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A network including TGFβ/Smad4, Gata2 and p57 regulates proliferation of mouse hematopoietic progenitor cells

Running head: NETWORK BETWEEN TGFb/Smad4, Gata2 AND p57 IN HPCs

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Highlights:

- Gata2 is a direct target of canonical transforming growth factor-β (TGFβ) signaling
- p57 is an indirect target of canonical TGFβ signaling regulated largely via Gata2
- Gata2 mediates a large part of the molecular programs downstream of TGFβ signaling
- Three factors are linked to the regulation of progenitor cell proliferation

Abstract

Transforming growth factor-β (TGFβ) is a potent inhibitor of hematopoietic stem and progenitor cell proliferation. However, the precise mechanism for this effect is unknown. Here, we have identified the transcription factor Gata2, previously described as an important regulator of hematopoietic stem cell (HSC) function, as an early and direct target gene for TGFβ-induced Smad signaling in hematopoietic progenitor cells. We also report that Gata2 is involved in mediating a significant part of the TGFβ response in primitive hematopoietic cells. Interestingly, the cell cycle regulator and TGFβ signaling effector molecule p57 was found to be upregulated as a secondary response to TGFβ. We observed Gata2 binding upstream of the p57 genomic locus, and importantly loss of Gata2 abolished TGFβ-stimulated induction of p57 as well as the resulting growth arrest of hematopoietic progenitors. Our results connect key molecules involved in HSC self-renewal and reveal a functionally relevant network regulating proliferation of primitive hematopoietic cells.

Introduction

Hematopoietic stem cells (HSCs) reside in a specialized micro-environment in the bone marrow (BM) known as the HSC niche, where numerous intrinsic and extrinsic regulatory factors combine to determine HSC fate [1]. One of these factors is transforming growth factor-β (TGFβ), an evolutionarily conserved growth factor with a well-documented, potent inhibitory effect on hematopoietic stem and progenitor cell (HSPC) proliferation *in vitro* [2-4], as well as a role in HSC self-renewal *in vivo* [5-7]. TGFβ signaling is initiated by ligand binding to the constitutively active serine/threonine kinase TGFβ receptor type II and subsequent recruitment of the TGFβ type I receptor to the ligand/receptor complex [8]. This initiates a phosphorylation cascade in which the downstream receptor activated (R)-Smads 2 and 3 are activated, allowing binding to the common (co)-Smad 4 and translocation to the nucleus where gene targets are regulated [9-11]. Central molecules in the TGFβ response are involved in all branches of Smad signaling, including those initiated by BMP and Activin. These are the inhibitory Smads (e.g. Smad7), which inhibit the whole signaling pathway, as well as Smad4, which is essential for transmitting the signal into the nucleus [9]. Interestingly, it has been demonstrated that the TGFβ signaling pathway is activated in HSCs *in vivo* [7, 12] and this activation seems to be critical for HSC maintenance as deletion of TGFβ receptor type II (TGFβRII) or Smad4 affects HSC function *in vivo* resulting in decreased repopulation capacity and self-renewal [6, 7]. While the mechanism mediating TGFβ signaling is well characterized, the downstream effector molecules in hematopoietic progenitors are poorly understood. The cell cycle inhibitor p57 has been reported to be transcriptionally activated by TGFβ and to play a critical role in TGFβ-induced cell cycle arrest of hematopoietic progenitor cells [13] and HSCs [14]. Additionally, HSCs have been reported to express high levels of p57, while hematopoietic progenitor cells do not [12, 15-17]. Furthermore, p57-deficient HSCs show a severely decreased self-renewal capacity and a reduced proportion of quiescent cells [18], suggesting that TGFβ has a role in keeping HSCs in a quiescent state through a mechanism involving the transcriptional activation of p57.

Here, we set out to elucidate the effector mechanisms involving p57 downstream of TGFβ signaling in primitive hematopoietic cells. However, even though p57 mRNA

levels were robustly upregulated following treatment of hematopoietic progenitor cells with recombinant human TGF-β1 (from now on referred to as TGFβ), the response was delayed and dependent on *de novo* protein synthesis, indicating that p57 is not an immediate effector molecule in the TGFβ response. Instead, we have identified the transcription factor (TF) Gata2, previously described as an important regulator of HSC function with similar functions as TGFβ and p57, [19, 20] as a Smad-dependent, direct target of TGFβ signaling in hematopoietic progenitor cells. Our results further reveal a transcriptional network involving Gata2, p57 and members of the TGFβ signaling pathway. This regulatory network is active in HSCs and downregulated upon differentiation. However, we can show that upon TGFβ treatment hematopoietic progenitors upregulate Gata2 and the network is restored. Importantly, Gata2 levels are critical for TGFβ-induced growth arrest demonstrating the functional importance for the TGFβ/Smad/Gata2/p57 axis in the regulation of hematopoietic progenitor cell proliferation. Thus, this study represents the first detailed mechanistic insight to TGFβ-induced growth arrest in the context of hematopoietic progenitor cells.

Methods

Mice

Wild type C57Bl/6 mice were used for bone marrow (BM) harvest. Cre expression under influence of the *Mx1*-promotor was induced with three intraperitoneal injections of polyinositolic polycytidylic acid (pIC) of 250 or 400 μg at 2-day intervals in adult conditional *MxCre/Smad4*$^{fl/fl}$ knockout mice [6] as well as in *MxCre/Gata2*$^{fl/fl}$ knockout mice [21]. Littermate mice lacking *Cre*-expression were used as controls. BM was harvested from *MxCre/Smad4*$^{fl/fl}$ mice and *MxCre/Gata2*$^{fl/fl}$ mice 7-10 days and 1-5 days, respectively, after the last pIC injection. All mice were maintained according to Swedish animal guidelines at the animal facility at BMC, Lund University. All experiments were approved by the Lund University Animal Ethical Committee.

Cell preparations and cell culture

Femur, tibiae and iliac crests from each mouse were crushed and BM was filtered through a 70 μm cell strainer (Becton Dickinson (BD) Falcon or Fisher Scientific), enriched for c-kit^{+} cells using positive magnetic selection (Miltenyi Biotec) and stained with fluorochrome conjugated antibodies for fluorescence-activated cell sorting (FACS). Cells were kept in PBS (Gibco) containing 2 % FCS (Gibco or Thermo Scientific). Murine BM was cultured in StemSpan Serum-free expansion medium (SFEM; StemCell Technologies) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (P/S, Gibco), 50 ng/ml murine stem cell factor (mSCF; PeproTech) and 50 ng/ml human thrombopoietin (hTPO; PeproTech). Lhx2 cells were cultured in Iscove's modified Dulbecco's medium containing L-glutamine and 25 mM HEPES (IMDM; Thermo scientific), supplemented with P/S, 10^{-4} M 2-Mercaptoethanol (Sigma-Aldrich), 5 % FCS, 100 ng/ml mSCF and 10 ng/ml human interleukin 6 (hIL-6; (PeproTech). When serum-free medium was required, FCS was replaced by 0.5 % BSA (StemCell Technologies). Where described, 0.05-10 ng/ml recombinant human TGF-β1 (TGFβ; R&D systems or Biovision) was added to cultures. An untreated control sample incubated in parallel with the TGFβ-treated sample was harvested at each indicated time point. Where cycloheximide (10 μg/ml) was used it was added to the cultures 3h prior to the start-point of the experiment.

Prior to global gene expression analysis, Lhx2 cells were serum-starved over night before treatment with 10 ng/ml TGF-β1 for 2 h. Untreated cells were used as controls.

Chromatin immunoprecipitation – sequencing (ChIP-Seq)

ChIP-Seq was performed as previously described [22]. Briefly, 1×10^8 Lhx2 cells were treated with 10 ng/ml TGFβ for 2h and then cross-linked with 1 % formaldehyde (Merck) for 15 min at room temperature before sonication using a Bioruptor. Sheared chromatin (150-500 bp fragments) was incubated with 10 μg anti-GATA-2 sc9008 (Santa Cruz Biotechnology) overnight and chromatin-antibody complexes precipitated with Protein-G agarose (Roche). Library preparation and sequencing was carried out as described[22]. Reads were aligned to the mm9 version of the mouse genome using bowtie (19261174), and peaks were detected using MACS (18798982) and selected on having a 15-fold increase over the IgG control with a -10*log10(pvalue)>90.

ChIP-PCR

For analysis of LSK and cKit$^+$ cells, the ChIP procedure [22] was modified as follows to accommodate the reduced cell number. Cells (300,000 – 6 million) were cross-linked and sonicated with solution volumes reduced as appropriate. Antibodies were pre-adsorbed to Protein G agarose, by incubating 2 ug of antibody (anti-GATA2 sc9008, anti-HA or normal rabbit serum IgG) with Protein G agarose (20 μl of a 50:50 slurry) in 100 ul RIPA buffer for 2 hours, before addition of BSA to 10 μg/μl for a further 30-60 min to block subsequent non-specific binding of chromatin. The agarose beads were recovered by centrifugation, supernatant removed, and 180 μl of sheared chromatin added, before incubation overnight at 4^{o}C with rotation. Each ChIP used chromatin equivalent to approximately 100,000-375,000 LSK or 3 million cKit$^+$ cells, based on the number of cells initially harvested. Wash volumes were reduced to 200 μl.

Real-time PCR was performed using either SYBR Green PCR or Taqman Master Mix (Life Technologies), using custom primers and probes; p57 PCR product was detected using Probe 76 (Universal ProbeLibrary, Roche Life Science); GAPDH product was detected using either SYBRGreen or the custom probe below (identified in Fig legends as “SYBR” and “plus probe” respectively). Custom primer/probe

sequences were: P57_F1 = gagctcccagaaagaccaca, P57_R1 = gaaaaagagctcctatggctgta; with probe: P57_F1A = gagggctgtggcaagactc, P57_R1A = tccagctttcaaattttatctcg, Smad7_F1 = aaaataagcaagggaagtgga, Smad7_R1 = ctggttctcagcctggtgtc; mGATA2_F = gtatgtcgtgggaggctgtt, mGATA2_R = taagcgccacctgaccat; GAPDH_F = caaggctgtgggcaaggt, GAPDH_R = tcaccaccttcttgatgtcatca, Custom GAPDH probe from Sigma Genosys = FAM-acgggaagctcactggcatggc_TAMRA.

RNA isolation and microarray hybridization

Total cellular RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's protocol. RNA concentration and integrity were determined by spectrophotometer (NanoDrop 1000, Thermo Scientific) and Bioanalyzer (2100 Expert, Agilent Technologies), respectively. Agilent's recommended procedures (Version 5.7, March 2008) were followed for the preparation and labeling of cDNA and hybridization, washing, scanning, and feature extraction of Agilent 60-mer oligonucleotide microarrays for microarray-based one-color gene expression analysis. Three independent RNA harvests were separately analyzed.

Analysis of microarray data

Arrays were normalized using cyclic-loess, and differentially expressed genes were identified using LIMMA (16646809) FDR< 0.2. Expression profiles were clustered using Genesis (11836235), and ontology analysis was carried out using DAVID (http://david.abcc.ncifcrf.gov/).

FACS

The following antibodies were used, either unconjugated or fluorochrome-conjugated: anti-B220, -CD3, -CD4, -CD5, -CD8, -Gr1, -Mac1, -Ter119, -Sca1, -cKit, -CD34, -CD48, -CD150 (BD, Biolegend or eBiosciences). Unconjugated antibodies were detected with tricolor conjugated goat $F(ab')_2$ anti-rat IgG (H+L) (Caltag Lab, Burlingame, CA). Samples were stained with 7-aminoactinomycin D (7-AAD; Sigma-Aldrich, St Louis, MO) to exclude dead cells. Cells were sorted on FACSAria or FACSVantage Cell Sorter (BD Biosciences) or analyzed on FACS Canto II (BD Biosciences).

Quantitative real-time PCR (qPCR)

RNA was extracted using RNeasy Micro Kit and reverse transcribed (Superscript III, Invitrogen) in the presence of random hexamers. Gene specific primers (Taqman; Applied Biosystems) were used to analyze the expression of *Gata2*, *Cdkn1c* (*p57*), *Id1* and *Smad7*, together with the housekeeping gene *Hprt*. Analyses were performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) according to manufacturer's protocol using the software SDS 2.2.1. Each assay was measured in triplicate and results were normalized to *Hprt* levels.

Lentiviral transduction

HA-Smad4 was cloned into the destination vector. Lentiviruses were produced at the vector unit at Lund University. Transfection of the HIV vector system into 293T cells was performed using Calcium Phosphate Transfection Kit (Sigma). Plates were coated with 40 μg/ml retronectin (Takara Bio Inc.) at RT for 2h, blocked with 2 % BSA solution at RT for 30 min and rinsed once with PBS. Freshly isolated BM was c-kit-enriched and kept in SFEM supplemented with 1 % P/S, 50 ng/ml mSCF, 50 ng/ml hIL-6 and 10 ng/ml murine interleukin 3 (mIL-3; Peprotech). Cells and virus supernatant (MOI: 15) were added to the pre-coated wells and incubated at 37°C overnight. After 24 hours 50 % of the medium was removed and fresh medium added to the culture. After additional 24 hours the cells were harvested for ChIP.

Colony-forming unit (CFU) assays

Whole BM cells or cells sorted on the basis of surface markers were plated in methylcellulose (Methocult 3231; Stem Cell Technologies), supplemented with 20 % IMDM, 1 % P/S, 50 ng/ml mSCF, 10 ng/ml mIL-3 and 10 ng/ml hIL-6, with or without TGFβ at indicated concentrations, in 6 well plates. Colonies were counted 10-15 days after plating.

Statistical methods

Statistics were determined using Mann Whitney test, Wilcoxon matched-pairs signed rank test or Student´s t-test and the following significance levels were used: **P*<0.05; ***P*<0.01; ****P* <0.001.

Data has been deposited in The Gene Expression Omnibus under accession numbers GSE73641 and GSM1899949.

Results

p57 expression is indirectly upregulated by TGFβ in primitive hematopoietic cells

The cell cycle inhibitor p57 is important for TGFβ-induced cell cycle arrest of HSPCs and its expression has been shown to be induced by TGFβ in primitive hematopoietic cells [13, 14]. To investigate how TGFβ affects p57 regulation in primary HSPCs over time, we performed a time course qPCR experiment where freshly sorted murine LSKCD34$^-$ cells (HSCs) or LSKCD34$^+$ cells (hematopoietic progenitor cells) were treated with TGFβ and harvested at different time points (Fig 1A). In agreement with earlier studies [13, 14], qPCR analysis of p57 expression revealed a strong upregulation at 5 h (2.8-fold, *P*=0.045) and 12 h (6.4-fold; *P*=0.018) following TGFβ treatment, in the hematopoietic progenitor population (Fig 1B). However, when protein translation was blocked by cycloheximide treatment, TGFβ-induced upregulation of p57 was effectively diminished (<1.3-fold; Fig 1C). Together, this data confirms previous findings of p57 as a downstream target of TGFβ but implies that p57 activation is a secondary effect dependent on the expression/activation of an additional transcriptional regulator. In HSCs (Fig 1D) p57 mRNA levels were higher than in progenitor cells (see Fig 1B) upon harvest, and unaltered by TGFβ-treatment (Fig 1D), possibly due to the previously reported HSC niche-induced TGFβ signaling activity in these cells [7]. In the presence of cycloheximide TGFβ had no effect on p57 expression in HSCs (Fig 1E).

Global gene expression profiling reveals Gata2 as a target of TGFβ signaling

To identify early gene targets of TGFβ signaling in hematopoietic progenitor cells we performed high throughput gene expression profiling of a primitive murine hematopoietic cell line overexpressing the oncogene Lhx2 (Lhx2 cells), previously demonstrated to have *in vivo* multilineage reconstitution potential [23]. Lhx2 cells have been shown to be highly similar to primary HSCs in the composition of TGFβ/Smad signaling molecules and in TGFβ response [23, 24], making them a good cell source for gene expression studies of TGFβ signaling, when large cell material is required. The 441 most differentially up- versus downregulated genes following 2 h TGFβ-treatment were clustered in pathways involved in HSPC regulation using the DAVID database. A subset of these genes are listed in Table S1 and presented in a heatmap

(Fig 2A). The microarray data confirm a change in expression of several genes previously known as TGFβ targets in other cell types, e.g. Smad7 [25], Skil [26], Id1 [27-30], Id2 [28, 29], Id3 [28, 30], Hes1 [31-33] and Cited2 [34-36], verifying the relevance of the array. The gene expression profiling additionally revealed a list of TFs (Table S2) responding early to TGFβ in primitive hematopoietic cells. Interestingly, among the TFs previously associated with stem cell activity only two genes (Hes1 and Gata2) had been related to an effect on the cell cycle [20, 37, 38]. To validate the results from the microarray we stimulated Lhx2 cells with TGFβ and harvested cells at different time points (using the same setup as for fresh HSPCs in Fig 1A) for qPCR analysis of the two well-known TGFβ targets Smad7 and Id1, as well as the TF Gata2. At 1- 2 h following TGFβ treatment an upregulation of Smad7 (Fig 2B), Id1 (Fig 2C) and Gata2 (Fig 2D) was observed. In the presence of the translation inhibitor cycloheximide Smad7 induction was partly blunted, but importantly, all three genes were still upregulated in response to TGFβ (Fig 2B-D), confirming that they are direct targets of TGFβ signaling. This has previously been reported for Smad7 [25] and Id1 [27], while Gata2 has not been implicated in this context.

Gata2 is a Smad-dependent direct target of TGFβ in primary hematopoietic progenitor cells

To investigate whether TGFβ signaling affects Gata2 expression in primary HSPCs we performed a second time-course qPCR experiment, using murine HSCs and progenitor cells in place of Lhx2 cells (Fig 1A). Intriguingly, in freshly isolated cells, Gata2 showed an expression profile comparable to p57, with higher mRNA levels in the HSCs than in the progenitor cell population (compare Figs 3A and 3B). Additionally, progenitor cells robustly upregulated Gata2 within 1-2 h following TGFβ-treatment (2.2-fold, $P<0.002$; Fig 3A). The fact that this early upregulation was detected also in the presence of cycloheximide, demonstrates that Gata2 is a direct target of TGFβ signaling in primitive hematopoietic cells (Fig 3C). Similar to p57, Gata2 expression was unchanged in HSCs at early time points and under cycloheximide conditions (Fig 3B and 3D).

Canonical TGFβ signaling has been shown to control proliferation of HSPCs through Smad proteins [5, 6]. To investigate if Gata2 and p57 are regulated via Smad signaling we used Smad4$^{-/-}$ LSK cells, deficient in all canonical TGFβ signal transduction and

resistant to TGFβ-induced growth arrest [6]. Following 2 h and 5 h TGFβ-treatment, Gata2 expression was significantly increased in WT total LSK cells compared to untreated control (1.4-fold and 1.8-fold respectively; *P*=0.004; Fig 3E), while cells deficient in Smad4 did not upregulate Gata2 (Fig 3E). Similarly, expression analysis of p57 in WT LSK cells showed a significant increase in gene expression after 5 h of TGFβ-treatment (2.1-fold; *P*=0.004; Fig 3F), whereas TGFβ-treated Smad4$^{-/-}$ LSK cells exhibited unaltered p57 expression (Fig 3F). This confirms that TGFβ-induced changes in Gata2 and p57 expression in hematopoietic progenitors are conveyed through Smad signaling.

Gata2 binds upstream the Cdkn1c (p57) genomic region

To gain further knowledge about the relationship between TGFβ and Gata2 we carried out GATA2- ChIP sequencing (ChIP-Seq) on 2 h TGFβ-treated Lhx2 cells (Fig 4A). Interestingly, there was a significant overlap (*P*<<0.001) between the GATA2 targets identified by ChIP-Seq and the downstream signature of TGFβ signaling as determined by microarray, comprising 110 genes (Fig 4B; genes are listed in Table S3). Both Smad7 and Gata2 were in this overlap while p57 was absent, possibly due to the short incubation (2 h) with TGFβ. Visual inspection of the ChIP-Seq data did however identify weak binding of GATA2 to the p57 promoter (data not shown). We verified these observations using qPCR of Gata2-bound chromatin in both Lhx2 and primary cells. The known -77kb Gata2 enhancer [39, 40] as well as the Smad7 intronic region and the p57 promoter region identified from the ChIP-Seq were all enriched by GATA2 immunoprecipitation in Lhx2 cells (Fig 4C-D). Binding to the p57 promoter was further confirmed in a second multipotent cell line (Fig S1) - as well as in primary cells (Fig 4E-F) where the minimal amount of material needed for reliable ChIP-PCR results limited us to the use of LSK cells. Together, this data implies that Gata2 binds to the regulatory region of p57 as well as a large number of additional TGFβ targets.

Smad4 binds the Gata2 genomic locus

Based on our findings that TGFβ has a direct effect on Gata2 through Smad4 (Fig 3B and C) we investigated possible binding of Smad4 to the regulatory regions of Gata2 in hematopoietic progenitor cells by ChIP-PCR. To this end an HA-tagged Smad4-expressing vector was transduced to progenitor-enriched, cKit^{+} BM cells to obtain

sufficient material for HA-ChIP analysis. Following treatment with TGFβ for 2 h (Fig 4A) we observed direct binding of Smad4, to the -77kb upstream enhancer of the Gata2 gene [39, 40] (Fig 4G), strengthening the observations from our gene expression analysis. We could also detect binding of Smad4 to a Smad7 intronic region (identified from our ChIP-Seq data) suggesting Smad7 as a direct target of TGFβ signaling in these cells (Fig 4G).

Gata2 is critical for normal TGFβ function

To investigate the relevance for the TGFβ/Smad/Gata2/p57 network in the HSC niche *in vivo* we analyzed HSPCs freshly isolated from MxCre-inducible Smad4 [6] knockout mice, deficient in TGFβ signaling (Fig 5A). Efficient out-floxing of Smad4 in this model has been shown previously [6]. Highly purified WT HSCs ($LSKCD34^-CD48^-CD150^+$) measured 4.7-fold ($P<0.001$) higher Gata2 mRNA levels as compared to the $LSKCD34^+$ cells (Fig 5B), and this differential expression was maintained in the $Smad4^{-/-}$ mice. Similarly, p57 was expressed 25.0-fold ($P<0.001$) higher in LSK $CD34^-CD48^-CD150^+$ cells compared to the less primitive LSK $CD34^+$ progenitor cells (Fig 5C), demonstrating a correlation between the primitiveness of the hematopoietic cells and the level of Gata2 and p57 expression. Surprisingly, although Smad4 deletion did not affect Gata2 expression, purified HSCs from $Smad4^{-/-}$ mice still exhibited a 1.9-fold lower baseline expression level of p57 compared to WT controls ($P= 0.04$, Fig 5C). These results imply that TGFβ signaling is dispensable for Gata2 expression in HSCs and that loss of TGFβ signaling leads to a reduction of p57 levels independent of Gata2 levels. Thus, to clarify the role of Gata2 in this pathway we performed similar experiments in BM of inducible Gata2 knockout mice [21]. To verify deletion of Gata2 in this MxCre-mediated conditional knockout mouse model, we analyzed Gata2 expression in freshly isolated Lin^-cKit^+ cells (Fig S2A); mice showing 41-99% reduction of Gata2 mRNA were included in further analysis, presented as Gata2 KOs. We also confirmed reduction/loss of Gata2 protein in bulk BM (Fig S2B) and performed PCR analysis of single colonies from CFU assays (Fig S2C). In concordance with previously reported findings of a reduction of HSCs in Gata2 haploinsufficient mice [19], Gata2 deletion resulted in a loss of the immunophenotypic LSK population (Fig S2D). However, in Lin^-cKit^+ progenitors we observed a significant reduction of p57 and Smad7 mRNA in the $Gata2^{-/-}$ background compared to littermate controls.

Importantly, TGFβ-induced upregulation of p57 was lost in Gata2$^{-/-}$ progenitors (Fig 5F) and Smad7 upregulation was significantly impaired (Fig 5G). To address the functional relevance for Gata2 in TGFβ-induced proliferation arrest we cultured lineage-depleted bone marrow cells from Gata2 heterozygous mice for four days *in vitro* with or without TGFβ. We found that loss of a single Gata2 allele rendered cells significantly less sensitive to TGFβ-induced proliferation arrest (Fig 5H). In accordance with this, Lin^{-}cKit^{+} cells purified from our GATA2 KO mice were less sensitive than WT cells to TGFβ-inhibition of colony formation (Fig S2E), despite incomplete outfloxing in the Gata2 KO cells (some colonies were heterozygote from pcr; not shown) and thereby probably a selection for remaining WT cells in the colony assay. It should be noted that, as might be expected, Gata2 deletion alone markedly reduced colony output (see Fig S2D) and survival (not shown) in bulk culture. In summary these results imply that maintenance of physiological p57 levels specifically in HSCs requires the presence of TGFβ signaling (via Smad4), while maintenance of Gata2 levels in HSCs seem to be Smad4-independent. However, in hematopoietic progenitor cells maintenance of p57 expression was found to be Gata2-dependent suggesting that both Gata2 and Smad4 are required for the maintenance of p57. In addition, in progenitor cells TGFβ stimulation lead to a Smad-mediated induction of both Gata2 and p57 levels and Gata2 was demonstrated to be critical for TGFβ-induced upregulation of p57 and subsequent proliferation arrest.

Taken together our results reveal a regulatory circuit between TGFβ/Smad4, Smad7, Gata2 and p57 critical for TGFβ-induced proliferation arrest of hematopoietic progenitor cells (Fig 6).

Discussion

The importance of the TGFβ pathway in HSC biology has been highlighted by observations of the Smad pathway being specifically activated in HSCs, as well as studies in genetic mouse models where deletion of TGFβRII or Smad4 results in increased proliferation and decreased self-renewal of HSCs [6, 7, 14]. Accordingly, recent work suggests that the physiological relevance for TGFβ in HSC biology is to keep HSCs quiescent and re-establish HSC homeostasis following hematopoietic stress [14, 41]. However, the molecular mechanism underlying TGFβ-induced proliferation arrest is largely unknown. Interestingly, TGFβ has been found to induce expression of the cell cycle inhibitor p57 in HSPCs [13, 14] and this activation is crucial for TGFβ-induced cell cycle arrest *in vitro* [13]. p57 is a likely downstream effector molecule of TGFβ signaling in HSPCs *in vivo*, since $p57^{-/-}$ HSCs purified from a p57 conditional knockout model lose quiescence and have severely impaired self-renewal capacity [18], similar to the phenotypes observed in TGFβ/Smad loss-of-function models. Here, we confirm that TGFβ induces upregulation of p57 in HPCs. However, in contrast to earlier observations in a cell line [13] we found that TGFβ-induced upregulation of p57 in primary hematopoietic progenitor cells was dependent on *de novo* protein synthesis, demonstrating that p57 activation is a secondary response to TGFβ. To identify early and possibly direct targets of TGFβ signaling we performed global gene expression analysis of Lhx2 cells shortly after TGFβ treatment, and generated a database of genes differentially expressed by TGFβ in HPCs. The TF Gata2 was revealed as one of the target genes. Interestingly Gata2 gene deletion in a mouse model results in similar effects on HSCs as a total blockage of TGFβ signal transduction with decreased abundance of LSK $CD34^-$ cells and impaired reconstitution potential [19]. Moreover, enforced Gata2 expression in $CD34^+CD38^-$ human cord blood cells mimics the effects seen after TGFβ stimulation of HSPCs with inhibited cell cycle both *in vitro* and *in vivo* [2, 4, 20]. These two seemingly contradictory scenarios of Gata2 haploinsufficiency and overexpression both resulted in a more quiescent state of primitive hematopoietic cells due to so far unknown mechanisms. Here we present evidence for Gata2 transcriptional regulation of the quiescence marker p57 downstream of the TGFβ signaling pathway. However, these findings do not exclude a role for Gata2 as a positive regulator of cell cycle downstream other

pathways. Importantly, we could confirm Gata2 as a direct target of TGFβ signaling in hematopoietic progenitor cells, as well as demonstrate a critical role of Smad4 for TGFβ induced Gata2 expression.

To identify TGFβ targets downstream of Gata2 in multipotent hematopoietic cells we carried out a ChIP-Seq experiment on TGFβ-induced Lhx2 cells. Interestingly, there was a large overlap between the GATA2 bound genes and the genes differentially expressed after 2 h TGFβ induction. In addition, ChIP-Seq revealed evidence for Gata2 binding to the p57 locus, indicating involvement in its regulation. This finding could be reproduced by several Gata2 ChIP-on-chip experiments on different hematopoietic cell lines (data not shown). Gata2 binding to the p57 regulatory region was confirmed by PCR analysis of GATA2-ChIPed material in multipotent hematopoietic cell lines, before extending this analysis into primary cells. In accordance, Gata2 bound to the same region upstream of the p57 transcriptional start site in freshly isolated LSK cells.

Inactive TGFβ is abundantly produced by several cell types in the BM, including HSPCs. However, Yamazaki *et al*. recently described how non-myelinating Schwann cells specifically express the TGFβ-activating integrinβ8, thereby creating an environment with active TGFβ in which HSCs reside [7]. It is believed that this co-localization of HSCs to the TGFβ-activating Schwann cells explains the observation of active Smad signaling in HSCs in contrast to progenitor cells [14]. These previous findings might explain our results that neither Gata2 nor p57 expression was clearly affected by TGFβ stimulation in HSCs. We speculated that the activated TGFβ signaling in HSCs *in vivo* result in already high baseline levels of Gata2 and p57 mRNA blunting the response to *ex vivo*-administered TGFβ. However, although Gata2 is a direct target of Smad4 (by ChIP), and TGFβ-induced upregulation of Gata2 in HSPCs *in vitro* is dependent on Smad4, Gata2 expression was unaltered in highly purified HSCs from Smad4 KO mice while p57 expression was significantly reduced. Still, deletion of Gata2 had strong impact on p57 baseline levels in hematopoietic progenitor cells as well as on TGFβ-induced upregulation of p57 and proliferation arrest. Together these observations suggest that even though the regulation of Gata2 and p57 expression is governed by multiple pathways, maintenance of physiological p57 levels requires the presence of both Gata2 and TGFβ signaling and that Gata2 is critical for TGFβ-induced proliferation arrest.

It is known that BM levels of TGFβ spike during recovery from hematologic stress directing the HSCs to return to quiescence, but the role of TGFβ in homeostasis is still controversial [41]. TGFβ signaling is also described to be adaptive [42] opening the possibility that Gata2 could be regulated differently by TGFβ during homeostasis than for example upon more sudden changes in TGFβ ligand concentrations. This theory is supported by our findings that steady state levels of Gata2 was unaffected in Smad4$^{-/-}$ cells while rapidly upregulated as a response to TGFβ treatment *in vitro*. Knocking out the Gata2 gene strikingly attenuated the phenotypic LSK compartment by loss of Sca1 expression and reduced c-Kit expression within the Lin$^-$ compartment. This could be due to a converted HSC signature or more likely to the loss of the Gata2-deficient HSCs in line with observations in haploinsufficient Gata2 mice [19] and with Gata2-deficient human BM showing a complete absence of the primitive CD38$^-$ cells within the CD34$^+$ progenitor compartment [43-45]. Even though we were limited to study progenitor-enriched Gata2-deficient cells we found these cells to exhibit a strong reduction of colony-forming ability and to be desensitized to TGFβ-induced growth arrest. Additionally, loss of one Gata2 allele was adequate to make the progenitor-enriched cells less sensitive to TGFβ-induced proliferation arrest. Thus, in hematopoietic progenitor cells, Gata2 is upregulated upon activation of TGFβ signaling and required for proliferation arrest through a mechanism that includes transcriptional activation of the cell cycle inhibitor p57.

GATA2 has previously been shown to physically interact with SMAD4 in various cell lines and to be involved in negative regulation of TGFβ-induced erythroid differentiation [46]. This is yet another example of that TGFβ signaling activity can result in different responses depending on cell type and context. Furthermore, in a previous protein-protein interaction screen where mouse and human TF cDNAs were transfected into hamster epithelial ovary cells, human but not mouse GATA2 was reported to interact with SMAD4 as well as several of the TGFβ-regulated TFs identified in our microarray, i.e. PML and CEBPA [47]. Interestingly, in our ChIP experiments we could observe that GATA2 binds to the regulatory region of Pml and Smad4 binds to the regulatory region of Gata2, implicating several levels of TF interactions in primary murine hematopoietic cells and a mechanistic connection of this TF network to TGFβ signaling and subsequent regulation of proliferation.

The Smad7 genomic region was highly enriched by GATA2 in Lhx2 cells, indicating a direct regulation of the main negative feedback molecule of TGFβ signaling by Gata2. We could show that Gata2 has an activating function on Smad7 since Gata2KO progenitor cells exhibited significantly dampened upregulation of Smad7 in response to TGFβ. The strong upregulation of Smad7 and modest increase of Gata2 levels in response to TGFβ in Lhx2 cells, along with the opposite observation upon translational block, make us speculate of the existence of a negative feedback mechanism between these genes that remains to be further investigated.

In our study we link together several molecules that have all been described to be of individual importance for HSC biology. Our approach is an important example of how studying interactions between regulatory molecules can provide a more comprehensive view of how these molecules are integrated and exert their downstream functions. Together, our data reveal a transcriptional network between TGFβ, Smad4, Smad7, Gata2, and p57, important for the regulation of hematopoietic progenitor cell proliferation.

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Authorship

Contribution: M.B. designed, performed and evaluated the majority of experiments and wrote the manuscript; E.R. performed several experiments; G.E.M. performed and supervised ChIP experiments and analysis and edited the manuscript, A.J.T. designed experiments, S.S. analyzed microarray and ChIP-Seq experiments; J.B. supervised microarray experiments; M.S. provided the Gata2 mice, G.K., T.E., S.K. designed and supervised the study and edited the manuscript.

Conflict of interest disclosure: The authors declare no competing financial interests.

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Figure legends

Figure 1. p57 is upregulated as a secondary response to TGFβ in primary hematopoietic progenitor cells. (**A**) Experimental setup. (**B**) Time course qPCR analysis of p57 expression in freshly isolated LSK $CD34^+$ cells treated with TGFβ (10 ng/ml) and/or cycloheximide (10 μg/ml) (**C**) and harvested at indicated time points. (**D**) Time course qPCR analysis of p57 expression in freshly isolated LSK $CD34^-$ cells treated with TGFβ (10 ng/ml) and/or cycloheximide (10 μg/ml) (**E**) and harvested at indicated time points. Expression data is normalized to HPRT. n=5, **P*<0.05; as analyzed by paired *t*-test comparing each treated sample to untreated cells at the same time point. Data represents mean values from independent experiments± SEM. HSPCs, hematopoietic stem and progenitor cells.

Figure 2. Lhx2 cells respond to TGFβ by direct upregulation of Smad7, Id1 and Gata2 mRNA levels. Lhx2 cells were serum-starved for 12h before the start-point of the experiment. **(A)** Selection of TGFβ-responsive genes separated into pathways of interest for HSC biology as annotated in the DAVID database (http://david.abcc.ncifcrf.gov/). Differentially expressed genes were identified with an FDR < 0.2 giving rise to a list of 441 genes (344 up- and 97 downregulated) from an expression microarray analysis of Lhx2 cells treated with TGFβ (10ng/ml) for 2h compared to untreated cells. n=3. (**B**) Time course qPCR analysis of Smad7 (n=4), Id1 (n=4) (**C**) and Gata2 (n=6) (**D**) expression in Lhx2 cells stimulated with TGFβ (10 ng/ml) and/or cycloheximide (10 μg/ml) and harvested at indicated time points. Expression data is normalized to HPRT and presented as fold change (+/-TGFβ). **P*<0.05; ***P*<0.01; ****P* <0.001 as analyzed by paired *t*-test comparing each treated sample to untreated cells at the same time point. Data represents mean values from independent experiments ± SEM.

Figure 3. Gata2 expression is directly upregulated as a Smad-dependent response to TGFβ in primary hematopoietic progenitor cells. Time course qPCR analysis of Gata2 in freshly isolated LSK $CD34^+$ cells (**A**) or $CD34^-$ cells (**B**) treated with TGFβ (10 ng/ml) and/or cycloheximide (10 μg/ml) (**C-D**) and harvested at indicated time points. Expression data is normalized to HPRT. n=5, **P*<0.05; ***P*<0.01 as analyzed

by paired *t*-test comparing each treated sample to untreated cells at the same time point. Data represents mean values from independent experiments ± SEM. (**E**) qPCR analysis of Gata2 and p57 expression (**F**) in LSK cells from WT mice and Smad4$^{-/-}$ mice. Cells were treated with TGFβ (10 ng/ml) for 2h or 5h. Expression data is normalized to HPRT and presented as fold change (+/-TGFβ). n=9, $^{**}P<0.01$ as analyzed by Wilcoxon matched-pairs signed rank test comparing each treated sample to untreated cells at the same time point. Data represents mean values from independent experiments ± SEM.

Figure 4. Transcription factor binding to TGFβ target regions. (**A**) Experimental setup. Chromatin of various hematopoietic cell fractions was immunoprecipitated with different antibodies and DNA subsequently analyzed by qPCR or sequencing. (**B**) Overlap analysis of Gata2 ChIP-Seq on 2h TGFβ-treated (10 ng/ml) Lhx2 cells and the most differentially expressed genes revealed by gene expression profiling (Microarray) of untreated versus 2h TGFβ-treated Lhx2 cells (10 ng/ml). Overlap represents 110 genes. $P<<0.001$ as analyzed by hypergeometric test. (**C**) qPCR analysis of GATA2-binding to loci for Gata2 (n=5 ChIPs from three independent chromatin preparations), Smad7 (n=4 ChIPs from two independent chromatin preparations) and p57 (n=6 ChIPs from three independent chromatin preparations) in Lhx2 cells treated with TGFβ (10 ng/ml) for 2h and immunoprecipitated with GATA2 antibody (black bars) or IgG control antibody (white bars). Enrichment is normalized to a region of the GADPH locus (SYBR). (**D**) Summary of C. Data represents mean values of fold enrichment of Gata2, Smad7 and p57 regions, in GATA2 ChIP compared to IgG, from independent experiments ± SEM. n=3. (**E**) qPCR analysis of p57 enrichment in freshly isolated LSK cells immunoprecipitated with GATA2 antibody (black bars) or IgG control antibody (white bars). Each pair of bars represents one independent experiment. Enrichment is normalized to a region of the GADPH locus (exp. 1 SYBR, exp. 2-3 plus probe). n=3. (**F**) Summary of E. Data represents fold enrichment of p57 region, in GATA2 ChIP compared to IgG, from independent experiments ± SEM. n=3. (**G**) qPCR analysis of Gata2 and Smad7 loci in freshly isolated cKit^{+} cells transduced with HA-Smad4 lentivirus, treated with TGFβ (10 ng/ml) for 2h and immunoprecipitated with HA antibody (black bars) or IgG

control antibody (white bars). Enrichment is normalized to a region of the GADPH locus (plus probe). n=1.

Figure 5. Gata2 is critical for normal TGFβ function. (**A**) Experimental setup. *MxCre/Smad4*$^{fl/fl}$ or *MxCre/Gata2*$^{fl/fl}$ and littermate control mice were injected with pIC three times and BM was harvested for cell sorting and subsequent experiments. (**B**) qPCR analysis of Gata2 and p57 expression (**C**) in freshly isolated LSK CD34^{-} CD48^{-} CD150^{+} (n=7) and LSK CD34^{+} (n=10) from WT mice and from Smad4$^{-/-}$ mice (n=4). Expression data is normalized to HPRT. **P*<0.05; ****P* <0.001 as analyzed by Mann Whitney test. Data represents mean values from independent experiments ± SEM. (**D**) qPCR analysis of p57 and Smad7 (**E**) expression in freshly isolated Lin^{-}ckit^{+} WT and Gata2 KO cells. Expression data is normalized to HPRT. n=10, **P*<0.05 as analyzed by Wilcoxon matched-pairs signed rank test. Data represents mean values from independent experiments ± SEM. (**F**) qPCR analysis of p57 and Smad7 expression (**G**) in freshly isolated Lin^{-}ckit^{+} WT and Gata2 KO cells treated with TGFβ (10 ng/ml) for 5h. Expression data is normalized to HPRT. n=10, **P*<0.05, ***P*<0.01 as analyzed by Wilcoxon matched-pairs signed rank test comparing each treated sample to untreated cells of the same cell type. Data represents mean values from independent experiments ± SEM. (**H**) Proliferation assay of lineage depleted WT and Gata2 heterozygote (Gata2 Het) BM cells. Cells were counted after 4 days of culture and data is shown as growth inhibition; fold growth of TGFβ-treated cells (10 ng/ml) compared to untreated cells, based on cell count. n=4. Data represents mean values from biological replicates ± SEM. **P*<0.05 as analyzed by unpaired *t*-test comparing growth inhibition in WT cells and Gata2 Het cells.

Figure 6. Model of the molecular mechanism behind the response to TGFβ signaling in hematopoietic progenitor cells. TGFβ ligands activate receptors that in turn activate R-Smads. R-Smads form a complex with Smad4, and this complex is responsible for gene regulation in the nucleus. Gata2 is an early target of TGFβ signaling, regulated directly (via Smad4) without the need for new protein synthesis, while p57 is a secondary target, dependent on protein translation. Smad7 is also directly upregulated by TGFβ signaling via Smad4. Gata2 is highly involved in the regulation of p57, which in turn plays a role in the control of proliferation arrest.

Figures

Figure 1

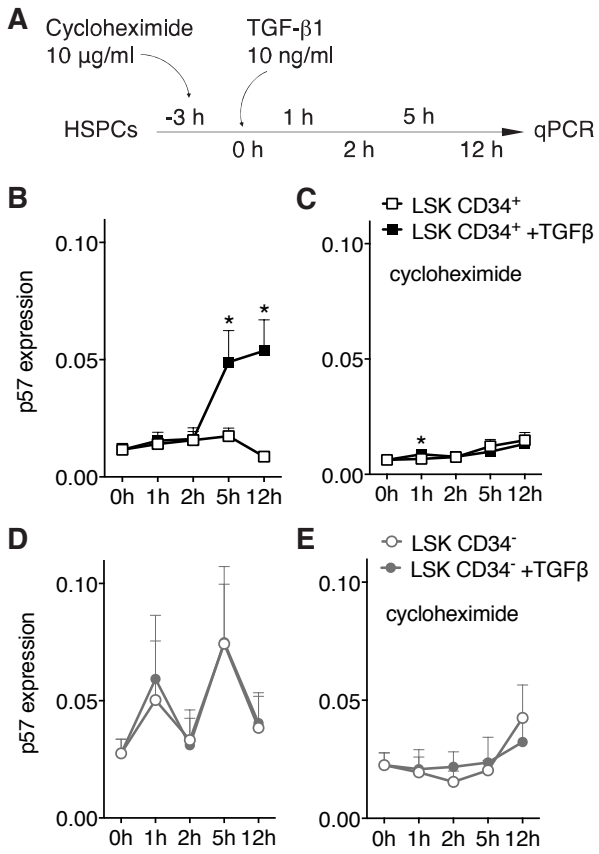
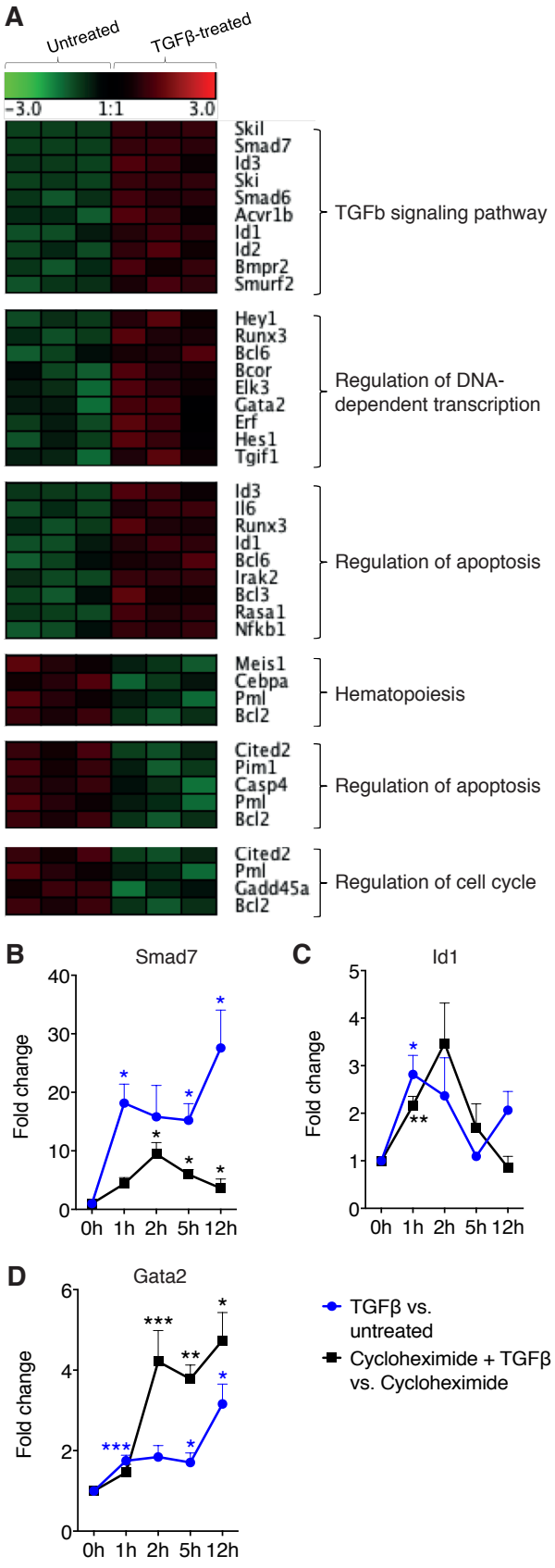


Figure 2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TGFβ-treated
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1:1
3.0
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Smad7
Id3
Ski
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Id1
Id2
Bmpr2
Smurf2
TGFb signaling pathway
Hey1
Runx3
Bcl6
Bcor
Elk3
Gata2
Erf
Hes1
Tgif1
Regulation of DNA-dependent transcription
Id3
Il6
Runx3
Id1
Bcl6
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Bcl3
Rasa1
Nfkb1
Regulation of apoptosis
Meis1
Cebpa
Pml
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Casp4
Pml
Bcl2
Regulation of apoptosis
Cited2
Pml
Gadd45a
Bcl2
Regulation of cell cycle
B
Smad7
Fold change
0h 1h 2h 5h 12h
C
Id1
Fold change
0h 1h 2h 5h 12h
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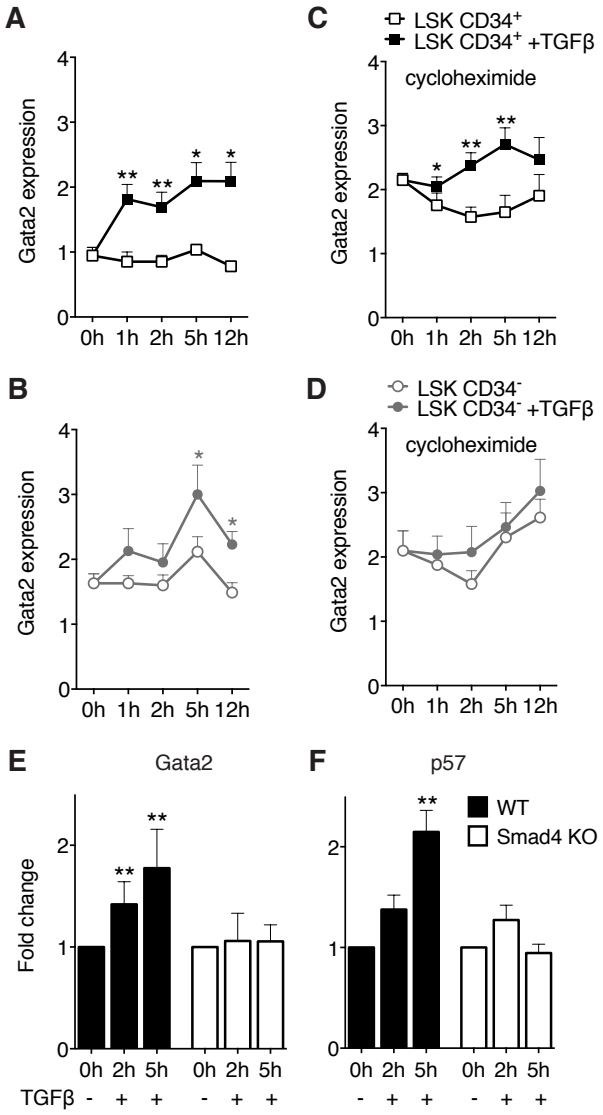
Gata2
Fold change
0h 1h 2h 5h 12h
TGFβ vs. untreated
Cycloheximide + TGFβ vs. Cycloheximide

Figure 3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C
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LSK CD34+ +TGFβ
cycloheximide
Gata2 expression
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B
D
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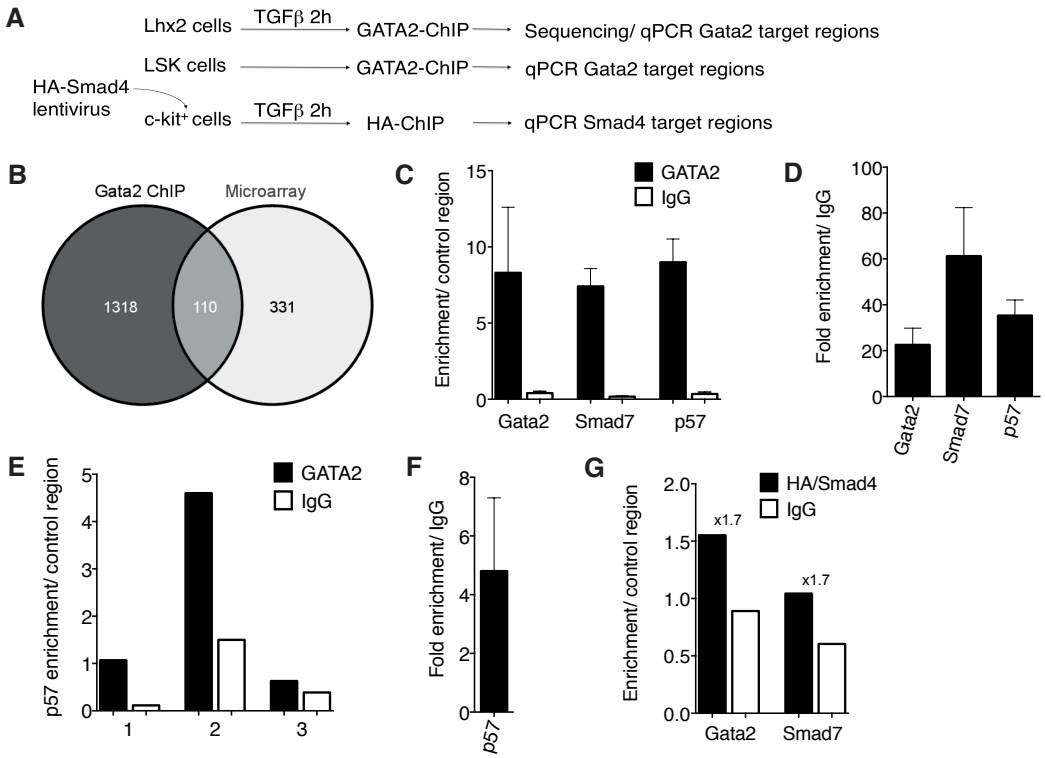
E
Gata2
F
p57
WT
Smad4 KO
Fold change
TGFβ - + + - + +

Figure 4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Lhx2 cells
TGFβ 2h
GATA2-ChIP
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LSK cells
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HA-Smad4 lentivirus
c-kit+ cells
TGFβ 2h
HA-ChIP
qPCR Smad4 target regions
B
Gata2 ChIP
Microarray
1318
110
331
C
GATA2
IgG
Enrichment/ control region
Gata2
Smad7
p57
D
Fold enrichment/ IgG
Gata2
Smad7
p57
E
GATA2
IgG
p57 enrichment/ control region
1
2
3
F
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G
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IgG
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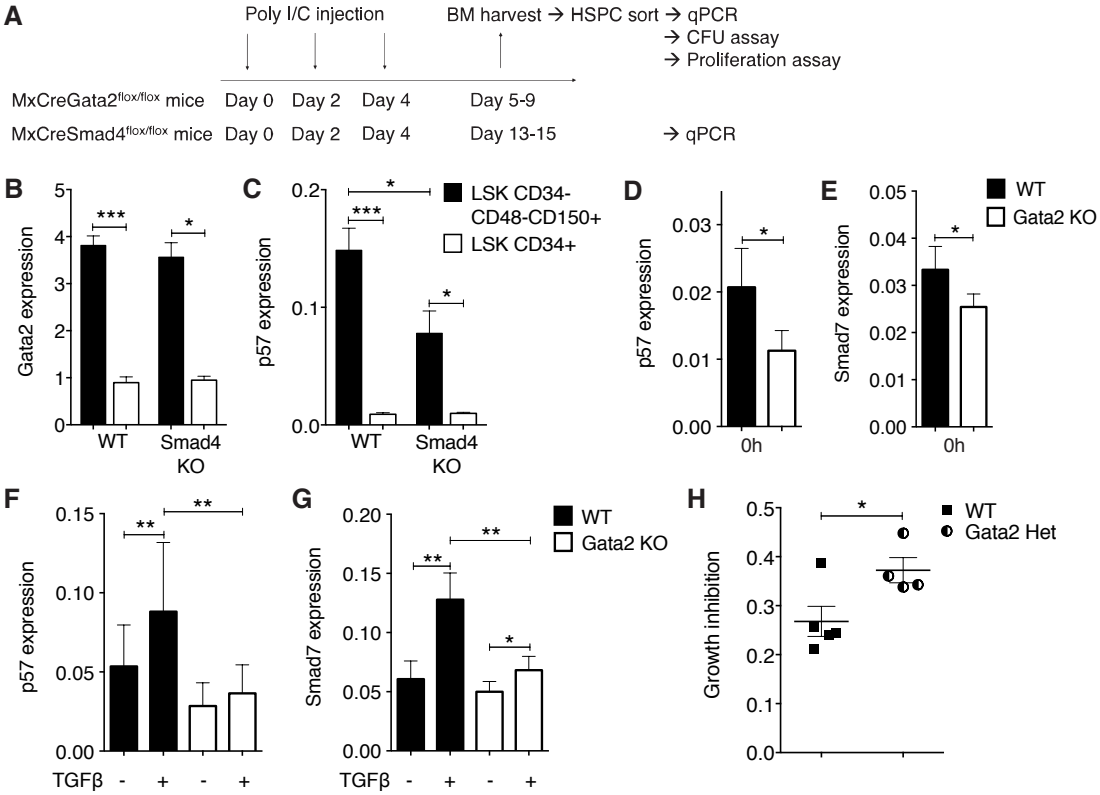
x1.7
x1.7
Gata2
Smad7

Figure 5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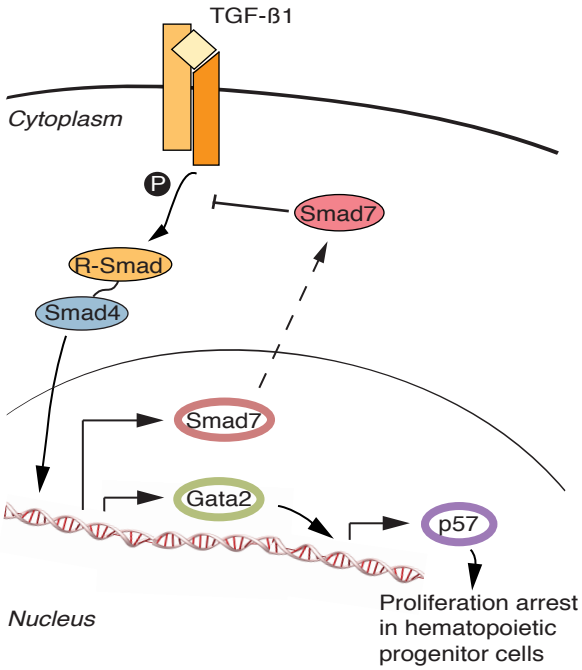
Poly I/C injection
BM harvest → HSPC sort → qPCR
→ CFU assay
→ Proliferation assay
MxCreGata2flox/flox mice Day 0 Day 2 Day 4 Day 5-9
MxCreSmad4flox/flox mice Day 0 Day 2 Day 4 Day 13-15 → qPCR
B
Gata2 expression
WT
Smad4 KO
C
p57 expression
LSK CD34-CD48-CD150+
LSK CD34+
D
p57 expression
0h
E
Smad7 expression
WT
Gata2 KO
F
p57 expression
TGFβ - + - +
G
Smad7 expression
H
Growth inhibition
WT
Gata2 Het

Figure 6


TGF-ß1
Cytoplasm
P
Smad7
R-Smad
Smad4
Smad7
Gata2
p57
Nucleus
Proliferation arrest
in hematopoietic
progenitor cells