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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of CTGF in chronic myeloid leukemia stem cell maintenance

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biological Sciences

by

Patrick C Chang

Committee in charge:

Professor Catriona Jamieson, Chair
Professor Dong-Er Zhang, Co-Chair
Professor Cornelis Murre

2015

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The Thesis of Patrick C Chang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2015

DEDICATION

This work is dedicated to the loving memory of my grandfather, Dan.
I am privileged to have been a part of your family.

EPIGRAPH

A fundamental problem, which remains to be solved in the whole cancer research effort, in terms of therapies, is that the preclinical models of human cancer, in large part, stink.

-Robert A. Weinberg

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LIST OF ABBREVIATIONS

HSC	hematopoietic stem cells
HSPC	hematopoietic stem and progenitor cell
CSC	cancer stem cell
LSC	leukemia stem cell
CML	chronic myeloid leukemia
ECM	extracellular matrix
BMME	bone marrow microenvironment
CTGF	connective tissue growth factor
IGFBP	insulin-like growth factor binding protein
VWC	von willebrand factor type C
TSP	thrombospondin
CT	cysteine knot
MMP	matrix metalloproteinases
LRP	lipoprotein-receptor related proteins
CP	chronic phase (CML)
BC	blast crisis (CML)
PV	polycythemia vera
ET	essential thrombocythemia
MF	myelofibrosis
AML	acute myeloid leukemia
TKI	tyrosine kinase inhibitor
BCL2_L	B-cell lymphoma 2 – long isoform

MCL1_L	myeloid cell leukemia sequence 1 – long isoform
BCLX_L	B-cell lymphoma-extra large – long isoform
BAX	BCL2 associated X protein
BAK	BCL2 homologous antagonistic killer
FACS	fluorescence activated cell sorting

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VITA

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|------|--|
| 2013 | Bachelor of Science, Physiology and Neuroscience,
University of California, San Diego |
| 2013 | Bachelor of Science, Management Science,
University of California, San Diego |
| 2015 | Master of Science, Biological Sciences,
University of California, San Diego |

FIELDS OF STUDY

Major Field: Biological Sciences

Studies in Hematopoiesis, Leukemia and Stem Cell Biology
Professor Catriona Jamieson

ABSTRACT OF THE THESIS

The role of CTGF in chronic myeloid leukemia stem cell maintenance

by

Patrick C Chang

Master of Science in Biological Sciences

University of California, San Diego, 2015

Professor Catriona Jamieson, Chair

Professor Dong-Er Zhang, Co-Chair

Chronic myeloid leukemia (CML) results from the BCR-ABL1 fusion protein that skews hematopoietic stem cell differentiation toward the myeloid lineage, creating an aberrantly self-renewing progenitor population that gives rise to leukemia stem cells (LSCs). While effective at reducing symptoms, current treatments target BCR-ABL1 with tyrosine kinase inhibitors (TKIs) but fail to

eradicate the niche-resident LSCs responsible for patient relapse and CML disease progression to its terminal stage, blast crisis (BC). Previous studies indicated that the extracellular matrix (ECM) could play a significant role in promoting LSC survival by demonstrating that niche-resident LSCs exhibit resistance to pharmacological inhibition by adopting stem cell-like behaviors such as quiescence, self-renewal and preferential expression of pro-survival BCL2 family isoforms. Analysis of LSCs through RT-PCR ECM array and RNA-seq identified a significant increase in BC versus chronic phase (CP) CML expression of connective tissue growth factor (CTGF), an ECM protein that has been linked to poor prognoses in multiple cancers. Lentivirally-enforced CTGF expression in the BC CML cell line, K562, increased pro-survival BCL2 family isoform expression and conferred chemoresistance to TKI treatment. Increased CTGF expression in CP CML LSCs was also associated with increased pro-survival BCL2 family isoform expression, and a trend for increased self-renewal. Cumulatively, these results indicate that CTGF could play a crucial role in CML LSC maintenance, enhancing survival, chemoresistance and, potentially, self-renewal. Therefore, delineating the role of CTGF in CML LSC maintenance could elucidate the process of BC transformation, resulting in biomarkers that predict patient outcome or novel targets for therapeutic intervention.

1. INTRODUCTION

1.1. Cancer

Cancer is a disease resulting from DNA mutations and epigenetic alterations that malignantly transform the physiology of individual cells^{1,2}. In 2015, the National Cancer Institute reported cancer as the second leading cause of death in the United States, with an estimated 1.66 million new diagnoses and 0.59 million cancer-related deaths^{3,4}. This estimation, however, is expected to rise, and cancer is expected to surpass heart disease as the leading cause of death. Despite advances in modern medicine, the number of fatalities due to cancer has not been significantly curtailed, indicating an urgent need to improve conventional therapies, or to begin devising alternative strategies.

Hanahan and Weinberg famously summarized the biological mutations leading to cancer formation in a review written in 2000. They defined six “hallmarks” of cancer that could be acquired throughout its development: the ability to induce angiogenesis, to become metastatic or invasive, to replicate indefinitely, to avoid apoptosis, to prevent growth suppression signaling or to proliferate uncontrollably¹. Hanahan and Weinberg updated their original review in 2011, citing advancements in cancer biology that had occurred in the decade since their original publication, and expanding their “hallmarks” by four additional features or capabilities². These additions, defined as emerging hallmarks, include altering cellular metabolism and avoiding immune destruction, and new characteristics of cancer development, encompassing genomic instability and

tumor promoting inflammatory responses².

While these hallmarks are representative of the progress made in cancer research over the past decade, they underscore the complexities of cancer biology that we have yet to unravel. Specifically, there is increasing evidence that points to the existence of a self-renewing subpopulation of cancer cells, defined as cancer stem cells (CSCs), that have the ability to form, propagate or recapitulate cancers (Figure 1.1)^{2,5-8}. In this model, the bulk population of cancer cells are derived from CSCs that have differentiated, giving rise to a hierarchical system of tumor heterogeneity. Subsequently, these cancer cells lack the ability to sustain or give rise to new tumors. Despite significant advances in understanding the biology of cancer, substantive solutions have yet to be developed for the public health problem at hand. In fact, these advancements only underscore that our understanding of the cellular mechanisms driving cancer development remain, at best, fragmented.

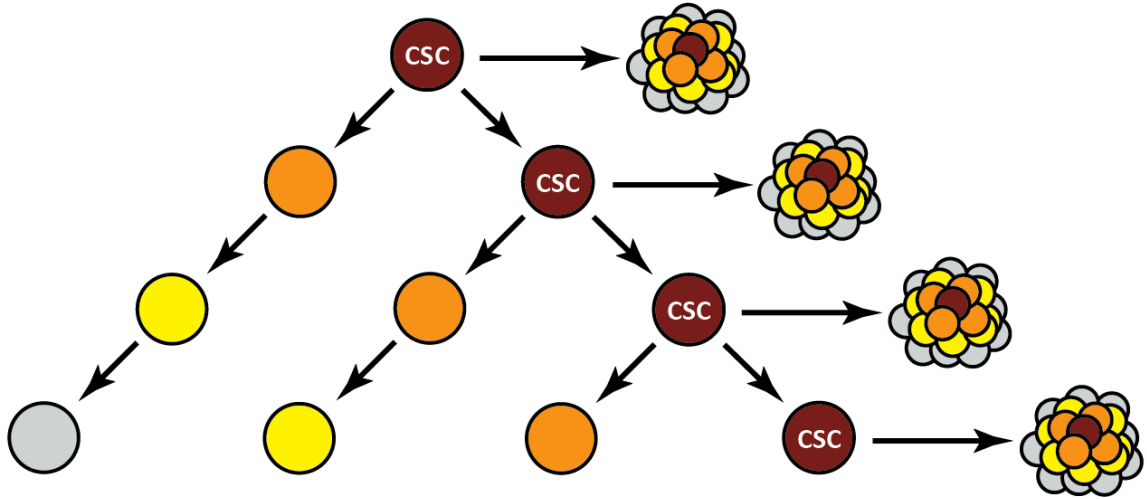


Figure 1.1 The cancer stem cell model of tumor heterogeneity. This model of tumor heterogeneity suggests that a specific subpopulation of cancer cells, CSCs, are responsible for maintaining tumors by self-renewing and differentiating, giving rise to the bulk population of tumor cells^{2,5-8}. Thus, tumor heterogeneity is derived from the hierarchical progression of CSC differentiation.

1.2. Cancer stem cells

CSCs were first described in acute myeloid leukemia (AML) as a rare population of cells expressing defined cell surface markers ($CD34^+CD38^-$) with the ability to maintain the bulk tumor of differentiated leukemic blasts in xenograft mouse models after serial transplantation⁶. CSCs have since been characterized in other hematologic malignancies, such as chronic myeloid leukemia (CML) and solid tumors, such as pancreatic, breast, colon and other cancers.^{2,6-9} Research into the behavior of CSCs suggests that they subvert stem cell-like properties such as quiescence, enhanced self-renewal and increased survival, effectively rendering them resistant to conventional forms of therapy^{2,6,10}. For instance, quiescent CSC populations were identified in CML and AML, and have been shown to be resistant to standard treatment¹¹⁻¹³. Studies have indicated that CML CSCs adopt aberrant self-renewal and increased survival through exploiting the WNT/ β -Catenin pathway, a critical regulator of normal hematopoietic stem cell (HSC) maintenance^{14,15}. Together, these results point to CSCs as the likely culprit in promoting drug resistance and patient relapse. Thus, it is imperative to elucidate the process of CSC evolution before further advancements in cancer therapeutics can be made.

1.2. Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a progressive cancer of the hematopoietic system resulting from the t(9,22)(q34;q11) translocation of the *c-abl* gene from chromosome 9 to the *bcr* gene located on chromosome 22,

forming the Philadelphia chromosome^{5,13,16}. The oncogenic BCR-ABL1 fusion protein that arises from this translocation prevents the proper differentiation of HSCs, leading to expansion of the myeloid progenitor pool that is a hallmark of chronic phase (CP) CML⁵. From the oncogenic activity of BCR-ABL1, some progenitors aberrantly acquire the ability to self-renew and increase their survival, resulting in the generation of blast crisis (BC) leukemia stem cells (LSCs) and transformation of CML to its acute stage^{5,14,16,17}.

CML has become an important paradigm for dissecting the molecular evolution of LSCs as its derivation can be traced back to the oncogenic BCR-ABL1 protein expression in HSCs and their progeny. Researchers were able to specifically target BCR-ABL1 with tyrosine kinase inhibitors (TKIs), turning CP CML into a manageable disease¹⁸. While TKI monotherapy represents a significant milestone in CML treatment and modern cancer therapeutics, it generally fails to produce curative results in patients with CML. Specifically, TKIs target the majority of BCR-ABL1 expressing cells but consistently fail to eliminate the quiescent, chemo-resistant LSCs responsible for driving relapse and BC CML transformation, which frequently occurs following discontinuation of TKI therapy^{11,19-23}. Therefore, patients must endure the physical and financial stresses of constant treatment or risk BC CML disease progression because the underlying mechanisms of malignant transformation in CML LSCs have yet to be elucidated. By elucidating the molecular drivers of BC transformation, perhaps curative results from CML therapy can be achieved.

1.3. Pro-survival BCL2 family isoform expression

Previous studies within our lab described a population of LSCs in BC CML that were resistant to TKI monotherapy when engrafted in the bone marrow microenvironment (BMME) or exposed to stromal conditioned media¹³. Both *in vivo* and *in vitro* exposure of LSCs to niche-specific factors resulted in the preferential switching to pro-survival B-cell lymphoma/leukemia 2 (BCL2) family isoform expression, leading to quiescence and TKI-resistance^{13,24}.

Pro-survival BCL2 family isoforms (BCL2_L, BCLX_L and MCL1_L) are anti-apoptotic gene splice isoforms that have been shown to contribute to leukemogenesis²⁵, disease progression²⁶, TKI resistance^{24,27,28}, hematopoietic stem and progenitor cell survival²⁹, and quiescence¹³. These proteins inhibit the intrinsic apoptosis pathway, or mitochondrial outer membrane permeabilization (MOMP) by preventing the oligomerization of pro-apoptotic BCL2 proteins, BCL2 associated X (BAX) and BCL2 homologous antagonistic killer (BAK), because of their structural similarities (Figure 1.2)³⁰⁻³³. In MOMP, p53 is activated by intrinsic apoptosis signals of cell stress, such as injury, hypoxia, DNA damage, chemotherapy or radiation, and oligomerizes BAK and BAX at the outer mitochondrial membrane³⁰⁻³³. This leads to cytochrome c spilling into the cytoplasm and the formation of the apoptosome with Apaf-1, cleaving procaspase-9 into caspase-9, which mediates the activation of executioner caspases (caspase-3, caspase-6 and caspase-7) and begins apoptosis³⁰⁻³³.

Even though we showed that the increased TKI resistance was a result of preferential expression of pro-survival BCL2 family isoforms, we did not define

the mechanisms through which the BMME or stroma-secreted factors increased LSC maintenance. Thus, we hypothesized that there must be elements within the LSC's extracellular matrix (ECM), its primary form of communication with its environment, that promoted the pro-survival BCL2 family isoform expression.

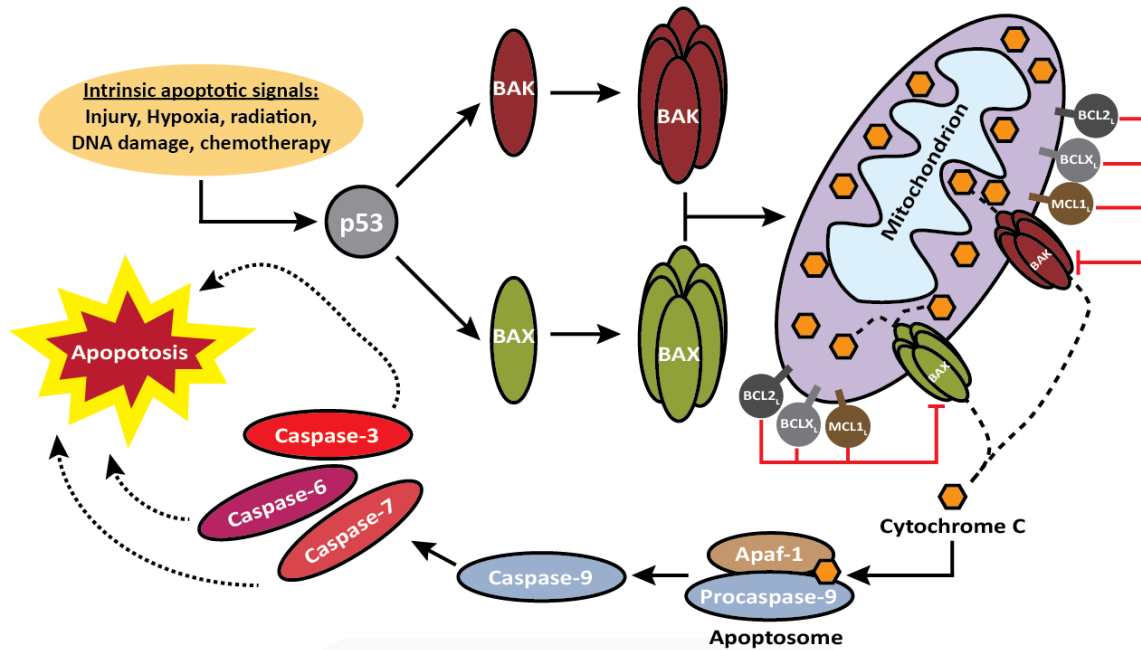


Figure 1.2 The intrinsic apoptosis pathway. Intracellular signals of apoptosis begin MOMP by activating p53, which signals BAK and BAX to oligomerize at the mitochondrial outer membrane and form pores. Cytochrome c is released into the cytoplasm and forms the apoptosome with Apaf-1 and procaspase-9, which is cleaved. Caspase-9 is released and activates executioner caspases, caspase-3, caspase-6 and caspase-7, which facilitate apoptosis. Pro-survival BCL2 family proteins, BCL2_L, BCLX_L and MCL1_L, can inhibit MOMP by associating with BAK and BAX and preventing their oligomerization³⁰⁻³³.

1.4. Connective tissue growth factor

Connective tissue growth factor (CTGF) is part of a family of matricellular proteins, which are secreted factors that mediate communication between cells and their ECM through binding multiple factors³⁴⁻⁴⁰. CTGF exists as a cleavable 36-38kDa protein with a signal peptide preceding four protein binding domains, listed respectively: insulin-like growth factor binding protein (IGFBP), von willebrand factor type C (VWC), thrombospondin (TSP) and cysteine knot (CT)³⁴⁻⁴⁰. After translation, CTGF is directed for secretion by the signal peptide, where it can either bind to factors in the ECM and the plasma membrane, such as Wnt, TGF- β , Fibronectin, vascular endothelial growth factor (VEGF), low density lipoprotein receptor-related proteins (LRPs) and integrins, or be released into the serum (Figure 1.3)³⁴⁻⁴⁰.

The hinge region separating the VWC and TSP domains in CTGF is cleavable by most proteases, such as matrix metalloproteinases, giving rise to N- and C- terminal halves, splitting the IGFBP and VWC from the TSP and CT domains (Figure 1.4)⁴¹⁻⁴⁴. These truncated halves of CTGF can be cleaved once more by elastase and plasmin, releasing all four of the individual domains (Figure 1.4)⁴¹⁻⁴³. Interestingly, the 11kDa C-terminal CT domain has been shown to preserve much of the bioactivity of the entire CTGF protein, which has been utilized for developing recombinant proteins⁴¹⁻⁴³.

While CTGF was originally identified as a component of injury and inflammation, such as wound healing and fibrosis, it has been increasingly identified in chronic diseases, such as chronic atherosclerosis, cardiac

hypertrophy, and even cancers⁴⁵⁻⁴⁷. Recent studies implicate CTGF as a biomarker of poor patient prognosis in cancers, such as breast cancer, pancreatic cancer and B-cell acute lymphoblastic leukemia⁴⁵⁻⁴⁷. It is believed that CTGF could play a crucial role in establishing the tumor microenvironment in multiple cancers through its ability to regulate inflammatory cytokine and chemokine expression, fostering cancer development^{45,48,49}. Thus, it is imperative to view CTGF not only as a secretable growth factor, but also a cytokine or inflammatory modulator^{43,48}.

Most importantly, CTGF modulates both intracellular and intercellular signaling through associations with key proteins involved in self-renewal, survival and chemoresistance of LSCs. While the effects of CTGF expression have been widely studied in stromal and endothelial cells, its effects in the hematopoietic system have yet to be elucidated. Specifically, the role of CTGF expression in regulating LSC maintenance has not been investigated. Thus, we profiled CTGF expression in LSCs to characterize its effects on maintenance in the diseased hematopoietic system.

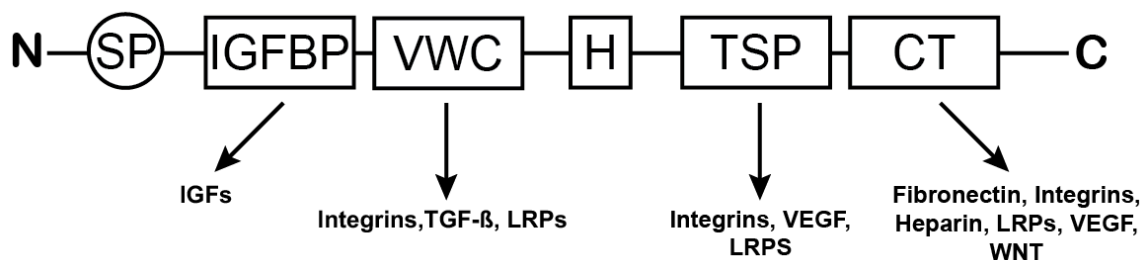


Figure 1.3 The functional domains of CTGF and their interactions with other molecules. The signal peptide (SP) directs CTGF for secretion, and the hinge region (H) is where most proteases can cleave CTGF. There are four protein-binding domains: insulin-like growth factor binding protein (IGFBP), von willebrand factor type C (VWC), thrombospondin (TSP) and cysteine knot (CT) ³⁴⁻⁴⁰.

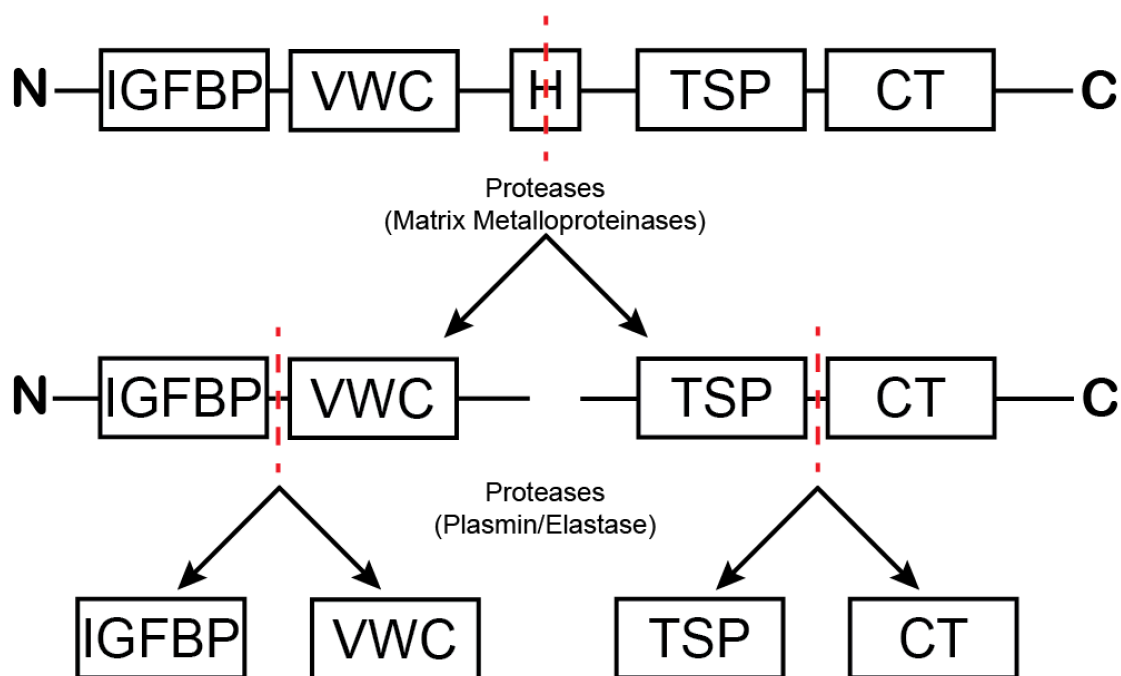


Figure 1.4 CTGF is cleaved into its individual domains by proteases. CTGF can be cleaved in half by many proteases, such as matrix metalloproteinases (MMPs) at its hinge (H) region. Further cleavage of these halves can be done by proteases such as plasmin or elastase, releasing each all four protein binding domains⁴¹⁻⁴³.

2. RESULTS

2.1. Introduction

The lack of curative results from tyrosine kinase inhibitor (TKI) monotherapy¹³ underscores the urgent need to map disease progression of chronic phase (CP) chronic myeloid leukemia (CML) to its acute stage, blast crisis (BC). Studies of chronic myeloid leukemia (CML) in the lab identified an underlying population of leukemia stem cells (LSCs), which we have shown to contribute to disease progression and therapeutic resistance^{13,14,50,51}.

Like normal stem cells, LSCs are capable of self-renewal, survival, differentiation, and quiescence in selective niches; however, they fail to regulate these processes adequately. Recent studies have indicated that LSCs from B-cell chronic lymphocytic leukemia (CLL)^{52,53}, acute myeloid leukemia (AML)^{54,55} and chronic myeloid leukemia (CML)^{13,56} exhibited therapeutic resistance when exposed to factors from the bone marrow microenvironment (BMME).

Specifically, we identified that these LSCs upregulated pro-survival BCL2 family isoform expression in the presence of stroma, or stromal secreted factors¹³. The mechanisms driving these alterations, however, remained unknown. Thus, we hypothesized that specific extracellular matrix (ECM) factors mediate signals between the bone-marrow niche and the LSCs, resulting in increased survival and chemoprotection.

2.2. CTGF is upregulated in the CP to BC CML transition

Purification of stem ($CD34^+CD38^-$) and progenitor ($CD34^+CD38^+$) populations from myeloproliferative neoplasms (MPNs), such as polycythemia vera (PV), myelofibrosis (MF), essential thrombocythemia (ET) and secondary acute myeloid leukemia (sAML), and CP and BC CML was performed by FACS on clinically annotated samples (Table 2.1-2). Total RNA was isolated from these cells and used for PCR microarray analysis of ECM and adhesion protein expression compared to normal peripheral blood (NPB) controls. Relevant ECM and adhesion proteins have been compiled and displayed, identifying significant overexpression of CTGF in most MPNs as well as CP and BC CML (Table 2.3).

Based on the demonstrable pattern of increased CTGF expression in microarray PCR assays, we analyzed our RNA-seq datasets^{13,16,57,58}, and performed quantitative RT-PCR (qRT-PCR) for CTGF mRNA expression in RNA isolated from MPN progenitors (Figure 2.1a-b). While CTGF expression was also increased in most MPN patient samples, CML samples displayed an interesting differential expression pattern, where CTGF expression increases during BC CML transformation.

To test if this was an effect isolated to a specific cellular population, we performed qRT-PCR on FACS-sorted stem ($CD34^+CD38^-$) and progenitor ($CD34^+CD38^+$) CML cells to probe for levels of CTGF mRNA expression. We observed a 10- to 1000-fold increase in CTGF expression versus CB controls during the transition from CP to BC CML, within both the stem and progenitor populations (Figure 2.1c-d).

Because of the pattern of increased CTGF expression in BC CML and its implications in poor patient prognosis for other leukemias and cancers⁴⁵⁻⁴⁷, we hypothesized that CTGF could promote CML progression by interacting with extracellular matrix proteins, modulating signals from the BMME and inducing LSC maintenance behaviors such as increased survival, self-renewal and chemoresistance.

Table 2.1 CML patient sample information.

ID	Date	Age	Sex	Sample Type	Treatment
CP-01	23 Nov 08	60	M	Chronic Phase	None
CP-02	23 May 08	63	F	Chronic Phase	None
CP-03	09 Dec 95	57	M	Chronic Phase	None
CP-04	14 Oct 08	44	M	Chronic Phase	None
CP-05	21 Sep 09	26	M	Chronic Phase	None
CP-06	25 Sep 09	62	F	Chronic Phase	None
CP-11	30 Oct 08	39	M	Chronic Phase	None
CP-12	26 Aug 09	N/A	N/A	Chronic Phase	None
CP-13	22 Sep 11	46	F	Chronic Phase	Imatinib
CP-19	20 Oct 10	40	M	Chronic Phase	None
AP-39	30 Oct 08	39	M	Accelerated Phase	Imatinib, Hydroxyurea
BC-02	26 Aug 04	34	M	Blast Crisis	None
BC-05	08 Dec 03	43	M	Blast Crisis	None
BC-06	26 Oct 93	30	M	Blast Crisis	Hydroxyurea
BC-07	29 Oct 93	48	M	Blast Crisis	Hydroxyurea
BC-08	26 Jul 00	53	M	Blast Crisis	Hydroxyurea
BC-09	16 Oct 91	65	M	Blast Crisis	None
BC-10	21 Sep 93	40	M	Blast Crisis	None
BC-17	02 Jul 10	29	M	Blast Crisis	Imatinib, Dasatinib
BC-19	23 Nov 07	46	M	Blast Crisis	Imatinib, Dasatinib

N/A = Data not available

Table 2.2 MPN patient sample information.

ID	Date	Age	Sex	Sample Type	Treatment
PV-575 (Early)	01 May 13	42	F	PV	Hydroxyurea
PV-575 (Late)	06 Nov 13	42	F	PV	Hydroxyurea
PV-584	08 May 13	53	M	PV	Hydroxyurea
PV-598	03 July 13	34	F	PV	None
PV-601	03 Jul 13	62	M	PV	None
PV-606	10 Jul 13	31	F	PV	Hydroxyurea
ET-222	04 May 11	47	F	ET	None
ET-234	25 May 11	76	M	ET	Hydroxyurea
ET-261	22 Jun 11	76	M	ET	Hydroxyurea
ET-580	08 May 13	74	F	ET	Hydroxyurea
ET-633	02 Oct 13	46	F	ET	Hydroxyurea
MF-206	06 Apr 11	70	M	MF	Shh inhibitor
MF-381	04 Apr 12	61	F	MF	TG101348
MF-382	04 Apr 12	62	F	MF	Shh inhibitor
MF-383	04 Apr 12	65	F	MF	Hydroxyurea
MF-624	14 Aug 13	81	M	MF	None
sAML-212	13 Apr 11	70	M	sAML	Shh inhibitor
sAML-216	27 Apr 11	70	M	sAML	Shh inhibitor
sAML-224	11 May 11	70	M	sAML	Shh inhibitor
sAML-228	13 May 11	63	M	sAML	TG101348

PV = Polycythemia vera

ET = Essential thrombocythemia

MF = Myelofibrosis

sAML = secondary acute myeloid leukemia

Shh inhibitor = Sonic hedgehog pathway inhibitor (Pfizer)

TG101348 = JAK-2 Inhibitor

Table 2.3 PCR microarray expression analysis of ECM and adhesion proteins in MPN and CML progenitors.

Symbol	Fold vs. NPB PV-54	Fold vs. NPB ET-356	Fold vs. NPB MF-177	Fold vs. NPB CP-568	Fold vs. NPB BC-55
ADAMTS1	-3.54	1.00	1.54	7.64	2.83
ADAMTS13	-3.52	-1.80	2.42	2.18	25.32
ADAMTS8	-6.16	-2.96	-4.01	-4.28	3.19
CTGF		263.56	336.86	121.43	1099.02
FN1	-1.56	1.73	9.89	-19.24	-4.95
ITGA1	-41.18	1.63	-2.03	-13.51	-1.20
ITGA2	3.38	2.97	3.55	2.52	5.70
ITGA3	1.03	-2.29	-1.50	-22.41	-1.96
ITGA4	-2.66	-1.90	-3.33	-1.70	1.45
ITGA5	-2.06	-2.48	-2.12	-1.72	1.42
ITGA6	-1.95	-1.26	-2.12	-1.65	1.22
ITGA7	4.07	2.24	4.04	-1.20	17.78
ITGA8					
ITGAL	-1.53	-3.60	-9.67	-36.15	-9.77
ITGAM	-1.33	-6.81	-8.78	-48.71	-3.48
ITGAV	-3.54	-2.08	-4.74	-3.24	1.13
ITGB1	-1.40	1.09	-9.15	-1.91	1.67
ITGB2	-2.56	-2.44	-11.27	-30.19	-12.36
ITGB3	19.51	6.74	4.13	-1.85	2.57
ITGB4	1.72	1.63	6.57	24.66	201.13
ITGB5	5.49	1.83	-3.87	-9.49	-6.05
TGFB1	-3.96	-4.82	-105.71	-966.08	-308.26
THBS1	-8.42	-34.25	-19.75	-659.85	-159.56
THBS2	-1.67	-3.29	-17.44	-2.71	1.39
THBS3	-2.94	-1.50	1.16	1.29	5.40
ACTB	-1.92	-1.56	-6.84	-5.49	-7.93
B2M	1.19	1.36	-2.59	-4.95	-3.86
GAPDH	-1.20	-1.08	1.60	1.45	1.52
HPRT1	1.76	-1.01	4.52	4.80	7.02

x < 100
10 < x < 100
3 < x < 10
-3 < x < 3
-10 < x < -3
-100 < x < 10
x < -100

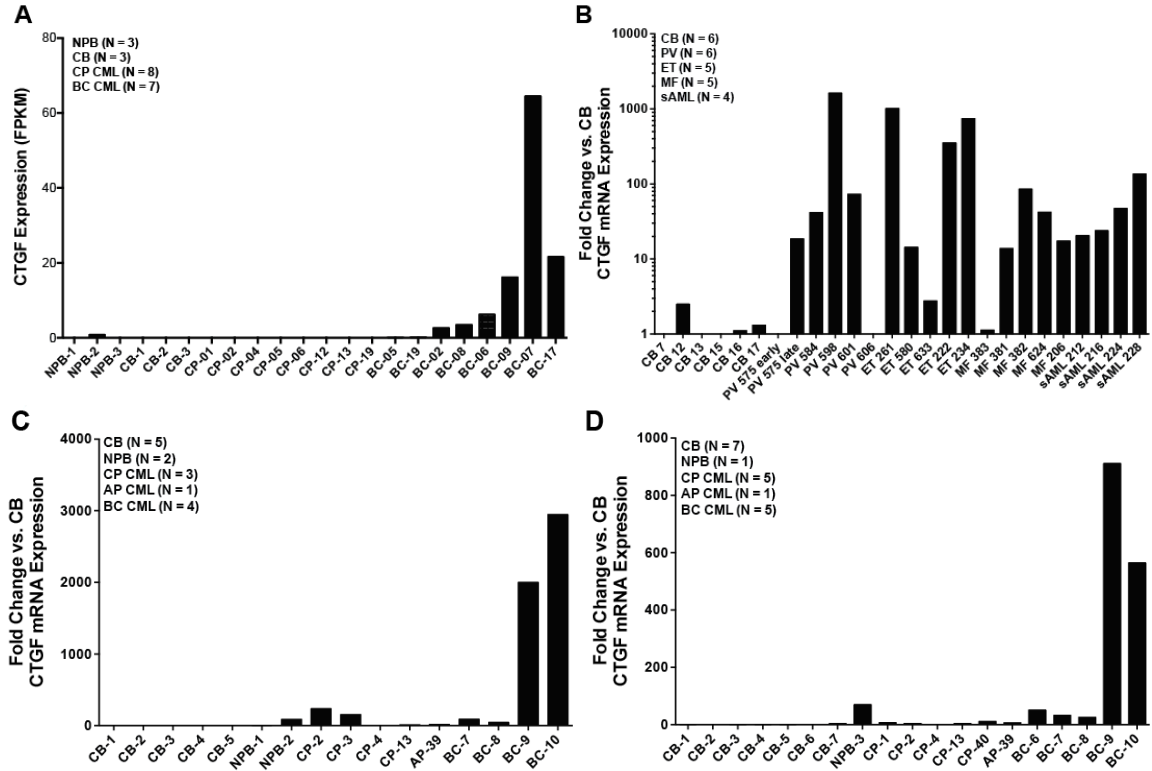


Figure 2.1 CTGF expression is increased in LSCs from MPNs and BC CML. **(a)** Graph shows relative CTGF expression in fragments per kilobase of exon per million fragments mapped (FPKM) from previously published RNA-seq datasets^{13,16,57,58}. **(b)** Fold change in CTGF mRNA expression by qRT-PCR in LSCs cells from PV (N=6), ET (N=5), MF (N=4) and sAML (N=5) versus control, CB (N=6). **(c)** Fold change in CTGF mRNA expression by qRT-PCR of FACS-sorted stem (CD34⁺CD38⁻) cells isolated from NPB (N=2), CP CML (N=3), AP CML (N=1) and BC-CML (N=4) versus control, CB (N=5). **(d)** Fold change in CTGF mRNA expression by qRT-PCR of FACS-sorted progenitor (CD34⁺CD38⁻) cells isolated from NPB (N=1), CP CML (N=5), AP CML (N=1) and BC CML (N=5) versus control, CB (N=7). All values from qRT-PCR are normalized to human HPRT mRNA expression.

2.3. Development of a lentiviral CTGF overexpression vector

The coding sequence (CDS) of CTGF was cloned into a fluorescently tagged lentiviral overexpression plasmid to better study its effects in the hematopoietic system, and to be able to sort for transfected and lentivirally transduced samples after potential *in vivo* engraftment (Figure 2.2c). The CTGF CDS was amplified through polymerase chain reaction (PCR) from the pCMV-SPORT6-CTGF plasmid without the 5'- and 3'- untranslated regions to prevent any post-transcriptional modulation of its expression. The 5'- PCR primer was designed to add an optimized overhang EcoRI restriction enzyme (RE) binding sequence, an EcoRI RE cut site and a Kozak consensus sequence to the beginning of the CTGF CDS, respectively (Figure 2.2a). The Kozak sequence, gccacc, was chosen to ensure proper translation initiation of the CTGF transcript⁵⁹ (Figure 2.2a). The 3'- reverse primer was complementary to the CTGF sequence, to ensure a double stranded PCR product, added the following features to the end of the CTGF CDS, respectively: a NotI RE cut site and an optimized overhang sequence for NotI RE binding (Figure 2.2a).

The resulting PCR product was double-digested and then inserted into the multiple cloning site (MCS) of the pCDH-Empty (pCDH-CMV-MCS-EF1-RFP) lentiviral overexpression plasmid (Systems Bioscience, Mountain View, California) (Figure 2.2b). Proper insertion of the CTGF CDS into the pCDH-CTGF plasmid (pCDH-CMV-CTGF-EF1-RFP) was confirmed through Sanger sequencing (Eton Biosciences, San Diego, California) (Figure 2.2c). Then, K562 BC CML cell line cells were transfected with pCDH-

Empty and pCDH-CTGF plasmids to validate functional qualities of the plasmid prior to lentivirus production (Figure 2.3a-b). RNA was extracted from whole cellular lysates 72 hours post-transfection, and analyzed to confirm CTGF overexpression by qRT-PCR (Figure 2.3c).

Lentivirus from the pCDH-Empty and pCDH-CTGF plasmids were synthesized according to previously established protocols and packaging plasmids from the Inder Verma Lab⁶⁰. First, 293T cells were either transduced with lentiviral particles from the pCDH-Empty or pCDH-CTGF packaging plasmids to confirm proper lentiviral functions and titer viral concentrations. Cells were imaged to confirm proper expression of fluorescent protein (Figure 2.4a-b). RNA was extracted from whole cellular lysates 72 hours after transduction to analyze CTGF overexpression by qRT-PCR (Figure 2.4c). The presence of fluorescent proteins and increased mRNA expression levels of CTGF from pCDH-CTGF lentivirus versus pCDH-Empty lentivirus controls confirmed proper functions of both plasmids in lentiviral form.

Then, K562 cells were transduced with a multiplicity of infection (MOI) of 50 with pCDH-Empty and pCDH-CTGF lentiviral particles to establish stable cell lines for *in vitro* characterization of CTGF expression. Signals from RFP were confirmed through microscopy (Figure 2.5a-b). CTGF overexpression was confirmed by qRT-PCR analysis (Figure 2.5c), and Western blot to ensure proper translation (Figure 2.4d). Through these experiments, we developed the pCDH-CTGF lentiviral plasmid and validated its functions of RFP and CTGF expression.

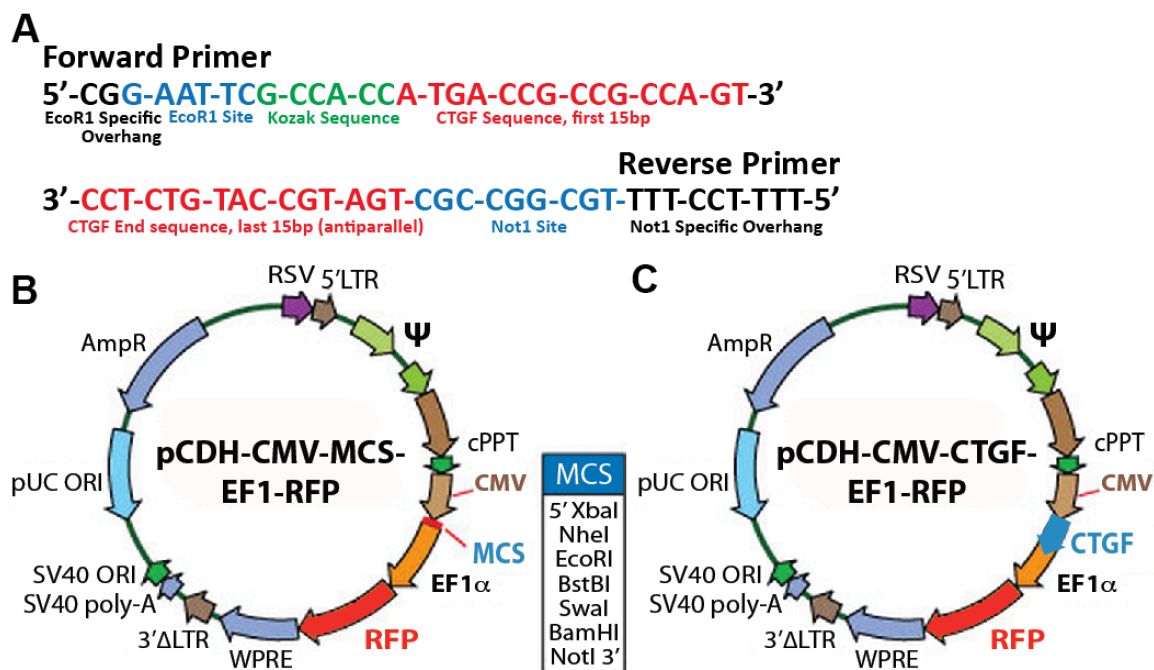


Figure 2.2 Cloning the pCDH-CTGF lentiviral overexpression plasmid. **(a)** Sequences of forward and reverse primers for amplifying CTGF CDS by PCR. **(b)** Vector diagram for the pCDH-Empty lentiviral packaging plasmid. **(d)** Vector diagram for the pCDH-CTGF lentiviral packaging plasmid. Vector diagrams are adapted from Systems Biosciences (Mountain View, California).

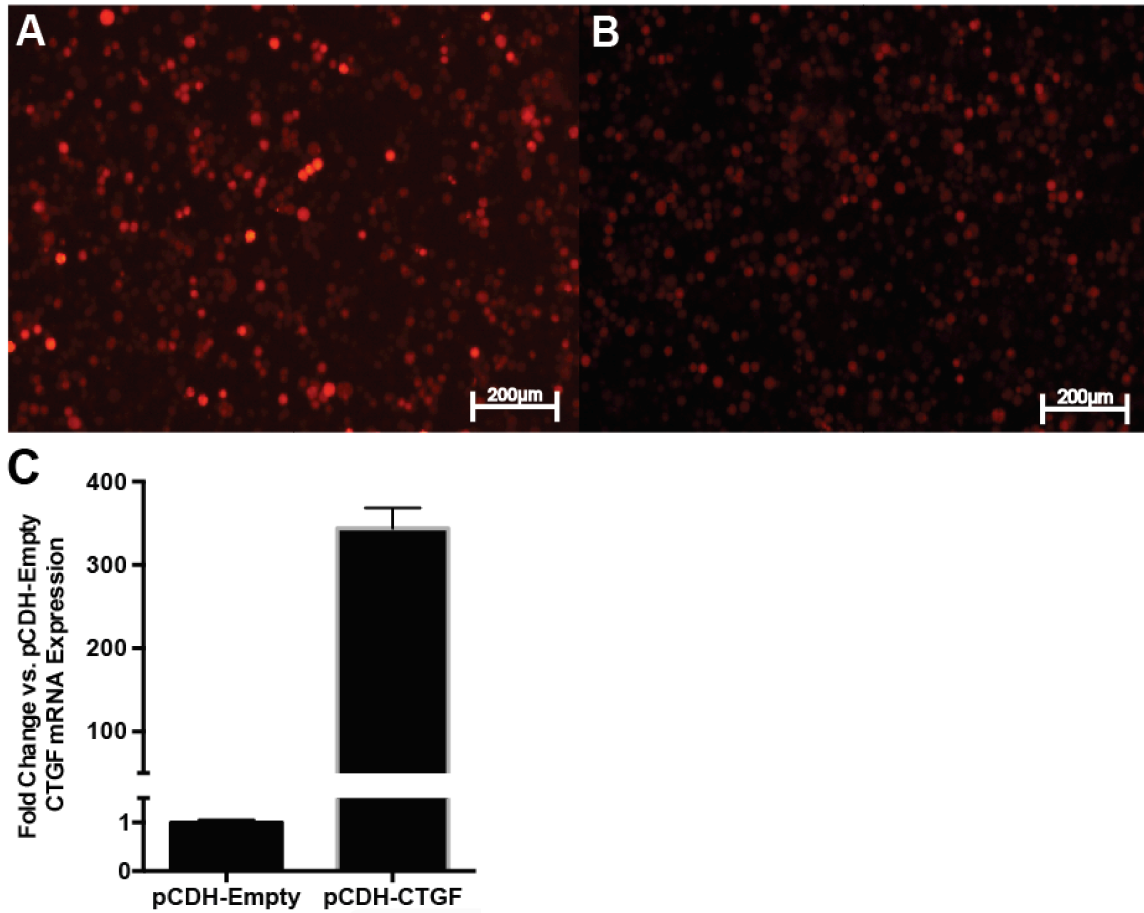


Figure 2.3 Transfection of K562 cells. After 72 hour, fluorescent images were taken of K562 transfected with (a) pCDH-Empty control and (b) pCDH-CTGF plasmids. (c) Fold change in CTGF mRNA expression by qRT-PCR of RNA extracted from whole cellular lysates of transfected cells versus pCDH-Empty control. All values from qRT-PCR are normalized to human HPRT mRNA expression. All graphs show mean $\pm$ SEM.

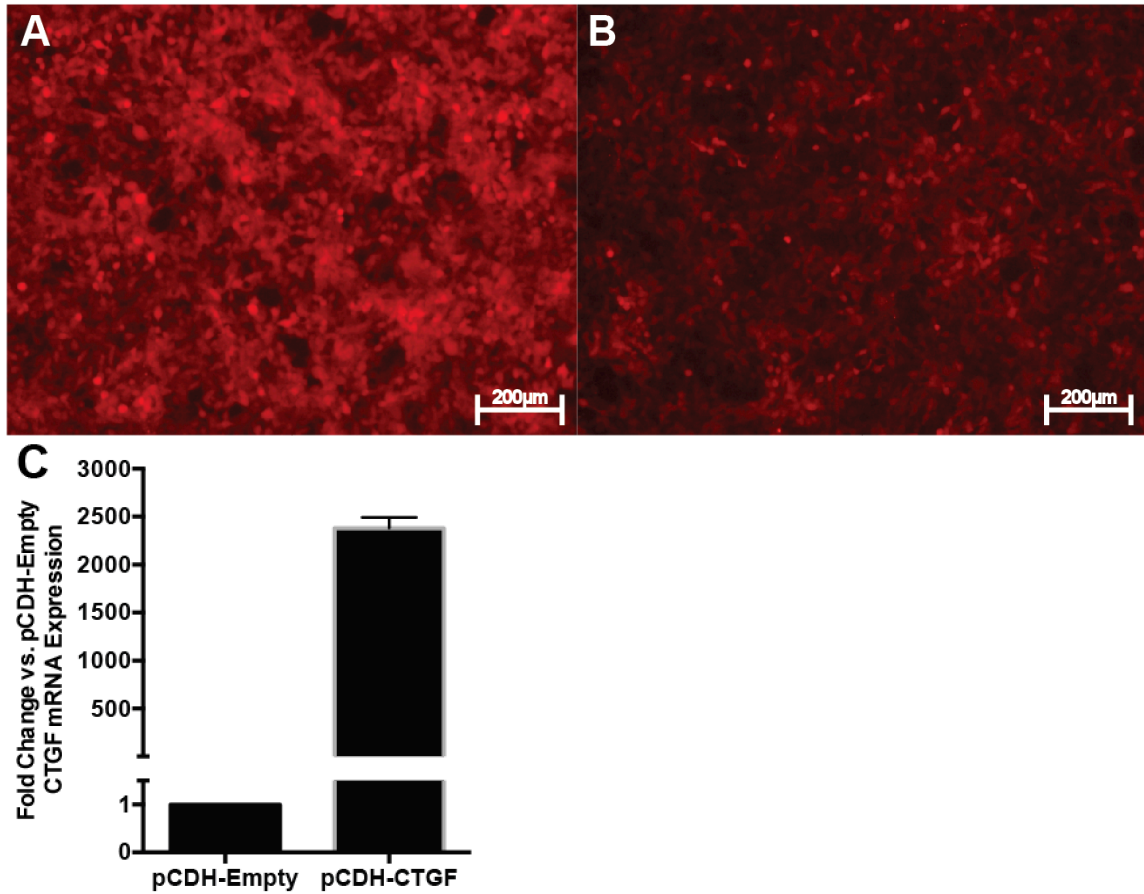


Figure 2.4 Lentiviral transduction of 293T cells. 293T cells were seeded at a density of 100,000 cells/well in a 24-well plate and allowed to reattach overnight before 72-hour transduction at a MOI of 50. Fluorescent images of (a) pCDH-Empty and (b) pCDH-CTGF transduced cells. (c) Fold change in CTGF mRNA expression by qRT-PCR of RNA extracted from whole cellular lysates of cells versus pCDH-Empty control. All values from qRT-PCR are normalized to human HPRT mRNA expression. All graphs show mean $\pm$ SEM.

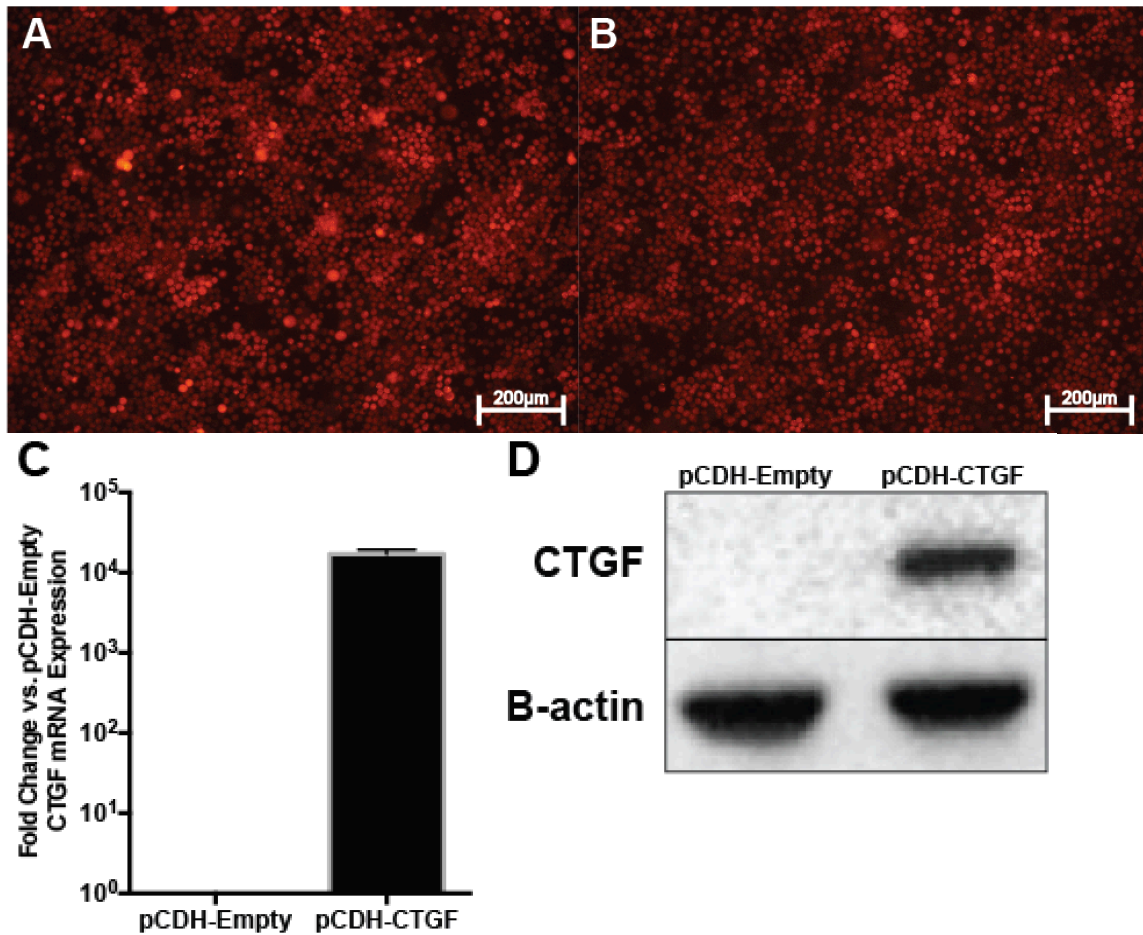


Figure 2.5 Lentiviral transduction of K562 cells. K562 cells seeded at a density of 50,000 cells per well in a 96-well u-bottom plate and then transduced for 72 hours at a MOI of 50. Fluorescent images of K562 cells transduced with **(a)** pCDH-Empty lentivirus and **(b)** pCDH-CTGF lentivirus after 72 hours transduction. **(c)** Fold change in CTGF mRNA expression by qRT-PCR of RNA extracted from transduced K562 versus pCDH-Empty control. **(d)** Western blot to measure changes in CTGF protein expression and β -actin loading control. All values from qRT-PCR are normalized to human HPRT mRNA expression. All graphs show mean $\pm$ SEM.

2.4. Lentivirally-enforced CTGF expression increases pro-survival factors in K562 cells

The effects of CTGF overexpression on CML were modeled in the K562 cell line because of its origin from a BC CML patient, as well as its basal expression of CTGF under wild-type conditions. K562 cells were stably transduced for 72 hours with pCDH-Empty and pCDH-CTGF lentivirus at a MOI of 50. Stable transductions were visually confirmed through fluorescent microscopy, and RNA was extracted from whole cellular lysates for qRT-PCR analysis. CTGF mRNA expression was confirmed (Figure 2.6a), and analysis of pro-survival BCL2 family isoforms revealed significant increases in the mRNA expression of pro-survival BCL2 family isoforms, BCL2_L, BCLX_L and MCL1_L, compared to transduction controls (Figure 2.6b).

Because of the pro-survival BCL2 family isoform expression, transduced K562 were cultured in reduced serum media to induce stress for FACS analysis of apoptosis markers (Annexin V, NearIR Live/Dead) and quantify changes in survival. FACS plots revealed significant increases in the population of live (AnnV-NearIR-) cells from CTGF overexpressing cells compared to pCDH-Empty controls (Figure 2.6c-d). These results indicate that increased CTGF expression from lentivirus transduction led to pro-survival BCL2 family isoform switching, conveying augmented survival capabilities to transduced K562.

As a proof of concept, we knocked down CTGF expression through shRNA transfection in stable K562 cell lines transduced with pCDH-CTGF lentivirus to test if the increase in pro-survival BCL2 family isoform expression

was a direct effect of its increased expression. Stable pCDH-CTGF expressing K562 cells were transfected with shCTGF (TRC Lentiviral shRNA, Thermo Fisher Scientific, Waltham Massachusetts) and control, shControl (MISSION pIKO.1-puro Non-Target shRNA Control, Sigma-Aldrich, St. Louis, Missouri), short hairpin knockdown plasmids. RNA was extracted from whole cellular lysates 72 hours post transfection. qRT-PCR analysis revealed an 80% reduction in CTGF mRNA expression and reduced levels of pro-survival BCL2 family isoforms (Figure 2.6e-f).

Through these experiments, we were able to demonstrate that increased CTGF expression led to the preferential expression of pro-survival BCL2 family isoforms and increased survival in K562. To confirm the ability of CTGF to confer increased survival was confined to CML cells, we moved to test lentivirally-enforced CTGF expression in hematopoietic stem and progenitor cells (HSPCs) selected from normal, cord blood samples.

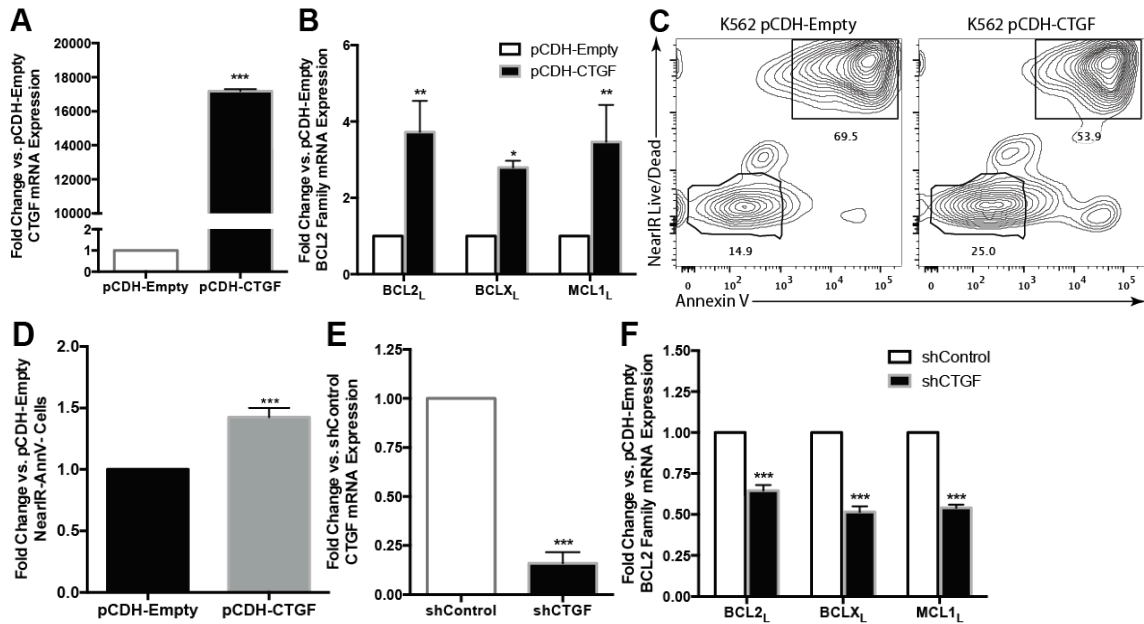


Figure 2.6 Lentivirally-enforced CTGF overexpression induces pro-survival gene expression in K562. **(a)** Fold change in CTGF mRNA expression by qRT-PCR of RNA extracted from K562 72 hours post transduction (N=4). **(b)** Fold change in pro-survival BCL2 family isoform expression by qRT-PCR of transduced K562 RNA (N=3). **(c)** Representative FACS plots of transduced K562 cells (10,000 cells/well) cultured in reduced serum media (FBS 0.5%) in a 96-well u-bottom plate for 7 days. **(d)** Fold change in the population of live, AnnV-NearIR-, cells (N=6). **(e)** qRT-PCR of RNA extracts for CTGF mRNA expression following 72 hour transfection of pCDH-CTGF transduced K562 with shControl and shCTGF knockdown vectors (N=2). **(f)** Fold change in pro-survival BCL2 family isoform expression after shRNA transfection by qRT-PCR (N=2). All graphs show mean $\pm$ SEM. All values from qRT-PCR are normalized to human HPRT mRNA expression. Panels **a**, **b** and **d** are normalized to pCDH-Empty control transductions. Panels **e** and **f** are normalized to shControl. Statistical analyses are by unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.5. CTGF does not alter pro-survival factors or differentiation in normal, cord blood derived HSPCs

To model the effects of CTGF overexpression in the normal hematopoietic system, we transduced HSPCs with pCDH-Empty and pCDH-CTGF lentiviruses. After 72 hours of transduction, we confirmed proper transduction through qRT-PCR of RNA extracted from whole cellular lysates for CTGF mRNA expression (Figure 2.7a). Further analysis of gene expression revealed no significant change in pro-survival BCL2 family isoform expression as a result of CTGF overexpression (Figure 2.7b).

Colony and replating assays were performed to characterize any changes in differentiation and self-renewal due to CTGF overexpression. The specific types of colony forming units (CFU) that arise as a result of hematopoietic differentiation were imaged for reference (Figure 2.8a-e). Typifying the colonies that formed after the first plating of the colony assays did not reveal significant changes in the number of colonies formed, as well as the types of colonies that formed (Figure 2.9a-b). Subsequent serial replating assays did present any changes in the number of colonies replated between experimental and control transductions (Figure 2.9c).

These results suggest that CTGF does not alter differentiation, self-renewal or pro-survival isoform switching in normal, cord blood derived HSPCs. Therefore, we hypothesized that the effects we had previously observed in K562 were mediated through BCR-ABL1, or mutations as a result of its oncogenic activity.

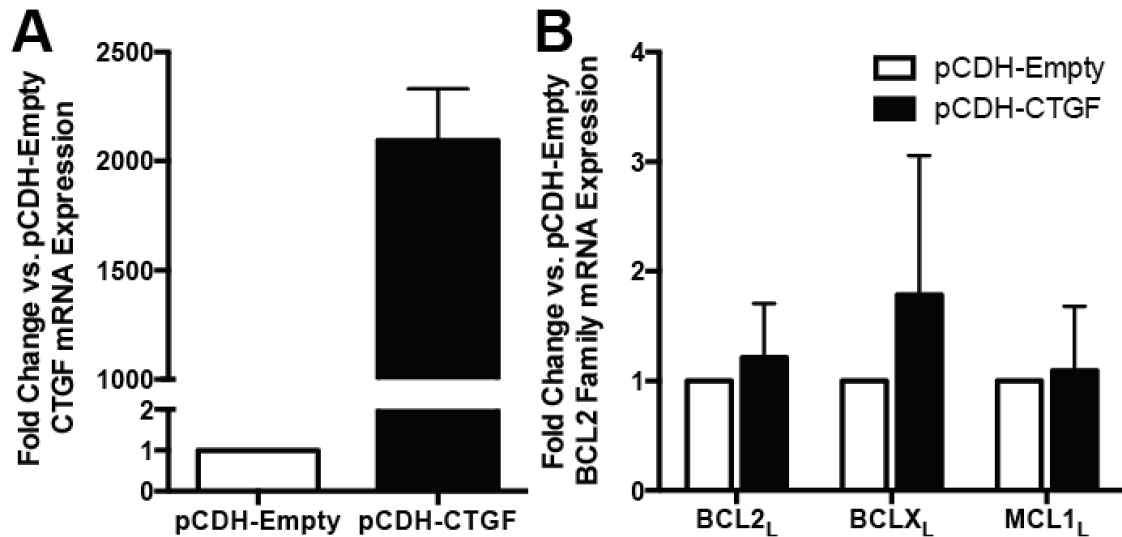


Figure 2.7 Lentivirally-enforced CTGF expression does not alter pro-survival gene expression in normal, cord blood derived HSPCs. Normal, cord blood samples were selected for HSPCs and transduced for 72 hours prior to extracting RNA from whole cellular lysates. **(a)** Fold change in CTGF mRNA expression versus control (N=4) by qRT-PCR. **(b)** Fold change in pro-survival BCL2 family isoform expression versus control (N=4) by qRT-PCR. All values from qRT-PCR are normalized to human HPRT mRNA expression, and then normalized to pCDH-Empty control transductions. All graphs show mean $\pm$ SEM from N=4 individual experiments. Statistical analyses are by paired t-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

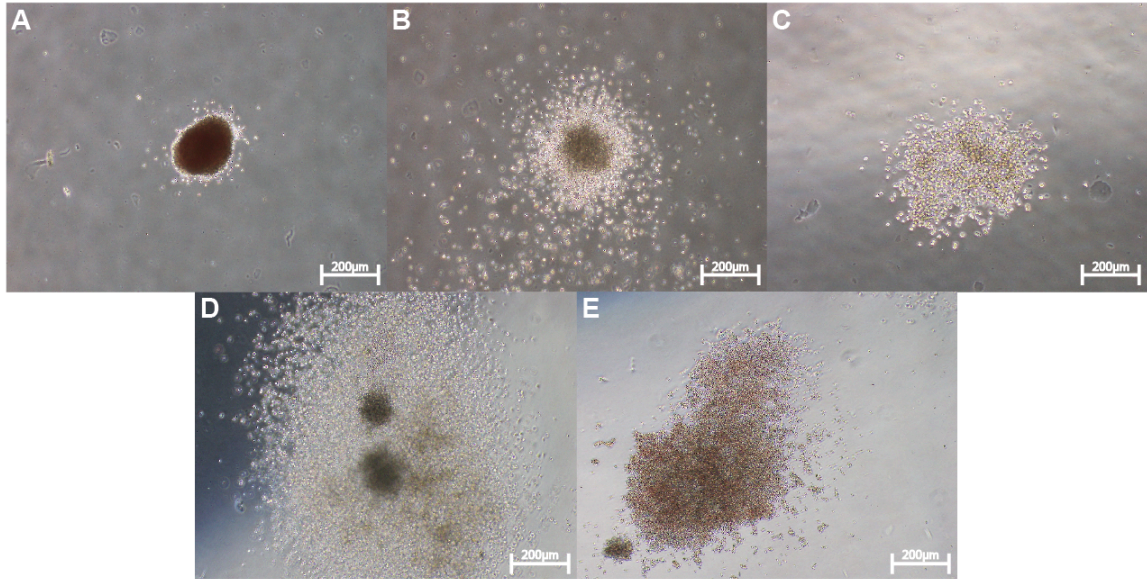


Figure 2.8 Representative images of specific hematopoietic colonies from the colony and replating assays. **(a)** Erythrocyte colony (BFU-E). **(b)** Granulocyte colony (CFU-G). **(c)** Macrophage Colony (CFU-M). **(d)** Granulocyte-Macrophage colony (CFU-GM). **(e)** Mixed colony (CFU-Mixed).

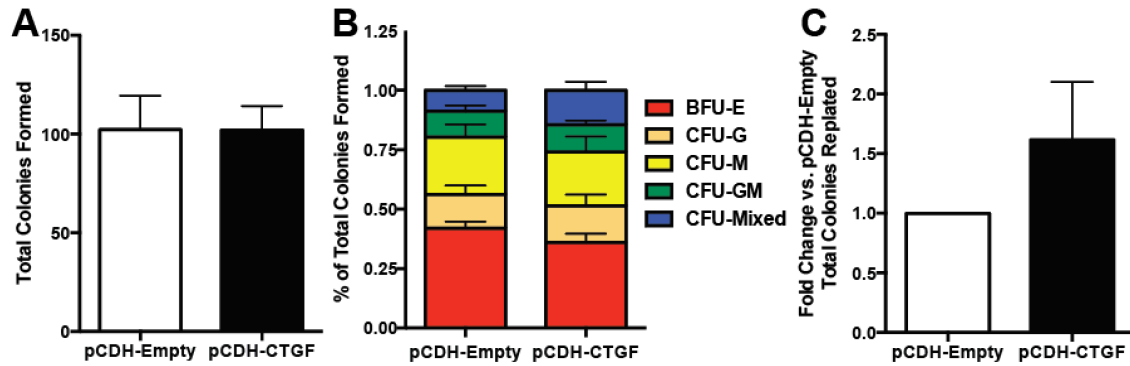


Figure 2.9 CTGF does not alter differentiation or self-renewal in normal, cord blood derived HSPCs. Colony assays were performed with 525 transduced cells in MethoCult from either experimental, pCDH-CTGF transduced, or control, pCDH-Empty transduced, cells. **(a)** Total number of colonies formed 14 days after plating in MethoCult. **(b)** Percentages of the types of colonies that formed 14 days after MethoCult plating. **(c)** Fold change in number of colonies that formed in self-renewal replating assay versus control, counted 14 days after replating. All graphs show mean $\pm$ SEM from N=4 individual experiments. Statistical analyses are by paired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.6. Lentivirally-enforced CTGF expression promotes LSC maintenance

Untreated CP CML patient samples were selected for LSCs and transduced with pCDH-Empty and pCDH-CTGF lentivirus to test if augmented CTGF expression would produce characteristic traits of BC CML transformation, such as increased survival and self-renewal. Increases in CTGF expression from lentiviral transductions were confirmed by qRT-PCR of RNA extracted from whole cellular lysates, 72 hours post transduction (Figure 2.10a). Consistent with the previous observations made in transduced K562, LSCs overexpressing CTGF also had increased levels of pro-survival BCL2 family isoform expression compared to pCDH-Empty controls (Figure 2.10b).

Remaining cells were used for colony and replating experiments to assess alterations in differentiation and self-renewal following modulation of CTGF expression. There were no observed changes in differentiation patterns or total number of colonies formed in transduced CP CML LSCs from the primary plating (Figure 2.11a). The serial replating assay, however, revealed a trend of increasing number of colonies replated in the CTGF expressing CP CML LSCs (Figure 2.11b).

The results from qRT-PCR analysis and serial replating assays indicate that CTGF expression increases survival, and potentially self-renewal in CP CML LSCs. These results were consistent with our predictions based off of our previous experiments in K562 and normal, cord blood derived HSPCs.

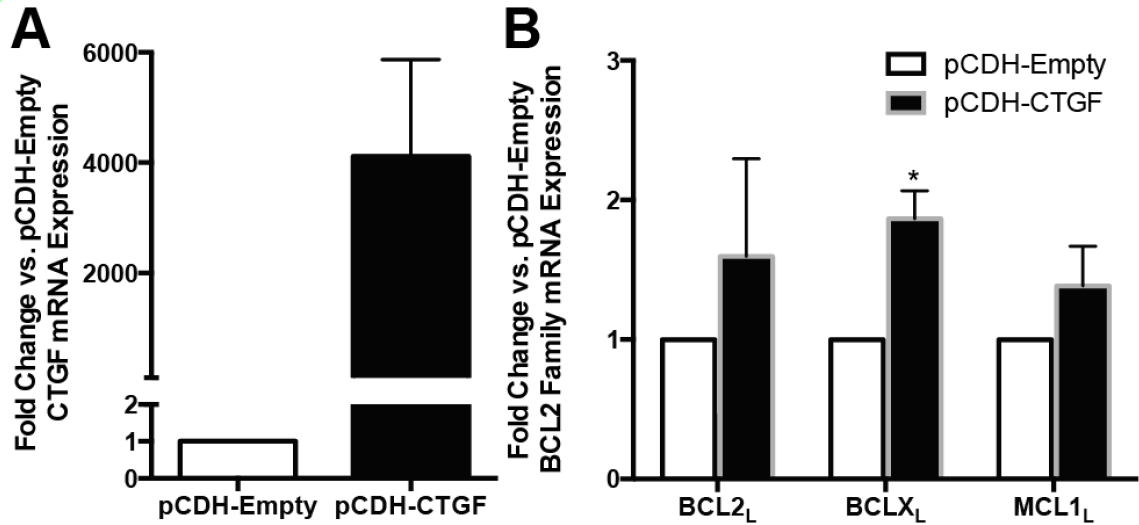


Figure 2.10 Lentivirally-enforced CTGF expression increases pro-survival BCL2 family isoform expression in CP CML LSCs. LSCs were isolated from CP CML patient samples and transduced for 72 hours prior to RNA extraction from whole cellular lysates. **(a)** Fold change in CTGF mRNA expression versus control (N=3) by qRT-PCR. **(b)** Fold change in pro-survival BCL2 family isoform expression versus control (N=3) by qRT-PCR. All values from qRT-PCR are normalized to human HPRT mRNA expression, and then normalized to pCDH-Empty control transductions. All graphs show mean $\pm$ SEM from N=4 individual experiments. Statistical analyses are by paired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

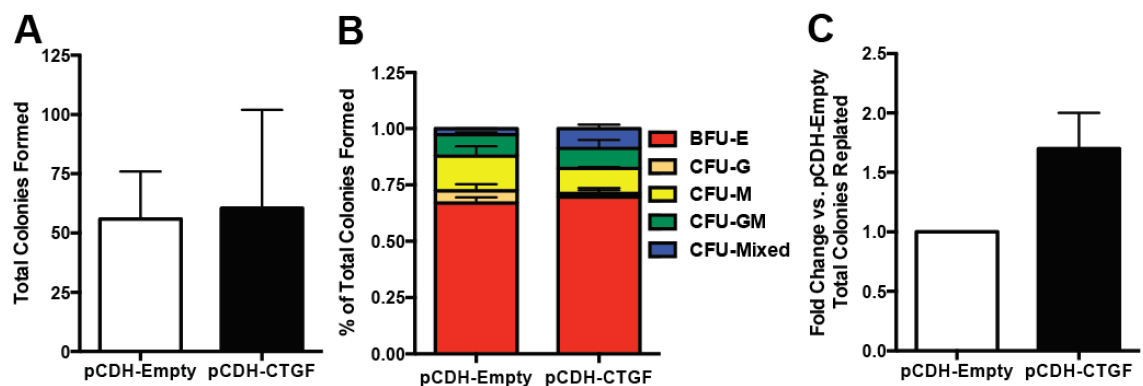


Figure 2.11 CTGF increases self-renewal, but not differentiation in CP CML LSCs. 525 transduced CP CML LSCs from each condition were plated in MethoCult for 14 days to perform colony assays. **(a)** Total number of colonies formed 14 days after plating in MethoCult. **(b)** Percentages of the types of colonies that formed 14 days after MethoCult plating. **(c)** Fold change in number of colonies that formed in self-renewal replating assay versus control, counted 14 days after replating. All graphs show mean $\pm$ SEM from N=2 individual experiments.

2.7. Lentivirally-enforced CTGF expression promotes TKI resistance

To validate the clinical significance of the observed increase in pro-survival BCL2 family isoform expression, we treated pCDH-Empty and pCDH-CTGF transduced K562 with dasatinib, a tyrosine kinase inhibitor (TKI) that potently targets BCR-ABL1 expressing cells.

Following overnight starvation, transduced K562 were inhibited with varying concentrations of dasatinib (0, 1, 5, 10 and 20nM) in DMSO. Treated K562 were stained for markers of apoptosis, Annexin V and DAPI, and analyzed by flow cytometry (Figure 2.12a). There was a significant increase in the percentage of live (AnnV-DAPI-) pCDH-CTGF K562 cells in comparison to controls (Figure 2.12b). Subsequently, there were decreased percentages of pCDH-CTGF K562 cells in the apoptotic (AnnV+DAPI-) and necrotic (AnnV+DAPI+) populations (Figure 2.12b). The fold change in live pCDH-CTGF K562 cells versus control, pCDH-Empty K562, was significant across all levels of dasatinib treatment (1, 5, 10 and 20nM) (Figure 2.12c).

The increase in pCDH-CTGF cells not expressing markers of apoptosis following dasatinib treatment allows us to conclude that CTGF expression confers a level of chemoresistance to TKI monotherapy. Therefore, we can confirm that our previous observations that the increased mRNA expression of pro-survival BCL2 family isoforms from increasing CTGF expression leads to chemoresistance in K562.

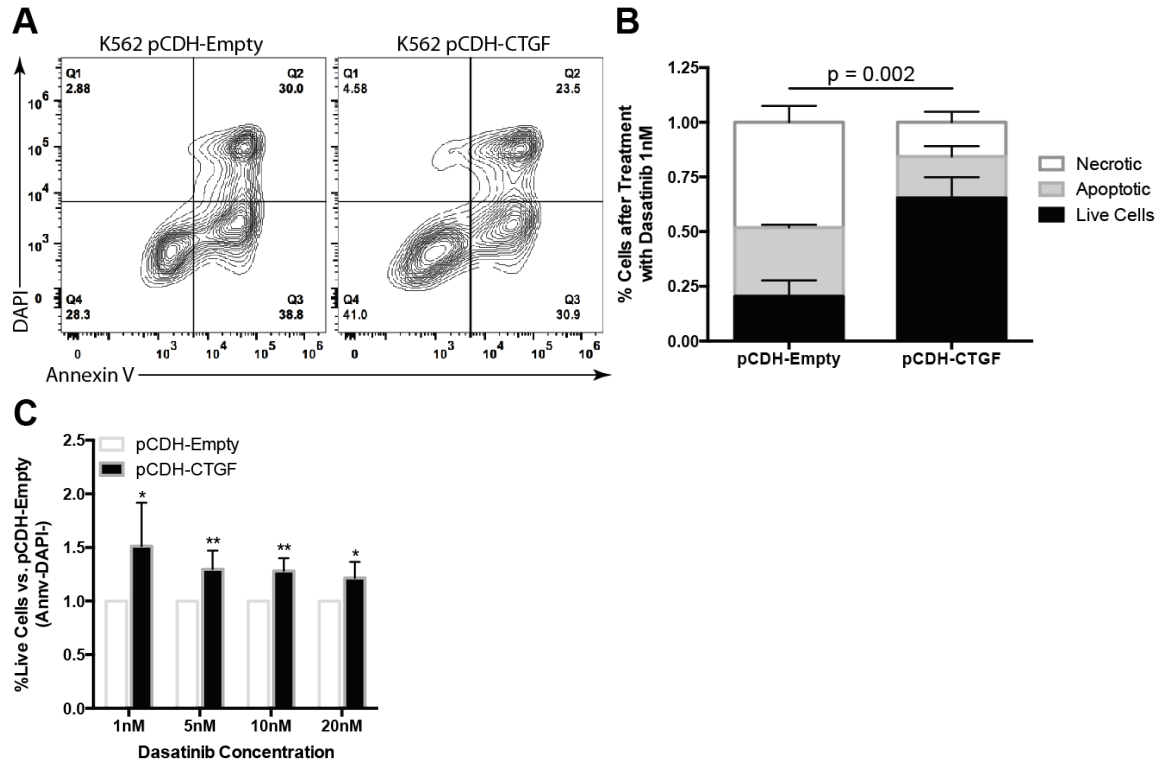


Figure 2.12 Lentivirally-enforced CTGF expression promotes drug resistance in K562. K562 transduced with pCDH-Empty and pCDH-CTGF lentivirus were serum-starved overnight in 0.5% FBS media prior to being seeded (400,000 cells/well) into a 24-well plate in the presence of dasatinib in DMSO (0, 1, 5, 10 and 20nM) for 48 hours. **(a)** Representative FACS plots of transduced K562 stained with apoptosis markers (Annexin V and DAPI) after 1nM dasatinib treatment. **(b)** Graph displaying the calculated percentages of transduced K562 in the live (AnnV-DAPI-), apoptotic (AnnV+DAPI-) and necrotic (AnnV+DAPI+) gates after 1nM dasatinib treatment. **(c)** Fold change in the percentage of transduced K562 cells in the live (AnnV-DAPI-) gate following dasatinib treatment (1, 5, 10 and 20nM) versus pCDH-Empty control. All graphs show mean $\pm$ SEM from N=5 individual experiments. Statistical analyses are by unpaired t-test or ANOVA with Tukey post-hoc test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. SUMMARY AND DISCUSSION

3.1. Introduction

In this study, we identified dysregulation of CTGF expression during blast crisis (BC) transformation of chronic phase (CP) chronic myeloid leukemia (CML), and developed a lentiviral CTGF overexpression vector to model its effects *in vitro*. Through these experiments, we observed that CTGF has the ability to increase CML LSC maintenance through inducing pro-survival isoform switching, resistance to TKI monotherapy and possibly self-renewal in diseased hematopoietic cells. Together, these findings demonstrate that CTGF can confer some of the malignant behaviors of BC transformation in CP CML LSCs, and set the stage for future investigations into CTGF as a biomarker of disease progression or a target for inhibitor development.

3.2. Summary

We profiled the expression of extracellular matrix (ECM) and adhesion proteins in LSCs from myeloproliferative neoplasm (MPNs) and CML, which all revealed increased levels of CTGF mRNA expression. With a larger sampling size, we discovered a curious correlation between CTGF and CML disease progression from the therapeutically manageable chronic phase (CP) to the terminal blast crisis (BC) stage. Specifically, there were elevated levels of CTGF expression in all of our untreated BC CML patient samples, suggesting that CTGF dysregulation may contribute to disease progression in CML.

We began probing the effects of CTGF *in vitro* by developing a fluorescently tagged lentivirus overexpression plasmid with only the coding sequence to prevent post-transcriptional regulation. After validating the lentivirus produced from the plasmid, we transduced the BC CML cell line K562 and observed that CTGF overexpression increased expression of pro-survival BCL2 family isoforms, BCL2_L, BCLX_L and MCL1_L, which were previously linked to chemoresistance in CML LSCs¹³.

Our transductions of hematopoietic stem (CD34⁺CD38⁻) and progenitor (CD34⁺CD38⁺) cells (HSPCs) selected from normal cord blood samples with CTGF overexpression lentivirus revealed no significant changes in pro-survival BCL2 family isoform expression, self-renewal or survival. These observations suggested that the previous changes in pro-survival gene expression in K562 could have been mediated by BCR-ABL1, or by the mutations resulting from its oncogenic activity.

CP CML LSCs transduced with CTGF overexpression lentivirus revealed the same pattern of increased pro-survival BCL2 isoform expression we had observed in K562. While there were not enough samples (N=2) to establish a clear significance, there is a clear trend of increased self-renewal from CTGF overexpression in CP CML LSCs. The results from mRNA analysis of transduced CP CML LSCs indicate that increased pro-survival BCL2 family isoform expressions were mediated by CML, perhaps through oncogenic BCR-ABL1 activity or another mutation. The potential increase in self-renewal capacity of these LSCs suggested that CTGF could be interacting with WNT/ β -catenin

signaling in the diseased CML hematopoietic system that previous studies have described^{9,14,51}; however, further experiments must be performed to confirm this trend.

Finally, we examined the effect of CTGF overexpression on resistance to tyrosine kinase inhibition (TKI) by dasatinib, the standard of care for CML, in K562. Following TKI treatment, flow cytometry analysis of apoptosis markers revealed an increase in the percent of live cells from CTGF overexpressing K562. This validated our observations from the initial transduction and previous experiments¹³, confirming that the increased pro-survival BCL2 family isoform expression confers chemoresistance in CML.

Our experimental results indicate that increased CTGF expression increases survival, chemoresistance and potentially self-renewal in CML (Figure 3.1). Specifically, transduced K562 exhibited pro-survival BCL2 family isoform expression and chemoresistance. Transduced CP CML LSCs had the same increase in pro-survival BCL2 family isoform expression and a trend of increased self-renewal, but no changes in differentiation. Finally, normal, cord blood derived HSPCs did not present with any of the aforementioned changes after transductions. Therefore, we conclude that lentivirally-enforced CTGF expression specifically alters LSC maintenance in the diseased CML hematopoietic system.

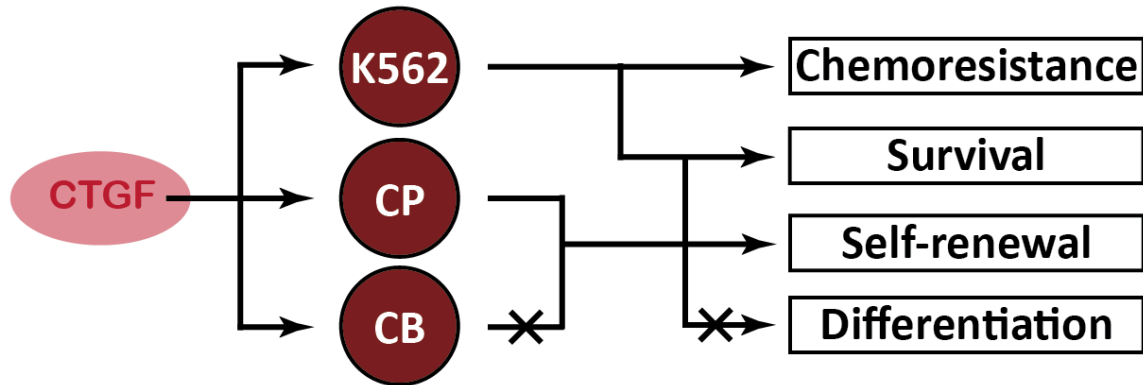


Figure 3.1 Summary of experimental outcomes from lentiviral-enforced CTGF expression in CML LSCs and normal, cord blood derived HSPCs. K562, CP CML LSCs and CB HSPCs were transduced with pCDH-CTGF lentivirus. As a result, K562 increased pro-survival BCL2 family isoform expression and chemoresistance. CP CML LSCs exhibited a trend for increased self-renewal and the same preferential expression for pro-survival BCL2 family isoforms, which could potentially lead to chemoresistance. Normal, cord-blood derived HSPCs did not exhibit any significant changes in survival or self-renewal. Neither CP CML LSCs nor normal, cord blood derived HSPCs showed altered patterns of differentiation.

3.3. Discussion

The dysregulation of CTGF expression in the transition of CP CML to BC led us to further investigate the effects of CTGF in the diseased hematopoietic system. Through increasing CTGF expression, we replicated the same signature of induced pro-survival BCL2 family isoform expression, chemo-resistance and, potentially, self-renewal that we previously described when CML LSCs were either engrafted into the bone marrow microenvironment (BMME) or treated stromal-secreted factors¹³. Cumulatively, these results indicate that CTGF could be one of the secreted factors from the BMME or stromal cells that promote maintenance in engrafted LSCs, enhancing survival, self-renewal and chemoresistance.

CTGF is a matricellular protein that can either remain bound to cellular components, such as the ECM or plasma membrane-bound ligands, or be released into serum³⁴⁻⁴⁰. It is well known that CTGF and other matricellular proteins are present during inflammatory responses, and that their expression can lead to extensive ECM remodeling^{45,48,49}. Thus, CTGF could allow cells to signal either themselves or their surrounding environment, much like releasing inflammatory or growth signals.

CTGF expression has also been well documented in the process of mesenchymal and epithelial cell development, which form the key regulatory components of the BMME^{34-36,38}. While CTGF has identified as a critical component in normal hematopoiesis⁶¹, specifically B-cell development, dysregulation of its expression in stromal cells, or LSCs, can lead to instances of

aggressive leukemia^{35,45,49}. Furthermore, we have shown that increased CTGF expression promotes LSC maintenance in CML by increasing survival, self-renewal and chemoresistance. Together, these findings suggest that CTGF plays a critical component of normal hematopoiesis, which can be hijacked in the process of LSC generation. Perhaps, further studies can investigate how LSCs can exploit CTGF's ability to induce extensive ECM remodeling and carve out a supportive niche in the BMME.

3.4. Future studies

Our future experiments would begin by repeating clonogenic assays in CP CML LSCs to establish proper statistical significance for the trend in self-renewal we had previously observed, and then attempt to establish clinical relevance through developing an *in vivo* model of CML with CTGF overexpression. CP CML LSCs would be transduced with lentivirus to overexpress CTGF and test whether or not they could achieve better levels of engraftment in respect to controls. Alternatively, BC CML LSCs could be inhibited with a CTGF monoclonal antibody, or by lentiviral shCTGF transduction, to test if CTGF knockdown would result in a reversal of malignant BC characteristics, such as increased self-renewal, survival, chemoresistance and serial transplantation. If successful, these experiments would develop an experimental mouse model for developing CTGF as a potential candidate for drug or monoclonal antibody inhibition. Specifically, the C-terminal cysteine knot (CT) domain of CTGF could be targeted as it has already been shown to preserve much of the protein's bioactivity^{37,43}.

The data from our experiments involving normal, cord-blood derived HSPCs suggested that lentivirally-enforced CTGF expression only increased survival, and potentially self-renewal, in the diseased hematopoietic system. We could perform double transductions of normal, cord blood derived HSPCs with pCDH-CTGF and BCR-ABL1 overexpression lentiviruses to test this hypothesis. Varying levels of BCR-ABL1 overexpression lentivirus could also be used to simulate the progressive increase of its expression during the BC transformation of CP CML. Through these experiments, we can observe the effects of CTGF on CML development and characterize its effects on survival, self-renewal and chemoresistance. Furthermore, these transduced HSPCs could be also used to model the effects of CML with CTGF overexpression *in vivo*, and develop CTGF as a potential candidate for therapeutic intervention.

Additionally, we could also elucidate the mechanism through which CTGF achieves its downstream regulation of pro-survival gene expression and chemoresistance (Figure 3). Research in breast cancer has linked BCLX_L expression, and subsequently chemoresistance, to CTGF by associating with integrins, which activated the ERK signaling pathway⁶². While this pathway was established in tissues from breast cancer, there could be a similar combination of integrins that achieves the same activation of the ERK pathway, leading to increased BCLX_L expression in LSCs from CML.

It may also be prudent to examine the possibility that CTGF plays an intermediary role in an inflammatory cytokine pathway (Figure 3). There are compelling pieces of evidence that suggest CTGF expression is an intermediary

in the IL-6 inflammatory pathway. Previous studies have linked STAT3 activation to chemoresistance and increased BCLX_L in CML^{24,63}. Interestingly, another study of epithelial regeneration linked the IL-6 inflammatory pathway to gp130's activation of YAP and STAT3, resulting in increased CTGF and BCLX_L expression^{64,65}. Furthermore, there is evidence that BCR-ABL1 increases IL-6 expression, establishing a paracrine signaling loop that contributes to CML pathogenesis⁶⁶. Our findings that indicate CTGF acts as a regulator of BCLX_L expression and chemoresistance, providing the basis for further investigation into its role in the IL-6 and STAT3 inflammatory pathways in CML cells. Together, these key mechanistic studies piece together a puzzle in which CTGF is the key intermediary signal transducer. By mapping the mechanism of upstream CTGF regulation and its downstream effectors, we can further elucidate the aberrant mechanisms BC CML uses to exploit regenerative pathways, and identify new targets for pharmacologic inhibition or biomarkers of disease progression.

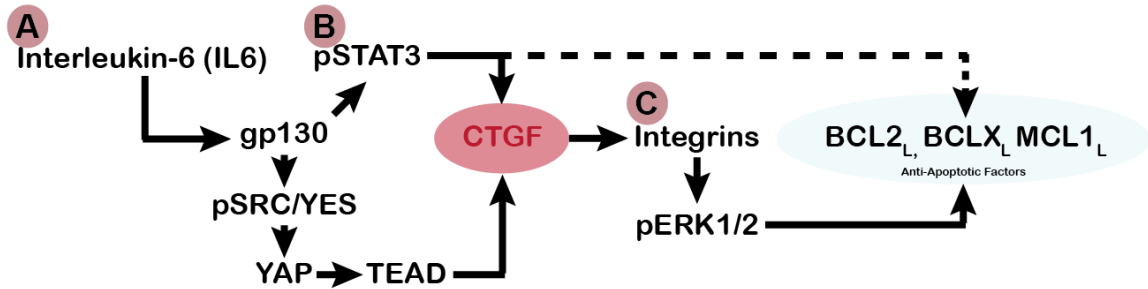


Figure 3.2 Summary of the proposed future studies into the mechanisms of CTGF induction and activity. **(a)** The proposed pathway of IL-6 as an upstream effector of CTGF expression, through gp130 activation of TEAD transcriptional activity. **(b)** Alternative pathway of CTGF induction through STAT3 activation, which has also been shown to increase chemoresistance, specifically through BCLX_L. **(c)** Proposed mechanism of CTGF downstream regulation of pro-survival BCL2 family isoform switching through binding integrins that phosphorylate ERK1/2.

4. MATERIALS AND METHODS

4.1. Patient sample preparation and FACS sorting

Normal cord blood and adult peripheral blood samples were purchased from All Cells (Alameda, California). MPN and CML patient samples were obtained from consenting patients at the University of California San Diego, Stanford University and the University of Toronto Health Network according to protocols approved by the institutional review board. Mononuclear cell fractions were extracted by Ficoll density centrifugation as previously described, and then selected for CD34⁺ cells by magnetic bead separation (Miltenyi, Bergisch Gladbach, Germany)^{5,13,51}. Progenitor purification was performed using CD34 and CD38 antibodies as previously described^{12,13,16,57,58} on a BD FACSAria II (BD Biosciences, Franklin Lakes, New Jersey).

4.2. Extracellular matrix RT² profiler PCR array

20,000-50,000 hematopoietic stem (CD34⁺CD38⁻) or progenitor (CD34⁺CD38⁺) cells (HSPCs) were sorted directly into RLT buffer with beta-mercaptoethanol on a BD FACSAria II (BD Biosciences, Franklin Lakes, New Jersey) using previously described methods^{12,13,16,57,58}. Total cellular RNA was extracted from whole cellular lysates with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Then, cDNA was pre-amplified with 1-100ng of RNA according to the RT² PreAMP cDNA Synthesis Kit (Qiagen, Venlo, Netherlands), and the products were plated into Extracellular Matrix RT² profile PCR array (Qiagen,

Venlo, Netherlands) for quantitative RT-PCR (qRT-PCR) on an iCycler (Bio-Rad Technologies, Hercules, California) platform.

4.3. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed on an iCycler (Bio-Rad Technologies, Hercules, California) using SYBR GreenER Super Mix (Life Technologies, Carlsbad, California). Total cellular RNA was extracted through the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and cDNA was synthesized using SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad, California). 5ng of cDNA template was amplified 0.2µM of each forward and reverse primer for qRT-PCR. Specific primers were designed for the coding sequence of CTGF. The threshold cycles were normalized to HPRT and compared through the delta-delta CT method⁶⁷. The following primers were used:

CTGF Forward: ttgcccagacccaacta

CTGF Reverse: gcaggaggcgttgcatt

HPRT Forward: cgtcttgctcgagatgtgatg

HPRT Reverse: ttatagcccccttgagcac

BCL2_L Forward: atgtgtgtggagagcgtcaa

BCL2_L Reverse: ttcagagacagccaggagaaa

MCL1_L Forward: agaccttacgacgggttg

MCL1_L Reverse: aatcctgccccagttgtta

BCLX_L Forward: catggcagcagtaaagcaag

BCLX_L Reverse: gaaggagaaaaaggccacaa

4.4. Polymerase chain reaction

Polymerase chain reactions (PCR) were performed with KOD Hot Start DNA Polymerase (EMD Millipore, Billerica, Massachusetts) using the Applied Biosystems 2720 Thermo Cycler (Life Technologies, Carlsbad, California). Each sample was amplified with 10 μ M of each forward and reverse primer, 2 μ L of DMSO and 100ng of template, pCMV-SPORT6-CTGF (MGC Accession: BC087839) (Life Technologies, Carlsbad, California). For a >1000KB insert, extension step was performed for 24s at 70^oC with the primers listed below:

CTGF Forward: cggaattcgccaccatgaccgccgccagt

CTGF Reverse: ttttcctttgcgcccgctgatgccatgtctcc

4.5. Bacterial transformation

2.5 μ g of pCDH-Empty lentiviral overexpression vector (pCDH-CMV-MCS-EF1-RFP CD512B-1, System Biosciences, Mountain View, California) or PCR inserts were double digested with 25 units of each restriction enzyme, EcoRI-HF and NotI-HF (New England Biosciences, Ipswich, Massachusetts). Alkaline phosphatase, Calf Intestinal (New England Biosciences, Ipswich Massachusetts) was used to treat digested pCDH-Empty plasmids prior to purification with QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands). Ligations were performed overnight with T4 DNA ligase (New England Biosciences #M0202, Ipswich, Massachusetts) with 25ng or 50ng of vector at varying vector:insert ratios (1:1, 1:3, 1:6, 1:10) in 20 μ L of total reaction volume. MAX Efficiency Stbl2 Competent Cells (Life Technologies, Carlsbad, California) were transformed with

15µL of ligation reactions, plated overnight in LB-AMP plates. Colonies were screened for the correct insert sequence (Sanger sequencing, Eton Bioscience, San Diego, California)

4.6. Transfection

Transfections of K562 erythroleukemia cell lines were performed with the Nucleofector 2b device (Lonza, Basel, Switzerland) and the Amaxa Cell Line Nucleofector Kit V (Lonza, Basel, Switzerland). 5.0µg of plasmid DNA was used in with 1,000,000 cells per nucleofection reaction. Cells were allowed to recover for 72 hours prior to harvest for mRNA expression analysis by qRT-PCR. pCDH-Empty and pCDH-CTGF vectors from cloning were used to test overexpression, and vectors from Thermo Scientific The RNAi Consortium Lentiviral shRNA were used for knockdown assays. Specifically, shCTGF refers to the TRC Lentiviral shRNA TRCN0000061952 (Thermo Fisher Scientific, Waltham, Massachusetts), and shControl refers to the MISSION pLKO.1-puro Non-Target shRNA Control SHC016 (Sigma-Aldrich, St. Louis, Missouri). The sequences of their hairpins can be found below:

shCTGF: ccggcccaaggaccaaaccgtggttctcgagaaccacggtttggtccttgggttttg

shControl: ccgggcgcgatagcgctaataatttctcgagaaattattagcgctatcgcgctttt

4.7. Western blots

1,000,000 cells were pelleted and PBS wash media was aspirated before being flash frozen. Cells were lysed with a radioimmunoprecipitation assay

(RIPA) buffer with 20mM Tris pH 8.0, 150mM NaCl and 1% Triton X, supplemented with Roche PhosStop Phosphatase Inhibitor Cocktail Tablets (Hoffman La-Roche, Basel, Switzerland), DL-Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, Missouri) and Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri). Protein quantification was performed with the Quick-Start Bradford Protein Assay (Bio-Rad Technologies, Hercules, California). Rabbit primary monoclonal antibodies specific to human CTGF (Abcam #ab5097), and β -action (Abcam #ab8227) and goat anti-rabbit secondary antibody conjugated to HRP (Abcam #ab6721) were used to visualize protein bands.

4.8. Cell lines and culture conditions

K562 cells (ATCC, Manassas, VA) were cultured in complete media, which includes DMEM (Life Technologies, Carlsbad, California), 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, California), 1% Penicillin-Streptomycin and 1% GlutaMAX (Life Technologies, Carlsbad, California). K562 cell lines were maintained in either T-25 or T-75 culture flasks and passaged at dilutions of 1:15 every 3-4 days. Stably transduced K562 were expanded and frozen (10,000,000 cells/mL) to maintain low passage aliquots, which were thawed every three months to ensure consistent biology.

4.9. Flow cytometry apoptosis analysis

K562 were collected and washed twice with PBS to remove trace amounts of FBS. Cells were stained with Annexin V, Alexa Fluor 488 conjugate (Life

Tehcnologies, Carlsbad, California), and DAPI (Life Technologies, Carlsbad, California) or LIVE/DEAD Fixable NearIR Dead Cell Stain Kit (Life Technologies, Carlsbad, California). Flow cytometry was performed on a BD LSFortessa (BD Biosciences, Franklin Lakes, New Jersey).

4.10. Lentiviral transduction

Lentiviral particles were prepared by transfecting packaging plasmids into HEK293T cells, concentrated and then measured according to previously established protocols^{13,16,50,51,58,60}. Cell lines were transduced with lentiviral particles prepared with the pCDH-Empty control and pCDH-CTGF (pCDH-CMV-CTGF-EF1-RFP) lentiviral packaging plasmids. 50,000 cells were plated into 96-well u-bottom plates in complete culture medium and transduced at a multiplicity of infection (MOI) of 50.

Chronic phase (CP) chronic myeloid leukemia (CML) patient samples were selected for leukemia stem cells (LSCs) by CD34⁺ magnetic bead separation (Miltenyi, Bergisch Gladbach, Germany) and cultured in StemPro media (Life Technologies, Carlsbad, California) supplemented with cytokines (IL-6 10ng/mL, FLT3 ligand 50ng/mL, SCF 50ng/mL and thrombopoietin 10ng/mL) as previously described^{13,16,51,57,58}. After 24 hours in culture, CP CML LSCs were transduced with pCDH-Empty and pCDH-CTGF lentivirus at a MOI of 50 for 72 hours. Normal, cord blood samples were selected for hematopoietic stem (CD34⁺CD38⁻) and progenitor (CD34⁺CD38⁺) cells (HSPCs) by CD34⁺ magnetic bead separation (Miltenyi, Bergisch, Gladbach, Germany) and cultured in the

same StemPro media and cytokine mix. After 24 hours of culture, cord blood HSPCs were transduced for 72 hours at a MOI of 50.

4.11. Colony and replating assays

Colony and replating assays were performed according to previously established protocols¹⁴. Following *in vitro* culture in Stempro Media (Life Technologies, Carlsbad, California), human cells were transduced for 72 hours and then harvested for counting by trypan blue exclusion. 525 cells were plated into a 12-well plate with Methycellulose Media (Stemcell Technologies, Vancouver, British Columbia, Canada).

After 14 days, total colonies typified and counted, and then selected for self-renewal replating assays. Equal numbers of the individual types of colonies were selected and resuspended in Myelocult Media (Stemcell Technologies, Vancouver, British Columbia, Canada), and then plated into Methocult media in a 96-well flat-bottom plate. After 14 additional days, each of the wells were scored for colony formation.

4.12. *In vitro* K562 dasatinib treatments

K562 transduced with pCDH-Empty and pCDH-CTGF virus were seeded at a density of 1,000,000/mL in reduced serum media: DMEM (Life Technologies, Carlsbad, California) with 0.5% FBS (Gemini Bio-Products, West Sacramento, California), 1% GlutaMAX and 1% Penicillin-Streptomycin (Life Technologies, Carlsbad, California). After overnight starve, K562 were plated into a 24-well

plate at 400,000 cells/mL and treated with dasatinib (0, 1, 5, 10 and 20nM) in DMSO. After 48 hours, cells were harvested for whole cellular lysates or FACS analysis of apoptosis markers.

4.13. Statistical methods

All statistical analyses were performed through the Graphpad Prism software, and individual tests are included in the figure legends.

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