STAT3 and STAT5 signaling in normal and leukemic hematopoietic cells
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Chapter 6

Maximal STAT5-induced proliferation and self-renewal at intermediate STAT5 activity levels

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Summary

The level of transcription factor activity critically regulates cell fate decisions such as hematopoietic stem cell self-renewal and differentiation. We have introduced STAT5A transcriptional activity into human hematopoietic stem/progenitor cells in a dose-dependent manner by overexpression of a tamoxifen-inducible STAT5A(1*6)-ER fusion protein. Induction of STAT5A activity in CD34+ cells resulted in impaired myelopoiesis and induction of erythropoiesis which was most pronounced at the highest STAT5A transactivation levels. In contrast, intermediate STAT5A activity levels resulted in the most pronounced proliferative advantage of CD34+ cells. This coincided with increased Cobblestone Area Forming Cell (CAFC) and Long-term Culture-Initiating Cell (LTC-IC) frequencies that were predominantly elevated at intermediate STAT5A activity levels, but not at high STAT5A activity. Self-renewal of progenitors was addressed by serial replating of CFUs, and only progenitors containing intermediate STAT5A activity levels contained self-renewal capacity. By extensive gene expression profiling we could identify gene expression patterns of STAT5 target genes that predominantly associated with a self-renewal and long-term expansion phenotype versus those that identified a predominant differentiation phenotype.
Introduction

STAT5 (Signal Transducer and Activator of Transcription 5) is widely expressed throughout the hematopoietic system, both in stem and progenitor cells as well as in committed erythroid, myeloid and lymphoid cells. Indeed, it is not surprising that STAT5 can be activated by a wide variety of cytokines and growth factors. These include those that act on the most immature human hematopoietic stem compartment such as Flt3-ligand (FL), Stem Cell Factor (SCF) and Thrombopoietin (TPO), as well as those that act on erythroid cells (Erythropoietin, EPO), lymphoid cells (Interleukin-7, IL-7; IL-2) or myeloid cells (IL-3; IL-5; Granulocyte/Macrophage-Colony Stimulating Factor, GM-CSF; Colony Stimulating Factors-1, CSF-1). Loss-of-function and gain-of-function experiments have revealed critical roles for STAT5 in several of these cell types. STAT5AB mice, which express an N-terminally truncated form of STAT5, have been used to assess stem cell function in the absence of wt STAT5 signaling. These mice were characterized by normal HSC numbers and stem cells isolated from the bone marrow or fetal liver were capable of engrafting irradiated recipients. Yet, competitive repopulating capacity of STAT5AB HSCs was severely impaired. The underlying mechanisms are not fully elucidated yet, but it has been observed that the responsiveness of STAT5AB HSCs to early-acting cytokines such as IL-3 and SCF was reduced, while the sensitivity to 5-fluoroacil was enhanced. More recently, mice completely devoid of STAT5 expression were developed as well, and data have indicated that STAT5 is required for HSC, lymphocyte, and erythrocyte development. We have observed that downmodulation of STAT5 expression impairs long-term self-renewal and maintenance of human stem and progenitor cells. Reversely, it has been shown that introduction of activating mutants of STAT5 in hematopoietic stem and progenitor cells enhances long-term self-renewal. Within the erythroid compartment, STAT5 fulfils an important anti-apoptotic role by upregulating Bcl-XL, although a more direct role in initiating erythroid commitment might exist as well. Although in the STAT5−/− mice myelopoiesis appears to be relatively unaffected, it is likely that in myeloid cells many of the signals initiated by e.g. IL-3 and GM-CSF are, at least in part, mediated by STAT5. STAT5 is also involved in eosinophil differentiation of human CB CD34+ cells in response to IL-5. Also, lymphoid development is severely impaired in STAT5−/− mice. These studies have highlighted that STAT5 signaling is involved at various stages throughout
hematopoietic development. Yet, little is known about the kinetics of cytokine-induced STAT5 signaling, or in which fashion STAT5 responds to lineage-restricted cytokine exposure. Also, it is currently unclear whether similar levels of STAT5 are required for the various functions that this transcription factor fulfils within the hematopoietic system.

Besides a role in normal hematopoiesis, elevated STAT signaling has been associated with leukemic transformation as well. Overexpression of a STAT5A S710F mutant (cSF5F) in murine bone marrow resulted in the development of multilineage leukemias upon transplantation into lethally irradiated wild-type or non-irradiated Rag2(-/-) mice\(^{31}\). The underlying mechanisms might involve STAT5 tetramer formation\(^{31}\) but cytoplasmic STAT5 functions have recently been described as well, whereby STAT5 forms a complex with PI-3K and Gab2, resulting in activation of the Akt/PKB pathway\(^{32}\). Other mechanisms by which STAT5 can contribute to the transformation process are the induction of genomic instability and DNA damage\(^{33}\), e.g. by upregulation of reactive oxygen species (ROS)\(^{34}\). Constitutive activation of STAT5 has been observed in the vast majority of Acute Myeloid Leukemia (AML) cases\(^{35-37}\). This may be attributed to the activating mutations in upstream kinases such as FLT3, c-KIT or JAK2\(^1\), or alternatively be due to autocrine growth factor production\(^{35}\). In primary AML CD34\(^+\) cells, we have recently been able to demonstrate that downmodulation of STAT5 expression by lentiviral RNAi impairs long-term expansion of leukemic stem/progenitor cells\(^{19}\). In murine cells, constitutive activation of STAT5 is sufficient to induce leukemia-like disease\(^{31,38}\). Elevated STAT5 signaling is also involved in the pathogenesis of other haematological malignancies, including Chronic Myeloid Leukemia (CML) induced by BCR-ABL\(^{39-44}\) or Polycythemia Vera (PV) induced by activated JAK2 V617F\(^{45-47}\).

Little information is currently available regarding mechanisms that are involved in STAT5-induced transformation. Recent reports highlighted the importance of transcription factor dosage that is associated with malignant transformation. Examples include PU.1, which is highly leukemic in murine models when expressed at 20% of normal endogenous levels, while complete loss or heterozygous dosage is not leukemic\(^{48}\). Furthermore, low levels of C/EBP\(\alpha\) are associated with leukemic transformation of myeloid lineages, while increased expression results in B-ALL\(^{49}\). Based on these findings we analyzed the effects of STAT5 in a dose-dependent manner on hematopoietic stem cell self-renewal and differentiation. We observed that at a low to intermediate STAT5 activity maximal effects on self-renewal of stem and progenitor cells were obtained in conjunction with long-term expansion. High STAT5 activity levels resulted in a predominant
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erythroid commitment with impaired myeloid development. Gene expression profiling over a range of STAT5 activities in human CB CD34+ cells revealed subsets of genes that were associated with the ‘self-renewal and long-term expansion’ phenotype. This subset contained (besides known growth-promoting genes such as PIM1 and CYCLIN D2) a large set of genes encoding membrane and membrane-associated proteins.

Materials and Methods

Cell culture and retroviral transductions

CD34+ cells were derived from neonatal cord blood from healthy full-term pregnancies from the Obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands, after informed consent. The protocol was approved by the Medical Ethical Committee of the UMCG. For all retroviral transduction experiments, the murine stem cell virus (MSCV) retroviral expression vector was used which contained an encephalo-myelocarditis virus (EMCV) derived internal ribosomal entry site (IRES2) in front of the truncated Neural Growth factor Receptor (NGF-R) (MiNR1 vector). The STAT5A(1*6) retroviral vector was described previously21,50. STAT5A(1*6)-ER was constructed by fusing the STAT5(1*6) cDNA, from which the stop codon was removed by PCR mediated mutagenesis, to the cDNA of the ligand binding domain of the murine estrogen receptor in pBlueScript (kindly provided by Hergen Spits, Department of Cell Biology and Histology, University of Amsterdam, The Netherlands). The resulting cDNA was cloned into the EcoRI site of pMSCV-iNGFR. pMSCV-STAT5A(S710F) was made by replacing the BamHI / NsiI fragment from pMSCV-STAT5(1*6) with the same fragment from pMSCV-STAT5A(wt). Cloning of STAT5A(S710F)-ER and STAT5A(wt)-ER was done in a similar way as pMSCV-STAT5(1*6) using cDNA from the corresponding pMSCV vectors. Details of the cloning and primer sequences are available upon request. pMSCV-STAT5B-ER was made by cloning the STAT5B-ER cDNA (kindly provided by Hergen Spits, Department of Cell Biology and Histology, University of Amsterdam, The Netherlands) from pLZRS into the EcoRI site of the pMSCV-iNGFR vector. Stable PG13 high titer retroviral producer cell lines were generated and CB CD34+ cells were transduced in three consecutive rounds as described previously21,50.
Stromal Coculture assays, Colony Forming Cell (CFC), Cobblestone Area Forming Cell (CAFC), LTC-IC and 2nd CFC assays

MS5 cocultures were performed as described previously. Briefly, MS5 stromal cells were preplated on T25 flasks in αMEM supplemented with 10% Horse serum (Sigma, Zwijndrecht, The Netherlands), penicillin and streptomycin and 200 mM Glutamine and cells were grown to full confluency. 3x10^4 transduced CB CD34+ cells were plated on MS5 in LTC medium (αMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% Horse serum (Sigma, Zwijndrecht, The Netherlands), penicillin and streptomycin, 200 mM Glutamine, 57.2 µM β-mercaptoethanol (Sigma) and 1 µM hydrocortisone (Sigma)). Half of the cultures was removed weekly and was replaced with fresh LTC medium. Harvested cells were counted and analyzed by FACS and progenitor content (CFCs) as indicated in the text. CFC assays were performed as described previously. Briefly, CFC assays were performed in MethoCult H4230 (StemCell Technologies, Grenoble, France) supplemented with 20 ng/ml Interleukin-3 (IL3), 20 ng/ml IL-6, 20 ng/ml G-CSF, 20 ng/ml c-Kit ligand (KL) and 6 U/ml EPO. 10^3-10^4 cells were plated in methylcellulose per plate in duplicate. For 2nd CFC assays, cells were harvested from methylcellulose after two weeks, washed three times with PBS, and replated into new methylcellulose for another two weeks after which 2nd CFCs were enumerated. CAFC and LTC-IC assays were performed by plating transduced CB CD34+ cells in limiting dilutions in the range of 6-1458 cells per well on MS5 stromal cells in 96-well plates in LTC medium. Cultures were weekly fed with new medium and after 5 weeks, methylcellulose was added to the wells. Two weeks later, wells containing CFCs were scored as positive. CAFC assays were performed as LTC-IC assays, but now CAFCs were counted at day 10 and at week 5 by microscopic evaluation of cocultures and phase dark colonies underneath the stroma were enumerated.

mRNA analysis

Total RNA was isolated using the RNeasy kit from Qiagen (Venlo, The Netherlands) according to the manufacturer’s recommendations. For real-time RT-PCR, cDNA was prepared by reverse transcribing the total RNA of 0.15 x 10^6 sorted cells using M-MuLV reverse transcriptase (Fermentas GmbH, St. Leon-Roth, Germany) according to the manufacturer’s instructions. cDNA was diluted 20 fold and 3 µl was then real-time amplified using iQ SYBR Green supermix (Bio-
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Rad, Veenendaal, the Netherlands) in a MyIQ thermocycler (Bio-Rad) and quantified using MyIQ software (Bio-Rad). HPRT, RPL27 and RPL30 expression levels\textsuperscript{51} were used to normalize between samples and to calculate relative expression levels. Primers and conditions are indicated in Supplementary Table 1. Genome-wide expression analysis was performed on Illumina (Illumina, Inc., San Diego, CA) BeadChip Arrays Sentrix Human-6 (46k probesets). Typically, 0.5-1 µg of mRNA combined from three independent transduction experiments was used in labeling reactions and hybridization with the arrays according to the manufacturer’s instructions. Data was analyzed using the BeadStudio v3 Gene Expression Module (Illumina, Inc.) and Genespring (Agilent, Amstelveen, The Netherlands).

Flow cytometry analysis

All antibodies were obtained from Beckton Dickinson (Alphen a/d Rijn, The Netherlands). Cells were incubated with antibodies at 4°C for 30 min. All fluorescence activated cell sorter (FACS) analyses were performed on a FACSCalibur (Becton Dickinson) and data was analyzed using WinList 3D (Topsham, USA). Cells were sorted on a MoFLo (DakoCytomation, Carpinteria, CA, USA).

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts of retrovirally transduced KG1a cells were made according to the mini scale procedure. EMSA analysis was performed by incubating 5 µg of nuclear extract with 5’-IRDye 700 labeled double stranded oligonucleotides for 30 minutes at room temperature. Binding reactions were run on non-denaturing 4 % Acrylamide gels in 1 x TBE and the gels were scanned using an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA). To check for specificity of the reactions, 50 fold molar excess of unlabeled oligonucleotide (either self or non-self) was added to the binding reactions. For supershift analysis, 1 µl of STAT5-specific antibody (C17, Santa Cruz Biotech, Santa Cruz, CA, USA) was added simultaneously with the probe.

Immunoblotting, histochemistry and cytoptins

Nuclear extracts were obtained as described above. Laemmli sample buffer was added to the samples, and samples were boiled for 5 min prior to separation on
12% SDS-acrylamide gels. Proteins were transferred to PVDF membrane (Millipore, Etten Leur, The Netherlands) by semidry electroblotting. Membranes were blocked in Odyssey blocking buffer prior to incubation with antibodies. Binding of antibodies was detected by incubating with Alexa680 or IRDye800 labeled secondary antibodies (Invitrogen, Breda, the Netherlands) and scanning of the membrane on an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA). Antibodies against STAT5 (C17) were obtained from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA, USA) and antibodies against tyrosine phosphorylated STAT5 (pY694, C11C5) were obtained from Cell Signaling Technologies (Leiden, The Netherlands) and were used in dilutions of 1:1000. May-Grünwald Giemsa staining was used to analyze cytospins.

**Luciferase assays**

For transactivation studies, 293T cells were transiently transfected in 12-well plates with the indicated constructs using Fugene6 (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Cells were transfected with a luciferase vector containing 3 STAT5-binding sites from the beta-casein promoter (3xGAS-luc), together with a pDM-LacZ vector as an internal control for transfection efficiency. 24 hours after transfection, cells were stimulated for 24 hours with 0-500 nM 4-hydroxy tamoxifen (Sigma) to induce STAT5 activation and cell extracts were made using Luciferase lysis buffer (Promega, Leiden, the Netherlands). Luciferase expression was measured according to the manufacturer's protocol (Promega, Leiden, the Netherlands). β-galactosidase expression was measured to correct for differences in transfection efficiency.

**Results**

**Dose-dependent induction of STAT5A activity in human CD34+ cells**

In order to generate a system in which STAT5 transactivity levels could be introduced into primary human CD34+ cells in a dose dependent manner, we constructed a fusion protein of the STAT5A(1*6) mutant with the modified ligand-
Figure 1. Dose-dependent induction of STAT5 activity using 4-OHT-inducible STAT5A(1*6)-ER fusion proteins. A, Schematic representation of the retroviral vectors used in this study. B, Western blot of nuclear extracts of KG1a cells transduced with MiNR1 control or STAT5A(1*6)-ER vectors. Cells were stimulated with 4-OHT in concentrations from 0-500 nM for 24 hrs as indicated and lysates were probed using antibodies against STAT5 and tyrosine 694 phosphorylated STAT5. C, Electrophoretic Mobility Shift Assay (EMSA) using nuclear extracts of MinR1 and STAT5A(1*6)-ER-transduced KG1a cells using a STAT5-specific probe from the beta-casein promoter. Cells were stimulated with 500 nM 4-OHT for 24 hrs or left unstimulated as indicated and nuclear extracts were prepared. Supershift experiments were performed using antibodies against STAT5. D, EMSA experiment as in C, but now cells were stimulated in a dose-dependent manner with 4-OHT as indicated. E, 293T cells were transiently transfected with MiNR1 or STAT5A(1*6)-ER vectors together with a luciferase reporter containing three STAT5 binding sites in the promoter. Cells were stimulated with 4-OHT in a dose-dependent manner as indicated for 24 hrs and cell lysates were prepared and analyzed for luciferase activity. F, CB CD34+ cells were transduced with STAT5A(1*6)-ER, stimulated with 4-OHT in a dose-dependent manner as indicated for 24 hrs and RNA was isolated. Q-PCR analysis was performed using primers against SOCS2. Q-PCR data were compared to results obtained from gene expression profiling using Illumina BeadChip Arrays. G, CB CD34+ cells were stimulated with a cocktail of cytokines (c-KIT ligand, FLT3-ligand, and Thrombopoietin (KFT)) in a dose-dependent manner as indicated. Total cell lysates were prepared and Western blotting was performed using antibodies against STAT5 and tyrosine 694 phosphorylated STAT5. H, CB CD34+ cells were transduced with wt STAT5A-ER and stimulated with 10 ng/ml IL3, 400 nM 4-OHT, or both. Total cell lysates were prepared and Western blotting was performed using antibodies against STAT5 and tyrosine 694 phosphorylated STAT5. I, CB CD34+ cells were stimulated with in creasing concentrations of KFT as in G, or transduced with STAT5A(1*6)-ER or wt STAT5A-ER after which cells were stimulated with 4-OHT as in B (for wt STAT5A-ER 0, 3.2, 16, 80, 400, and 2000 nM 4-OHT was used). RNA was isolated and Q-PCRs were performed using primers against CISH.
binding domain (LBD) of the Estrogen Receptor that is responsive to 4-hydroxy tamoxifen. STAT5A(1*6)-ER was inserted into the retroviral MiNR1 vector (schematically depicted in Fig.1A). Dose-dependent STAT5A inducibility was first tested in 293T human kidney and KG1a cells and later in transduced CB-derived CD34+ cells. Western blotting of nuclear extracts of transduced KG1a cells revealed that STAT5 tyrosine 694 phosphorylation and nuclear translocation could be induced in a dose-dependent manner upon treating cells with increasing concentrations of 4-OHT (Fig.1B). Specific STAT5A DNA-binding was determined by Electrophoretic Mobility Shift Assays (EMSA) and supershift experiments (Fig.1C), which could also be induced in a dose-dependent manner upon increasing concentrations of 4-OHT (Fig.1D). STAT5A transactivation was determined by transient transfections of 293T cells with the STAT5A(1*6)-ER fusion together with a 3x-GAS-luciferase reporter, and these studies also revealed a dose-dependent induction of STAT5A transactivation upon treatment with increasing concentrations of 4-OHT (Fig.1E). To study the effects of STAT5 transactivation on the expression of endogenous target genes, CB CD34+ cells were retrovirally transduced with STAT5A(1*6)-ER and RNA was isolated from cells that were treated with increasing concentrations of 4-OHT for 24 hrs. Illumina microarray analysis as well as Q-PCR analysis revealed that STAT5 target genes were induced in a dose-dependent manner and an example for SOCS2 expression is shown in Fig.1F.

Finally, we wished to determine how the induced STAT5A(1*6)-ER transactivity levels compare to normal endogenous STAT5 activity levels induced by cytokines as well as to wt STAT5A-ER activity levels. CB CD34+ cells were stimulated with the cytokines c-KIT-ligand, FLT3-ligand and Thrombopoietin (KFT) in increasing concentrations and STAT5 tyrosine 694 phosphorylation was determined by Western blotting. As shown in Fig.1B and Fig.1G, 4-OHT-induced STAT5A(1*6) tyrosine phosphorylation was within a similar range and never exceeded endogenous STAT5 phosphorylation levels induced by cytokines. Furthermore, endogenous IL-3-induced STAT5 signaling was compared to wt STAT5A-ER signaling in CB CD34+ cells. IL-3-induced STAT5A tyrosine phosphorylation was comparable to wt STAT5A-ER tyrosine phosphorylation induced by 4-OHT (Fig.1H). Moreover, similar observations were done in Q-PCR analyses for STAT5 target genes and an example is shown in Fig.1I in which cytokine-induced CISH expression was within a similar range compared to STAT5A(1*6)-ER and wt STAT5A-ER-induced CISH expression, whereby somewhat higher 4-OHT concentrations were required to activate the wt STAT5A-ER molecules.
Figure 2. Maximal proliferative advantage of CB CD34\(^+\) cells at intermediate STAT5A activity levels while differentiation is most affected at high STAT5A transactivation levels. A, CB CD34\(^+\) cells were transduced with MiNR1 control or STAT5A(1*6)-ER vectors and cocultures were initiated on MS5 bone marrow stromal cells in the presence of 4-OHT concentrations ranging from 0-500 nM as indicated. Cultures were weekly demidepopulated and cumulative cell counts of a representative experiment (out of three) are indicated. B, Experiment as in A, but now cells were transduced with MiNR1 control or STAT5A(S710F)-ER vectors. C, Experiment as in A, but now cells were transduced with MiNR1 control or wt STAT5A-ER vectors and cocultures were initiated on MS5 bone marrow stromal cells in the presence of 4-OHT concentrations ranging from 0-2000 nM. D, experiment as in A, but now cells that were harvested by weekly demidepopulation were analyzed by FACS for expression of myeloid (CD14, CD15) and erythroid (GPA) antigens. Data from a representative week 2 experiment is shown. E, Experiment as in A, but now cells harvested at week 2 were analyzed by cytospin and May-Grunwald-Giemsa staining. F and G, Experiments as in B and C, but now cells that were harvested by weekly demidepopulation were analyzed by FACS for expression of myeloid (CD14, CD15) and erythroid (GPA) antigens. Data from representative week 2 experiments are shown.

Proliferative advantage of CD34\(^+\) cells is most pronounced at intermediate STAT5A activity levels

CB CD34\(^+\) cells were transduced with MiNR1 empty vectors or STAT5A(1*6)-ER vectors, and cocultures on MS5 bone marrow stroma were initiated. 4-OHT was added to the cocultures in concentrations from 0-500 nM. Cultures were weekly demidepopulated and expansion of suspension cells was monitored. Control MiNR1 cultures expanded about 15 to 20-fold over a period of three weeks, which
was not affected by addition of 4-OHT to the cultures (Fig.2A). Activation of STAT5A resulted in a proliferative advantage as we have observed before\(^\text{21}\), but surprisingly the maximum fold expansion of over 80-fold within three weeks was not reached in the groups with the highest STAT5 activity levels (Fig.2A). Rather, transduced CD34\(^+\) cells treated with 20nM 4-OHT resulting in intermediate STAT5 activity levels displayed the maximal proliferative advantage (Fig.2A). In order to determine whether the effects on proliferation were specific for the STAT5A(1*6)-ER mutant, we repeated the co-culture experiments with STAT5A S710F-ER and wild-type STAT5A-ER (Fig.2B and B) as well as with STAT5B-ER (data not shown) and basically similar results were observed, albeit with somewhat different kinetics. STAT5A(S710F)-ER reached maximal long-term expansion at 20 nM (Fig.2B) whereby the maximal number of CAFCs was observed. While at 500 nM a strong expansion was observed at week 1, this expansion was transient and all cells differentiated towards an erythroid fate and no long-term cultures could be maintained after three weeks of MS5 coculture. Maximal wt STAT5A-ER induced proliferation was reached at 400 nM 4-OHT, which was not further increased upon further activation of STAT5 (Fig.2B).

**Impaired myelopoiesis and induction of erythropoiesis at high STAT5A transactivation levels**

Hematopoietic differentiation was monitored in suspension cells from MS5 cocultures by FACS and morphological analysis of cytospins by MGG staining. As observed previously\(^\text{21}\), STAT5A activation impairs myelopoiesis, as demonstrated by a reduction in cells that express monocytic and granulocytic antigens CD14 and CD15 (Fig.2D), while erythroid differentiation was induced as demonstrated by an increase in cells expressing Glycophorin A (Fig.2C), CD36 and CD71\(^{\text{bright}}\) (data not shown). Analysis of cytospins of suspension cells at week two yielded similar results, with an impairment of myelopoiesis and an induction of erythropoiesis upon STAT5 activation (Fig.2E). Importantly, maximum inhibition of myelopoiesis was observed at the highest STAT5 activity levels (Fig.2D). These data contrast our observations related to the proliferative capacity of cells which were maximal at intermediate STAT5 activity levels. Maximal erythroid differentiation and impaired myeloid differentiation were also achieved at the highest STAT5A S710F-ER and wt STAT5A-ER (Fig.2F and G) and STAT5B-ER (data not shown) activity levels.
Self-renewal of progenitors at intermediate STAT5A activity levels

To address the self-renewal capacity of hematopoietic progenitors, CB CD34⁺ cells were transduced with MiNR1 control or STAT5A(1*6)-ER vectors, and 10⁴ cells were plated in methylcellulose in duplicate in the absence or presence of increasing concentrations of 4-OHT. As depicted in Fig.3A, increasing STAT5 activity levels resulted in a dose-dependent reduction in CFCs. No apoptotic cells were observed, suggesting that reduced proliferation or differentiation beyond the progenitor stage was involved rather than programmed cell death (data not shown). After two weeks, CFC colonies were harvested and replated into fresh methylcellulose. Only cells that were cultured with low concentrations of 4-OHT were capable of giving rise to 2nd CFCs (Fig.3A, right panel). Secondary colonies were exclusively of myeloid origin, while erythroid colonies lacked replating capacity. Thus, low to intermediate STAT5 activity levels could confer self-renewal properties to myeloid progenitors, but self-renewal of erythroid progenitors was not observed.
Figure 4. CAFC and LTC-IC frequencies are increased at intermediate, but not high, STAT5A activity levels. A, CB CD34+ cells were transduced with MiNR1 control or STAT5A(1*6)-ER vectors and cocultures were initiated on MS5 bone marrow stromal cells in limiting dilution on 96-well plates in the presence of 4-OHT concentrations ranging from 0-500 nM as indicated. Early cobblestone areas were enumerated at week 2 (n=3). B, Experiment as in A, and representative images of cocultures at week 2 are shown. C, Stem cell frequencies were determined in LTC-IC assays in limiting dilution in 96-well plates on MS5 stroma by plating MiNR1 or STAT5A(1*6)-ER-transduced cells in concentrations of 6-1458 cells per well. Cultures were weekly fed with new medium, and at week 5 methylcellulose was added and LTC-IC frequencies were determined two weeks later. Data from two representative experiments is shown (in C and D). Panels on the left display all LTC-IC data over a range of plated cell densities without 4-OHT or in the presence of 500 nM 4-OHT. The panel on the right displays LTC-IC frequencies over a range of 4-OHT concentrations as indicated. E, Similar experiments as in C and D, but now stem cell frequencies were determined in LTC-IC assays in limiting dilution in 96-well plates on MS5 stroma by plating wt STAT5A-ER-transduced cells.
CAFC and LTC-IC frequencies are increased at intermediate, but not at high STAT5A activity levels

To determine the effects of dose-dependent STAT5 signaling on the human hematopoietic stem cell population, CAFC and LTC-IC assays were performed. Previous studies have shown that induction of STAT5A activity facilitates the interaction of human CD34+ cells with bone marrow stroma. These data indicated that activation of STAT5 induces HSCs into a ‘hyperactive’ state with the capacity to form CAFCs underneath bone marrow stromal cells that retain the capacity for long-term ex-vivo self-renewal21. First we determined the formation of CAFCs at week 2 in sorted transduced cells that were plated in limiting dilution in 96-well plates on MS5 bone marrow stromal cells. Control MiNR1 cells normally do not form CAFCs until week 5 in these cocultures and indeed no CAFCs were observed in MiNR1 control cells at week 2 (Fig.4A). In contrast, upon induction of STAT5A activity early CAFCs appeared within 1 week after plating which persisted to grow for over 5 weeks (Fig.4A and B). Importantly, the highest CAFC frequencies were observed at intermediate STAT5A activity levels (20 nM 4-OHT) and CAFC frequencies were significantly lower at high STAT5A activity levels (500 nM 4-OHT). Representative photographs of MS5 cocultures at week 2 are shown in Fig.4B. LTC-IC frequencies were determined in limiting dilution in 96-well plates, and in line with our CAFC data at week 2, LTC-IC frequencies were significantly enhanced upon induction of STAT5A activity, which were maximal at intermediate, but not the highest, STAT5A activity levels (2 representative experiments are shown in Fig.4C). Similar results were obtained in experiments in which CB CD34+ cells were transduced with wt STAT5A-ER vectors whereby maximal LTC-IC frequencies were observed at intermediate STAT5 activity levels (Fig.4E).

Genome-wide changes in gene expression induced by dose-dependent STAT5 signaling

In order to correlate global changes in gene expression with levels of STAT5A transactivity, Illumina BeadChip array analysis were performed using STAT5A(1*6)-ER-transduced CD34+ cells that were stimulated with increasing concentrations of 4-OHT (0-500 nM) for 24 hrs. mRNA was isolated from transduced and MoFlo-sorted cells which was used to hybridize human Illumina BeadChip Arrays that contain 46k probe sets. STAT5 expression levels, as well as the number and location of retroviral integration sites, were identical in all groups
as one batch of CD34+ cells was divided into six groups after transduction and MoFlo sorting that then received a certain dose of 4-OHT. Data was first analyzed by identifying significant changes in gene expression that were induced at intermediate STAT5A activity levels (20 nM versus 0 nM 4-OHT; fold change>2; p<0.05). Thus, 437 changes in gene expression were identified, which are presented in Fig.5 and Supplemental Table 2. Of the 437 changes, 220 genes were upregulated and 217 were downregulated (Fig.5, Supplemental Table 2). A number of the changes in gene expression were verified by independent Q-PCR analysis and we observed that the Illumina BeadChip Array analyses were in good agreement with data obtained by Q-PCR (26 out of 29 changes identified by Illumina BeadChip Arrays were confirmed by Q-PCR, Supplemental Fig.1). To determine whether the identified target genes were indeed potential STAT5 target genes or whether they were specific for the STAT5A(1*6)-ER mutant we also performed gene expression studies on CB CD34+ cells transduced with wt STAT5A-ER vectors that were stimulated with 0 or 500 nM 4-OHT. Fold changes are included in Supplemental Table 2 and Supplemental Figure 1 and we observed that the expression of the vast majority of identified genes was affected by both the STAT5A(1*6)-ER and wt STAT5A-ER in a similar manner, although the fold changes were typically somewhat lower in the wt STAT5A-ER transduced cells. As we observed different phenotypes at 20 nM (predominant long-term growth and self-renewal) and 500 nM (predominant erythroid differentiation, schematically represented in Fig.6), we analyzed our datasets in order to determine which differences in gene expression between these groups might exist. We noted that within the upregulated genes, two subgroups could be identified. In one subgroup maximal gene expression induced by STAT5 was reached at 20 nM 4-OHT ([STAT5]int group, defined by 20 nM > 0 nM (>2-fold) and 500 nM = 20 nM (+/-20%). This group contained 138 changes (Supplemental Table 3). A second subgroup contained genes that responded in a more linear fashion upon increasing STAT5 activity (‘linear up’ group, defined by 20 nM > 0 nM (>2-fold) and 500 nM > 20 nM (>1.2 fold)). This group contained 60 changes (Supplemental Table 4). The [STAT5]int group contained genes such as PIM1, GATA2, Insulin Growth factor Binding Protein 4 (IGFBP4), IGFBP5, CISH, Calbindin 2 (CALB2), Interleukin 3 Receptor alpha (IL3Ra), Integrin 2 (ITGA2) and MUC1. The oncogene MAF was also upregulated maximally at intermediate STAT5 activity levels. The ‘linear up’ group contained genes such as Suppressor of Cytokine Signaling 2 (SOCS2), and Regulator of G-protein Signaling 1 (RGS1), as well as various novel potential STAT5 target-genes including members of the CCL chemokine family (CCL2, -3, -
Figure 5. Genome-wide changes in gene expression induced by dose-dependent STAT5 signaling. CB CD34+ cells were transduced with MiNR1 control or STAT5A(1*6)-ER vectors and cells were stimulated with 0, 0.8, 4, 20, 100 or 500 nM 4-OHT for 24 hrs as indicated. RNA was isolated and genome-wide gene expression patterns were determined by Illumina Sentrix Human-6 BeadChip Arrays. The displayed data indicate gene expression changes in the 20 nM versus 0 nM groups (more than 2-fold up- or downregulated). Of the 437 changes, 217 genes were downregulated and 220 genes were upregulated. The upregulated genes were further subdivided into genes that reached their maximal expression levels at 20 nM (500 nM=20 nM +/- 20%, 138 changes) and genes that were further upregulated in response to further increasing STAT5 activity levels (500 nM>20nM, at least 1.2-fold, 60 changes). Gene lists are indicated in the supplemental data files. A number of gene expression changes were verified by Q-PCR analysis (all Q-PCR analysis are shown in Supplemental Fig.1). Gene expression changes were annotated according to cellular component and significant GO cellular component annotations are presented in pie charts.
Q-PCR analysis also revealed that Bcl2 and p21 are induced by STAT5 in a linear fashion (Fig.5). 217 genes were found to be downregulated by induction of STAT5A activity in CD34+ cells (20 nM < 0 nM 4-OHT (>2-fold)) and this group included CXCR4, Neutrophil Elastase (ELA2) and C/EBPα, genes that we previously identified as being downmodulated via STAT5A21,50 as well as various novel STAT5-regulated genes including Gap Junction protein Alpha 4 (GJA4) and Rho GTPase activating protein 21 (ARHGAP21) (Fig.5, Supplemental Table 2). We annotated the groups of genes according to biological process, cellular component and molecular function. It was striking to observe that genes within the [STAT5]int group were strongly enriched for membrane (p=0.0152), integral to membrane (p=0.00548), and intrinsic to membrane (p=0.00563) categories (Table 1, Figure 5). Genes within this group are listed and further annotated in Supplemental Table 3. These categories were absent from the ‘linear up’ group, which was most strongly enriched for genes belonging to the extracellular space (p=4.80E-05) category (Table 1, Fig.5). The downmodulated group was enriched for plasma membrane categories (p=0.000586), and also contained actin cytoskeleton (p=0.00574) and cell junction (p=0.00941) categories, amongst others (Table 1).

Discussion

The balance between hematopoietic stem cell self-renewal and differentiation needs to be tightly controlled, as a shift towards differentiation might exhaust the stem cell pool while a shift towards self-renewal might mark the onset of leukemic transformation. A number of transcription factors have been proposed to be critically involved in governing stem cell fate and lineage commitment, and these include HOX transcription factors52, c-MYC53, NOTCH154, β-CATENIN55, C/EBPα56, PU.157 and STAT518,20,21. It is therefore no surprise that dysregulation of these transcription factors can contribute to the development of leukemias. Over the past few years it has become increasingly evident that the dose of transcription factor activity is highly critical in determining cellular fates, whereby low versus high levels of activity might associate with very different phenotypes. Here, we describe that maximal self-renewal and long-term expansion is induced in human stem/progenitor cells by relatively low STAT5 transcriptional
activity levels, while high STAT5 activity levels predominantly result in erythroid commitment without long-term growth.

Maybe the clearest example to date that illustrates transcription factor dosage is PU.1. This transcription factor fulfils a variety of functions within the hematopoietic system, including maintenance of the stem cell pool, the generation of myeloid and lymphoid progenitors, as well as monocytic differentiation\(^\text{57}\). While a 50% reduction in PU.1 expression did not result in severe phenotypes, a reduction to 20% of normal PU.1 levels was shown to be highly leukemogenic\(^\text{48}\). Complete deletion of the \(PU.1\) locus did not induce leukemia, and these data convincingly indicate that the levels of PU.1 are highly important in determining the phenotype. Other examples include c-MYC, which is thought to be expressed at very low levels in resting HSCs, but its expression increases upon HSC activation and is high in proliferating progenitors\(^\text{53}\). Enforced overexpression of c-MYC in HSCs resulted in a quick loss of stem cells presumably due to the induction of differentiation.

NOTCH1, when expressed at high levels in neural stem cells leads to growth arrest, while low levels of the active form promote proliferation\(^\text{59}\). In human CD34\(^+\) cells, overexpression of the active form of NOTCH1 has resulted in opposing phenotypes, ranging from the induction of apoptosis\(^\text{59}\) to stem cell expansion\(^\text{54}\) and it is well plausible that the dose of active NOTCH1 is critical in determining the phenotype. Activation of \(\beta\)-CATENIN by WNT3A was suggested to enhance stem cell self-renewal\(^\text{65}\), although a loss of hematopoietic stem cell repopulation and a
block in multilineage differentiation was reported by others\textsuperscript{60,61}, which might well be explained by different dosages of $\beta$-CATENIN activity that were introduced. These examples illustrate that the level of transcription factor activity that is introduced within the hematopoietic stem/progenitor compartment strongly influences the decision whether to self-renew, proliferate or differentiate. In the present study we have used a model system in which the transcription factor activity of STAT5 could be tightly controlled by making use of 4-OHT-inducible chimeras of the modified Estrogen Receptor ligand binding domain fused to STAT5. Even though this 30 kDa domain is relatively large and we therefore cannot exclude the possibility that target gene expression might be altered by for instance changing STAT5 tetramer formation\textsuperscript{31}, we have observed induction of the known previously described STAT5 target genes with our ER fusion molecules. Thus, this approach allowed us to perform gene-dosage experiments in transduced human CD34\textsuperscript{+} stem/progenitor cells in relation to self-renewal, long-term expansion and differentiation. Our studies revealed that low to intermediate STAT5 activity, but not high levels of STAT5 activity, are required to induce optimal self-renewal and long-term expansion on stroma. This intermediate STAT5 activity was associated with the formation of cobblestone area (CA) formation underneath the bone marrow stroma within a few days after plating, and these CAs contain self-renewing cells as demonstrated by their serial replating capacity\textsuperscript{21,25,62}. In fact, this interaction with bone marrow stroma was required for self-renewal as no long-term cultures could be established in cytokine-driven liquid cultures using cells transduced with either activated STAT5 or FLT3-ITD\textsuperscript{21,63}. Genome-wide gene expression analysis revealed that at intermediate STAT5 activity levels this group was significantly enriched for genes encoding for membrane proteins. These include membrane proteins that have been associated with adhesion, such as Protocadherin 21, CEECAM1 and MUC1. While loss-of-function experiments are still required to determine whether these membrane proteins are indeed involved in self-renewal and long-term growth by mediating the interaction between the bone marrow microenvironment and HSCs expressing STAT5, we have already performed studies focusing on the role in MUC1 in normal and leukemic CD34\textsuperscript{+} cells. These studies indeed indicate that normal CD34\textsuperscript{+} cells can be further subfractionated into MUC1\textsuperscript{+} cells to enrich for stem and progenitor cells, that overexpression of MUC1 is sufficient to elevate stem cell self-renewal of human CD34\textsuperscript{+} cells, and finally that MUC1 expression is increased in CD34\textsuperscript{+} cells from AML patients in the majority of cases\textsuperscript{64}. Besides an enrichment for adhesion molecules in the group of genes that is upregulated by intermediate STAT5 activity
levels, we find that the group of downmodulated genes is also enriched for membrane-associated genes. Together, these data argue that STAT5 might strongly alter the membrane composition of stem/progenitor cells, such that interactions with the niche that allow long-term self-renewal are facilitated. This would be in line with our observations indicating that cells expressing activated STAT5 cannot be maintained long-term outside of a bone marrow microenvironment.

Besides membrane-associated genes, we also identified a number of growth promoting oncogenes as STAT5 targets. These include the serine/threonine kinase PIM1, which was initially identified as a MYC-cofactor that predisposes to lymphomagenesis\(^\text{65}\). Recently, it was shown that MYC recruits PIM1 to target genes to phosphorylate the serine 10 residue of histone H3 on the nucleosome, thereby contributing to transcriptional activation\(^\text{66}\). PIM1 has previously been identified as a STAT5 target and can induce cytokine-independent growth of cell lines\(^\text{22}\). MAF was also identified as a STAT5 target. In Multiple Myeloma, the oncogene MAF not only stimulates cell cycle progression, but also improves the interaction between tumor and stromal cells\(^\text{67}\). C/EBP\(\alpha\) expression was reduced by STAT5 as we have observed previously\(^\text{50}\), and in a variety of cases low expression levels or impaired signalling of C/EBP\(\alpha\) has been associated with enhanced self-renewal and leukemic transformation\(^\text{56,68-71}\). Importantly, the effects of STAT5 on these oncogenes, as well as on the cluster of genes that was enriched for membrane proteins, were all maximally induced or repressed at intermediate STAT5 activity levels and did not significantly change upon further increases in STAT5 activity. Yet, the phenotype did change upon a further increase in STAT5 transactivity from a predominant growth and self-renewal phenotype at intermediate levels, towards a predominant differentiation phenotype upon high STAT5 activity. The group of target genes at high STAT5 activity levels was significantly enriched for secreted extracellular factors. Within this group, we identified the chemokine family members CCL2, -3,-5, and -22 as novel STAT5 targets, and several potential STAT5 binding sites were found in their 5\(^\prime\)-promoter sequences (unpublished observations). It has been described that several of these chemokines have growth inhibitory effects on murine progenitor cells as determined by colony assays in methylcellulose\(^\text{72}\). These genes respond in a linear fashion in response to STAT5 activity and reach their highest expression levels at maximal STAT5 activity. The same is true for the cell cycle inhibitor p21CIP, and it is plausible that these growth inhibitory genes might shift the balance from proliferation towards differentiation at the highest STAT5 transactivation levels.
In conclusion, we propose a model which is summarized in Fig.6. Intermediate STAT5 activity levels are sufficient to induce maximal levels of growth promoting proto-oncogenes such as PIM1 and MAF, or low levels of C/EBPα, as well as maximal levels of membrane-associated genes such as MUC1 that might mediate the enhanced interaction between human hematopoietic stem/progenitor cells with their bone marrow microenvironment. This pattern of gene expression will drive long-term expansion and self-renewal. Upon further increases in STAT5 activity, only specific subsets of STAT5 target genes will be further enhanced, and these include p21CIP and members of the chemokine family. This set of genes will negatively affect cell cycle progression and expansion, resulting in a shift towards a differentiation phenotype along the erythroid lineage. On the basis of this model we would predict that relatively low, but not high, levels of STAT5 transcription factor activity would favour leukemic transformation, and it will be challenging to perform these gene-dosage studies in an in vivo setting to confirm these hypotheses. Also, it will be intriguing to decipher why genes respond so differently to increasing levels of STAT5

**Acknowledgements**

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**Supplemental data (available upon request)**

Supplemental Table S2: gene expression changes in group I
Supplemental Table S3: gene expression changes in group I intermediate
Supplemental Table S4: gene expression changes in group I linear up
Table I. GO annotations related to gene expression changes induced by STAT5A(1*6)-ER

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and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for 
Chapter 6


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Supplemental Figure 1. Validation of STAT5A(1*6)-ER Illumina gene expression data by Q-PCR and comparison with wt STAT5A-ER Illumina data. Q-PCR analysis were performed using primers and conditions listed in Supplemental Table 1. 26 out of 29 gene expression changes identified by Illumina were confirmed by Q-PCR. Also, STAT5A(1*6)-ER Illumina gene expression data was compared to wt STAT5A-ER Illumina data (500 nM 4-OHT).
### Supplemental table 1. Primers sequences and q-PCR conditions

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**Q-PCR conditions:**

Initial denaturation: 3’, 94 °C  
40 cycles of:  
  - denaturation: 15”, 94 °C  
  - annealing and extension: 45”, 58 °C  

Fluorescent data acquisition during annealing and extension.