cancer self-renewal where both HIF-1 α and HIF-2 α either promote or inhibit the self-renewal of leukemia (10, 20, 21, 50). MYC is a key regulator of pluripotency & differentiation that acts downstream of NOTCH (51), a developmental pathway that co-operates with HIFs to maintain self-renewal of T-ALL (43, 49, 50, 52, 53) Also, MYC maintains self-renewal in stem cells and CSCs through effects on cellular metabolism (14, 24, 29). How MYC cooperates with HIFs to regulate CSC self-renewal as well as frequency is less understood.

To investigate the role of HIF-2 α in the self-renewal of MYC dependent CSCs, we characterized Sca-1⁺ CSCs in mouse, and human T-lymphoblastic lymphoma patient derived ABCG2⁺ CSCs. In these CSCs, HIF-2 α a transcription factor known to maintain stem cell robustness (15), negatively regulates p53 expression and ROS levels to confer self-

renewal capacity. Importantly, our findings reveal that high HIF-2 α expression in Sca-1+ CSCs is enabled by distinct MYC binding to HIF-2 α promoter regions. Pluripotency factors such as Nanog and Sox2 together facilitated this binding, thereby conferring selective or exclusive self-renewal capacity to CSCs versus non-CSCs. Nanog and Sox2 are broadly expressed in human cancers (15, 54, 55). Nanog is known to maintain self-renewing T-cell leukemia cells by suppressing p53 (13) however the molecular mechanism was not clear. Now, our findings in human ABCG2+ CSCs suggest that Nanog mediated p53 suppression was HIF-2 α dependent. Furthermore, in Sca-1+ cells, we found that Nanog cooperates with Sox2 to facilitate MYC mediated selective upregulation of HIF-2 α in CSCs versus non-CSCs. Selective or exclusive MYC binding to HIF-2 α promoters in human ABCG2+ CSCs versus non-CSCs was facilitated by Nanog alone. Further experiments are required to understand the independent role of Nanog in self-renewal of human CSCs.

Involvement of the iPS factors Oct4, Nanog and Sox2 in CSC self-renewal may be tumor type specific (6,15). Only Nanog was required to regulate human ABCG2+ CSCs self-renewal, whereas both Nanog and Sox2 regulated the mouse CSCs self-renewal. Also, MYC protein levels were higher in human ABCG2+ CSCs as compared to mouse CSCs. Interestingly, Oct4, a downstream mediator of HIF-2 α pathway (31,33) was neither involved in human or mouse T-ALL derived CSCs. These observations indicate that the role of these stemness effectors might be tumor specific (6,11,12,15,17).

Our study is suggestive but does not confirm the possibility of CSCs asymmetric self-renewal at single cell level. Indirect evidences indicate that single Sca-1+ cell may give rise to tumors, representing a clonal population of both Sca-1+ and Sca-1- cells with distinct phenotype. Stemness factors Nanog and Sox2 were involved in regulating HIF-2 α expression in Sca-1+ versus Sca-1- cells, suggesting the possibility of asymmetric self-renewal and maintenance of CSC frequency. Further, previous observations illustrate that tumors maintain a stable population of CSCs (1) that may be attributed to asymmetric cellular division (2). Similarly, in a MYC-induced T-ALL model in zebrafish and in human CD7+ lymphoma stem cells, CSCs maintained their frequency during serial transplantation (47, 49). Therefore, CSCs may maintain their frequency by undergoing asymmetric self-renewal. Our T-ALL CSCs model of Sca-1+ cell self-renewal may serve as a model to understand the underlying mechanisms that maintain asymmetric self-renewal in cancer.

We identified that MYC and HIF-2 α negatively regulate p53 and ROS in CSCs and this appears to be required to maintain stemness. Nanog and Sox2 interacted with MYC and HIF-2 α to maintain balance between stemness and differentiation (Figure 7H). Thus, targeting this MYC-HIF-2 α stemness pathway could be a targeted therapy against CSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance:

Findings show that the HIF-2 α stemness pathway maintains leukemic stem cells downstream of MYC in human and mouse T cell leukemias.

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Figure 1: Sca-1+ versus Sca-1- cells of MYC-driven T-cell lymphoma exhibit self-renewal
Hypothesis: HIF-2α pathway is differentially activated in CSC versus non-CSCs in MYC driven T-ALL. **B.** Representative flow cytometry panels of Sca-1+/CD4+/CD8+/CD19- cells in MYC-induced thymic tumor (D9476 parental monoclonal tumor, supplementary table 1). **C.** qPCR analysis of stemness related gene expression; five independent samples from each parental tumor. *p<0.05 ** p<0.001 comparing to control (student t test). **D.** Demarcated tumor images from monoclonal serial transplantation assay. Number of mice with tumor/ total number of injection is given below each image (additional images in supplementary Figure 2). **E.** Flow cytometry quantification of Sca-1+ cell frequency in parental, primary (1^o), secondary (2^o) and tertiary (3^o) tumors (five independent samples, details in supplementary table 3A). **F.** Ratio of CSC frequency in serially transplanted tumors. ****p<0.00001 (analyzed by ELDA, supplementary table 4). Data in C&E represents mean ± SEM.

Figure 2: Fate of Sca-1+ cells following HIF-2α gene silencing

HIF-2α silencing was performed by treating Sca-1+ cells with 1μM of accell HIF-2α siRNA for 3-days and various assays (B-H) were performed on Day-4. **A.** Flow cytometry analysis reports expression of the indicated transcription factors in primary (1^o) and secondary (2^o) single Sca-1+ cell derived tumors (detail in supplementary table 3B). **B.** qPCR analysis of indicated genes in HIF-2α silenced Sca-1+ cells. NT: non-targeted siRNA. **C.** Alamar blue proliferation assay result. **D.** Sca-1+ cell phenotype following HIF-2α inactivation. Values were compared with NT group. **E.** Representative flow cytometry panels of GSH (monochlorobimane, mBCL), DCFH-DA (ROS) and p53 stained Sca-1+ cells. T- denotes targeted (siRNA HIF-2α treated), grey dots: isotype control. Quantification of the results is given in Supplementary table 5A. **F.** Levels of p53, and Sca-1 (ELISA), and GSH and ROS (calorimetric methods) following HIF-2α silencing. **G.** Western blot to quantitate p53 levels in siRNA HIF-2α and p53 silenced cells. **H.** siRNA HIF-2α treated cells were co-treated with NAC (1mM) or siRNA p53 to perform rescue experiment (additional data in supplementary figure 3). Data in A-D, and F-H are presented as mean ± (SEM) of at least 4 independent samples, *p<0.05, **p<0.001, ***p<0.0001 (ANOVA and Dunnett post hoc test).

Figure 3: HIF-2α maintains self-renewal of Sca-1+ cells by negative regulation of p53 and ROS

A. Sca-1+ cells were treated with siRNA HIF-2α with or without NAC (1mM) or siRNA p53 for 3 days and on Day-4 cells were injected to syngeneic mice to perform limiting dilution analysis (see details in supplementary table 5). **B.** NAC or siRNA p53 silencing rescued CSC frequency of HIF-2α silenced Sca-1+ cells (Supplementary table 5B). ***p < 0.0001 (ELDA). Error bar represents 95% confidence intervals. **C-E.** Self-renewal capacity was evaluated by performing serial transplantation assay. 5 × 10³ cells of each treatment groups were injected i.v., and mice were sacrificed after 10 weeks to find thymic tumors. Thymic tumors were dissociated and Sca-1+ cells were flow cytometry sorted and subjected to limiting dilution assay to estimate CSC frequency. Representative images of recovered thymic tumors are presented with demarcated circle. Insert shows number of mice with tumors per total number of injection. **D.** Representative flow cytometry panels indicate % of Sca-1+ cells in recovered tumors. **E.** CSC frequency of tumor cells recovered from the tumors shown in C (Supplementary table 5C–D). ***p < 0.0001, ELDA. Error bar represents 95% confidence intervals.

Figure 4: *In vivo* inhibition of HIF-2α in Sca-1+ cells by FM19G11 small molecular inhibitor
 Three weeks after the injection of 5000 Sca-1+ cells i.v., syngeneic mice were treated with FM19G11 (1.5 mg/kg; thrice weekly) or vehicle for two weeks. Animals were either observed for long-term (A) or sacrificed to recover Sca-1+ cells from the treated tumors (B-G). **A.** Kaplan-Meier survival estimation; n= 10 animals in each group. MYC dependency was confirmed by doxycycline treatment. Difference between vehicle and FM19G11 treatment was calculated using log-rank test; p<0.001. **B.** Sca-1+ cells frequency as measured by flow cytometry (five independent samples; **p<0.001, student t test). **C.** Western blot indicates sustained decrease of intracellular HIF-2α and gradual increase of p53 levels. **D.** CSC frequency following FM19G11 treatment (Supplementary table 6A-B). ***p<0.0001, Error bar represents 95% confidence intervals. **E.** Flow cytometry analysis of FM19G11 ± NAC (10mg/kg) treated tumor cells. Anti-cleaved Caspase-3 (Asp175, Cell Signaling) was used to stain apoptotic cells. **F.** Sca-1+ cells in E were evaluated for shown values, and compared with untreated Sca-1+ cells. **G.** To the right, phenotypes, and to the left, limiting dilution assay of tumors that escaped FM19G11 sensitivity (as shown in 4A). F & G phenotype data are presented as mean ± SEM, 4 independent samples. *p<0.05, **p<0.001, ***p<0.0001 (student t test). Protein levels were measured by ELISA. Raw data of limiting dilution is given in supplementary table 6C) ***p<0.0001. Error bar represents 95% confidence intervals.

Figure 5: MYC interacts with Nanog and Sox2 to induce HIF-2α in Sca-1+ versus Sca-1- cells

A. ELISA based analysis of Sca-1 and MYC proteins in Sca-1+ and Sca-1- cells derived from a monoclonal tumor (#D9476, Figure 1D). **B** qPCR analysis of MYC ChIP data in Sca-1+ and Sca-1- cell samples at the indicated promoters. 1GX1A served as negative control, while ODC1, a MYC target gene, served as positive control. The IgG panel represented matched isotype. Data was quantified as percentage of input DNA (N= 3 independent samples; student t test). **C.** qPCR data of indicated genes in Sca-1+ and Sca-1- cells following MYC inactivation upon doxycycline treatment (N = 4 independent experiments, student t test). **D.** qPCR analysis of MYC ChIP in Sca-1+ cells at the HIF-2α promoter following siRNA silencing of Nanog and Sox2. **E.** HIF-2α protein (ELISA) following co-silencing of Nanog and Sox2 (N = 4 independent experiments, student t test). **F.** Clonogenic capacity following co-silencing of Nanog and Sox2 (Supplementary Figure 5). **G.** ELISA for Nanog and Sox2 co-silenced Sca-1+ cells. E-G, N = 4 independent samples; ANOVA and Dunnett post hoc test. Data are presented as mean ± (SEM). *p<0.05, **p<0.001.

Figure 6: ABCG2+ cells in the human T-lymphoblastic leukemia (T-LBL) subjects are HIF-2α dependent

A. Representative flow cytometry panel indicates enrichment and purity analysis of ABCG2+/CD7+ cells from the cervical lymph nodes of T-LBL subject #5 ($90 \pm 4.5\%$; $n=4$; supplementary figure 6D). **B.** Engraftment of human CD45 cells in the bone marrow (BM) of NOD/SCID mice injected with 5×10^3 ABCG2+ or ABCG2- cells of subject #5 (quantification data is in supplementary figure 7A–B). **C.** Serial transplantation of ABCG2+ cells in NOD/SCID mice (details in supplementary Figure 7C–D). **D.** qPCR analysis of indicated gene expression in cells obtained during serial transplantation (five independent samples from each patient; student t test). **E.** Effect of NAC or siRNA p53 co-treatment in HIF-2α silenced ABCG2+ cells (four independent experiments, patient #5 & 6; ANOVA with Dunnett test). **F & G.** Co-silenced ABCG2+ cells (1×10^4) were subjected to serial engraftment (details in supplementary figure 8B). At least 3 independent samples, student t test. Data in D–G are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.001$

Figure 7: MYC interacts with Nanog to induce HIF-2α in ABCG2+ versus ABCG2- cells

A. qPCR analysis of ChIP-MYC in ABCG2+ and ABCG2- cell samples at the indicated promoters. ODC1, a MYC target gene, served as positive control. The IgG panel represented matched isotype. Data was quantified as percentage of input DNA (N= 4 independent samples; student t test). **B.** qPCR data of indicated genes in MYC silenced ABCG2+ cells. **C.** qPCR analysis of MYC ChIP in ABCG2+ cells at the HIF-2α promoter following siRNA silencing of Nanog and Sox2. (N= 4 independent samples; student t test). **D.** Chromatin materials pulled down with MYC primary IP from Fig 7A was used for Re-chip with Nanog antibody or IgG. Re-ChIP quantitative PCR signal is shown as fold enrichment relative to the IgG control. **E, F&G.** siRNA Nanog silenced ABCG2+ cells were evaluated for the reversal of GSH, p53, and self-renewal following introduction of a constitutively active mutant HIF-2α transgene (Supplementary Figure 8E). ELISA, and calorimetric assay measured proteins and GSH respectively. For *in vivo* engraftment, 1×10^4 ABCG2+ cells, patient #5 and #6 were used. Data in D-F are presented as mean $\pm$ SEM of four independent experiments. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ (student t test). **H.** A model figure summarizing the results on the identification of the MYC mediated HIF-2α stemness pathway.