Connective tissue growth factor is expressed in bone marrow stromal cells and promotes interleukin-7-dependent B lymphopoiesis

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ABSTRACT

Hematopoiesis occurs in a complex bone marrow microenvironment in which bone marrow stromal cells provide critical support to the process through direct cell contact and indirectly through the secretion of cytokines and growth factors. We report that connective tissue growth factor (*Ctgf*, also known as *Ccn2*) is highly expressed in murine bone marrow stromal cells. In contrast, connective tissue growth factor is barely detectable in unfractionated adult bone marrow cells. While connective tissue growth factor has been implicated in hematopoietic malignancies, and is known to play critical roles in skeletogenesis and regulation of bone marrow stromal cells, its role in hematopoiesis has not been described. Here we demonstrate that the absence of connective tissue growth factor in mice results in impaired hematopoiesis. Using a chimeric fetal liver transplantation model, we show that absence of connective tissue growth factor has an impact on B-cell development, in particular from pro-B to more mature stages, which is linked to a requirement for connective tissue growth factor in bone marrow stromal cells. Using *in vitro* culture systems, we demonstrate that connective tissue growth factor potentiates B-cell proliferation and promotes pro-B to pre-B differentiation in the presence of interleukin-7. This study provides a better understanding of the functions of connective tissue growth factor within the bone marrow, showing the dual regulatory role of the growth factor in skeletogenesis and in stage-specific B lymphopoiesis.

Introduction

Hematopoiesis is a tightly regulated multi-stage process, predominantly occurring in fetal liver before birth and in the bone marrow (BM) postnatally.[1] All hematopoietic cells are derived from a small population of hematopoietic stem cells. The differentiation and self-renewal properties of these stem cells are governed by signals derived from cellular and acellular components that constitute the complex BM microenvironment, comprising osteoblasts, osteoclasts, adipocytes, endothelial cells, stromal cells, extracellular matrix (ECM) and factors secreted by many cell types.[2,3] Additionally, factors involved in bone formation have also been shown to play an important role in hematopoiesis.[4] Several cellular components, cytokines and growth factors have been identified as being involved in B-cell development in the mouse. Adherent BM stromal cells were shown to be indispensable for continuous culture of B cells, suggesting the necessity of secreted factors to support B-cell development.[5] The generation of pre-pro-B cells from multipotent hematopoietic progenitors has been shown to require CXC chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells, while interleukin-7 (IL-7) secreted by BM stromal cells is absolutely essential for the proliferation and differentiation of pro-B cells in the adult mouse.[6-8] These BM stromal cells form specific cellular niches for early B-cell development.[9-11]

Connective tissue growth factor (CTGF), also known as CCN2, belongs to the CCN family of proteins and is a cysteine-rich secreted protein composed of four modules: an insulin-like growth factor-binding domain, a von Willebrand factor type C domain, a thrombospondin type I domain, and a C-terminal cystine knot domain.[12] CTGF is associated with a broad spectrum of cellular functions, including cell adhesion, proliferation, migration, differentiation, survival, collagen deposition, and synthesis of ECM.[12,13] CTGF is highly expressed in bone cartilage during development and healing and is indispensible for bone formation.[14,15] The significance of CTGF in skeletogenesis, chondrogenesis and angiogenesis was demonstrated in *Ctgf*$^{-/-}$ mice, which exhibit defects in chondrocyte proliferation, ECM composition within the hypertrophic zone, and growth plate angiogenesis, as well as impaired endochondral ossification, distorted cartilages and disorganized hypertrophic zones of long bones.[16,17] In addition, CTGF regulates cellular components in the BM microenvironment. For example, CTGF is secreted by osteoblastic cells, which are known to play a critical role in hematopoietic stem cell niches, and *in vitro* studies using recombinant CTGF suggested that CTGF enhances proliferation and differentiation of these cells.[14,15,18-20]

Apart from its physiological roles, CTGF has been implicated in fibrosis and cancer. High expression of *CTGF* has been consistently identified in several cohorts of patients with

 doi:10.3324/haematol.2013.102327
The online version of this article has a Supplementary Appendix.
Manuscript received on December 9, 2013. Manuscript accepted on April 7, 2014.
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acute lymphoblastic leukemia (ALL).[21-25] Specifically, high *CTGF* expression is exclusive to B-lineage ALL and is secreted by pre-B ALL cells, but is not found in T-cell ALL.[21] Moreover, high levels of *CTGF* expression in ALL are linked to poor outcome in patients[22,24] and a recent study suggested that *CTGF* promotes leukemia cell engraftment and growth in the BM.[26] To date, at least 21 different types of cancer have been associated with either low or high *CTGF* expression, and linked to distinct clinical outcomes.[27]

Since CTGF has been documented to play an important role in the BM microenvironment, we investigated whether this growth factor is involved in hematopoiesis. Our data show for the first time that absence of *Ctgf* impairs hematopoiesis and that *Ctgf* is expressed in BM stromal cells to support normal B lymphopoiesis.

Methods

Additional details on the design and methods of this study are provided in the *Online Supplementary Methods*.

Mice

Ctgf$^{+/-}$ mice were generated on a 129SvJxBalb/cJ background.[16] For this study, mice were backcrossed onto the C57BL/6J (CD45.2) strain for at least nine generations. Newborn *Ctgf*$^{-/-}$ mice were collected soon after birth and were used in phenotypic analysis and transplantation experiments. Seven- to 12-week old C57BL/6J mice and B6.SJL-Ptprca Pep3b/BoyJ (C57BL/6J-CD45.1) mice were purchased from the Animal Research Centre, Perth. Animals were housed under pathogen-free conditions and all studies were approved by the Animal Ethics Committee, Telethon Kids Institute, Perth.

Flow cytometry and cell sorting

All flow cytometry and cell sorting (FACS) studies were performed on single-cell suspensions and cells were stained using standard protocols. The flow cytometry details are provided in the *Online Supplementary Methods.* All antibodies were purchased from BD Bioscience unless otherwise described and the details are presented in *Online Supplementary Table S1*. Flow cytometry was performed on a BD LSRII (BD Bioscience) and FACS on a FACSAria (BD Bioscience). Data were analyzed with FlowJo software (TreeStar).

Fetal liver transplantation

For fetal liver transplantation, 2x10^6 neonatal liver or 1x10^6 E13.5 fetal liver cells were transplanted into the recipients by tail vein injection. For hematopoietic stem cell transplantation, liver cells were extracted from E13.5 fetal livers and 1x10^4 lin^{neg}c-kit$^+$Sca-1$^+$ (LSK) cells were sorted by FACS. For competitive transplantation, 5000 CD45.2 *Ctgf*$^{-/-}$ or wild-type (WT) LSK cells were transplanted together with 5000 competitor LSK cells (CD45.1$^+$) into the recipients. Analysis of hematopoiesis was performed 16 weeks after transplantation and data were derived from gated CD45.2$^+$ reconstituted donor cells.

Quantitative reverse transcription polymerase chain reaction expression analysis

Total RNA was extracted from adult and neonatal BM and purified cell preparations using the RNeasy Micro Kit with RNase-free DNase I (Qiagen). cDNA was synthesized using Omniscript RT kit (Qiagen) with anchored oligo-dT primer (Life Technologies). Taqman Gene Expression Assays for mouse *Ctgf* (Mm01192933_g1) and mouse eukaryotic translation elongation factor 1 alpha 1 (*Eef1a1*) (Mm01973893_g1) were performed on an Applied Biosystem Prism 7000 sequence Detection System using Taqman Universal PCR Master Mix (Life Technologies). Results were obtained with the relative standard curve method and *Ctgf* mRNA levels in each sample were normalized to the levels of *Eef1a1*.

B-cell proliferation assay

B-cell proliferation was measured by [^{3}H]thymidine incorporation. B220$^+$ BM cells, pro-B (B220$^+$CD43$^+$IgM^{neg}), pre-B (B220$^+$CD43^{neg}IgM^{neg}) and IgM$^+$ B (B220$^+$CD43^{neg}IgM$^+$) cells from adult C57BL/6J mice were isolated by FACS with a purity >95%. Cells were cultured in 96-well plates, at a density of 1.5x10^5/well for B220$^+$ cells or 5x10^4/well for pro-B, pre-B, or IgM$^+$ B cells, with indicated concentrations of recombinant human CTGF[28] and recombinant mouse interleukin-7 (IL-7) for 72 h; 0.5 μCi/well of [^{3}H]thymidine (PerkinElmer) was added to the cultures for the last 24 h. Cells were harvested and [^{3}H]thymidine incorporation was measured in a MicroBeta® TriLux Microplate Scintillation and Luminescence Counter (PerkinElmer). Results are presented as fold-change normalized to the reading from cells cultured in medium.

Statistical analysis

Data were analyzed using a two-tailed unpaired Student *t*-test. The results are presented as means ± standard error of mean (SEM). A P value <0.05 is considered statistically significant.

Results

Absence of Ctgf impairs hematopoiesis in newborn mice

Previous studies in *Ctgf*$^{-/-}$ mice on a 129Sv/J x Balb/cJ background demonstrated that *CTGF* played a role in skeletal development.[16] After backcrossing this strain onto C57BL/6J, we confirmed a critical role for *CTGF* in skeletal development: the mice exhibited multiple skeletal defects, including disorganized and enlarged hypertrophic zones in femora (Figure 1A,B), and *Ctgf*$^{-/-}$ but not *Ctgf*$^{+/-}$ mice died perinatally, as previously published.[16] Despite the profound defects in bone and ECM content in the BM of *Ctgf*$^{-/-}$ mice, it was not known whether these mice have normal hematopoiesis. To address this, we enumerated three major types of hematopoietic cells: B cells (B220$^+$), T cells (CD3$^+$), and myeloid cells (Gr-1$^+$CD11b$^+$) in BM, spleen, and liver of newborn *Ctgf*$^{-/-}$, *Ctgf*$^{+/-}$ and C57/BL/6J WT mice. Flow cytometric analysis revealed significantly lower proportions of B cells in BM (Figure 1C) and spleen (Figure 1D) from *Ctgf*$^{-/-}$ mice compared to those from *Ctgf*$^{+/-}$ or WT mice. In the liver (Figure 1E), *Ctgf*$^{-/-}$ mice showed significantly increased proportions of B cells and decreased myeloid cells compared to *Ctgf*$^{+/-}$ and WT mice. These data suggest that newborn *Ctgf*$^{-/-}$ mice have altered hematopoiesis. While the findings from newborn BM and spleen compared to liver are seemingly paradoxical, they are likely attributable to context-dependent actions that have been noted for CTGF and other CCN proteins.[29]

Absence of Ctgf in fetal or neonatal liver cells impairs their hematopoietic potential after transplantation into wild-type recipient mice

To gain insight into the role of *CTGF* in hematopoiesis,

we used a chimeric mouse model to compare the hematopoietic potential of *Ctgf*$^{-/-}$ and WT liver cells. Neonatal or E13.5 fetal liver cells (the stage when hematopoietic stem cells are highly enriched[30]) from *Ctgf*$^{-/-}$ or WT donors (CD45.2) were transplanted into lethally irradiated WT recipients (CD45.1). Sixteen weeks after transplantation, we examined BM and spleen cells. We observed the same reconstitution potential in neonatal and fetal liver cells. Results in Figure 2 and *Online Supplementary Figure S1* are from fetal liver transplants.

Analysis of the hematopoietic stem and progenitor cell compartment of the recipients transplanted with *Ctgf*$^{-/-}$ or WT cells (*Online Supplementary Figure S1A-C*) demonstrated no significant differences in either cell numbers or frequencies (*Online Supplementary Figure S1D*). Compared to WT cells, the absence of *Ctgf* did not affect the capacity to regenerate total cell numbers in BM and spleen in the transplant recipients (*Online Supplementary Figure S1E*). Of note, recipients transplanted with *Ctgf*$^{-/-}$ cells had significantly lower numbers and frequencies of B cells in both BM and spleen compared to mice transplanted with WT cells (Figure 2A). Additionally, it was observed that the myeloid population in the BM of recipients transplanted with *Ctgf*$^{-/-}$ cells was more abundant (Figure 2A).

Further analysis of the B-cell subpopulation was conducted using previously described gating strategies[31] (Figure 2B). While the proportions of pre-B, immature B and mature B cells were unchanged within the B220^{+}CD43^{neg} fraction (Figure 2B), we found a significantly lower proportion of B220^{+}CD43^{neg} cells in the BM and spleen, leading to lower total numbers and frequencies of pre-B, immature B, and mature B cells in BM as well as B220^{+}CD43^{+} and B220^{+}CD43^{neg}IgM^{neg} populations in the spleen of recipients transplanted with *Ctgf*$^{-/-}$ compared to WT cells (Figure 2B,C). In contrast, the absence of *Ctgf* did not affect the B220^{+}CD43^{+} population, which is likely to be pro-B cells, although plasmacytoid dendritic cells cannot be definitively excluded.

We then sought to test whether B-cell function was altered in the absence of *Ctgf*. B220^{+} spleen cells were isolated from recipients of *Ctgf*$^{-/-}$ or WT cells 16 weeks after transplantation and stimulated with 20 μg/mL of lipopolysaccharide for 48 h. The repopulated *Ctgf*$^{-/-}$ B cells appeared to have normal responses following activation with lipopolysaccharide and showed no difference in CD80^{+} up-regulation (*Online Supplementary Figure S1F*). Taken together, these data suggest that *Ctgf* is required in cells from fetal or neonatal liver to maintain normal B lymphopoiesis upon transplantation into WT recipient mice.

Absence of Ctgf in hematopoietic stem cells does not affect development of blood cell lineages

We next investigated whether *Ctgf* has a cell autonomous effect in hematopoietic stem cells. We isolated hematopoietic stem cell-enriched lin^{neg}Sca-1^{+}c-kit^{+} (LSK) cells from E13.5 fetal livers of *Ctgf*$^{-/-}$, WT, and C57BL/6J-CD45.1 (competitor) mice and performed both non-competitive and competitive transplantation experiments using the chimeric model as above. No significant differences were found between the recipients of *Ctgf*$^{-/-}$ and WT hematopoietic stem cells in non-competitive transplants (*Online Supplementary Figure S2A-D*), suggesting that *Ctgf*

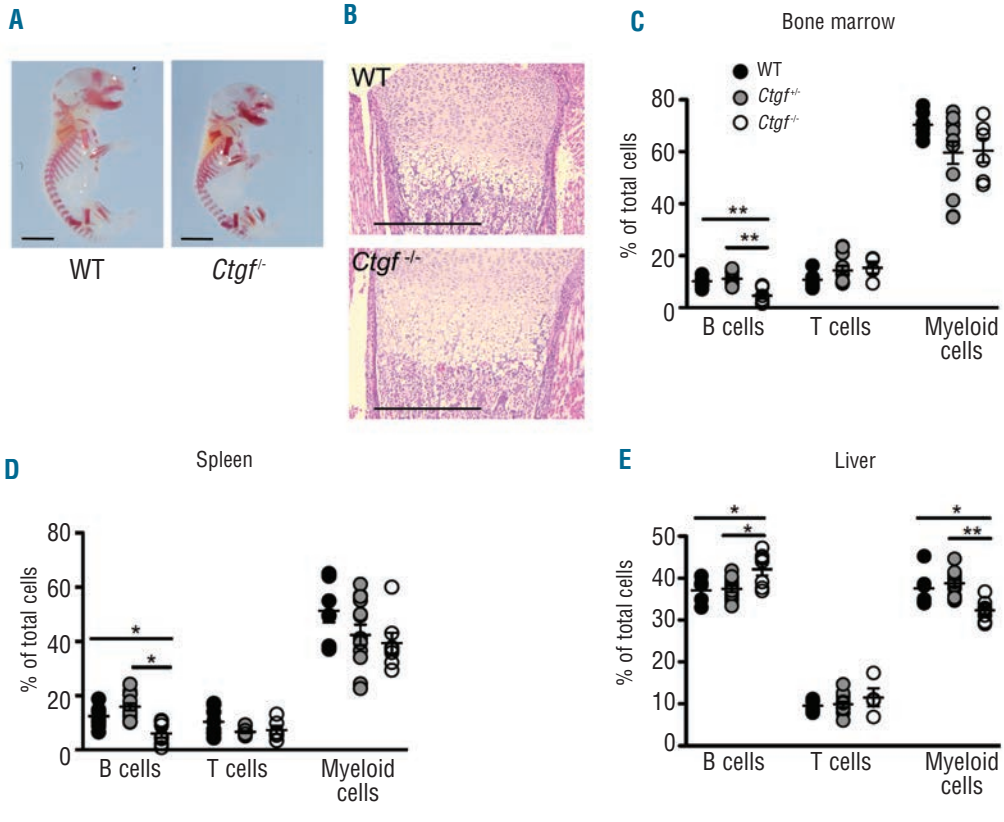

Figure 1. Newborn *Ctgf*$^{-/-}$ mice exhibit skeletal defects and impaired hematopoiesis. (**A**) Representative photomicrographs depicting alizarin red staining of skeletal structure in *Ctgf*$^{-/-}$ and WT C57BL/6J newborn mice. Bars, 5 mm. (**B**) Representative photomicrographs showing hematoxylin and eosin staining of upper femora from *Ctgf*$^{-/-}$ and WT newborn mice. Bars, 500 μm. (**C-E**) Data shown are from individual newborn mice (● WT, ◉ *Ctgf*$^{+/-}$, ○ *Ctgf*$^{-/-}$), as % B cells (B220^{+}), % T cells (CD3^{+}), and % myeloid cells (CD11b^{+}Gr-1^{+}) of total cells from (**C**) BM, (**D**) spleen, and (**E**) liver. Data were collected from four independent experiments with at least one neonate per group. Error bars represent SEM, **P*<0.05; ***P*<0.01.

has minimal cell autonomous effects in hematopoietic stem cells. For competitive transplants, equal numbers of donor cells (*Ctgf*$^{-/-}$ or WT) and competitor cells were transplanted into the recipients and we did not observe any competitive disadvantage of *Ctgf*$^{-/-}$ hematopoietic stem cells in hematopoietic reconstitution compared to WT hematopoietic stem cells (*Online Supplementary Figure S2E,F*). Collectively, these findings demonstrate that the absence of *Ctgf* in hematopoietic stem cells does not affect hematopoiesis or stem cell properties.

Abundant Ctgf expression in bone marrow stromal cells from adult and newborn mice

Because these *in vivo* data suggest that the *Ctgf*-dependent effect on hematopoiesis is not cell autonomous in hematopoietic stem cells, we examined the expression of *Ctgf* in the BM compartment using quantitative reverse transcriptase polymerase chain reaction analysis. Adult BM cells and fragments of tibiae and femora were treated with collagenase,[32] followed by FACS. In unfractionated adult BM cells, *Ctgf* was barely detectable (Figure 3A). Consistent with this, we did not detect any *Ctgf* expression in pro-B (B220^{+}CD43^{+}), pre-B (B220^{+}CD43^{neg}IgM^{neg}), or IgM^{+} (B220^{+}CD43^{neg}IgM^{+}) B cells isolated from adult BM (*data not shown*). Remarkably, in the purified CD45^{neg}Ter119^{neg}CD11b^{neg} population, *Ctgf* expression was readily detectable (Figure 3A), indicating that BM stromal cells endogenously express *Ctgf*. To further investigate this observation, we isolated subpopulations from neonatal femora and tibiae, chosen over adult cells to provide increased yields of cells, including mesenchymal stem cells, CAR cells, endothelial cells and vascular cell adhesion molecule (VCAM)-1^{+} reticular cells (*Online Supplementary Figure S3A*).[9,32-34] *Ctgf* expression was readily detected in each of these purified populations, with CAR cells showing the highest expression (Figure 3B). Thus, bone stromal cells are responsible for the bulk of *Ctgf* expression within the bone or BM. As assessed by quantitative reverse transcriptase polymerase chain reaction analysis, adult BM stromal cells and all subpopulations of neonatal BM stromal cells expressed IL-7 mRNA (*Online Supplementary Figure S3B*).

Given the high expression of *Ctgf* in BM stromal cells and because CTGF plays a significant role in skeletogenesis, we wondered whether the absence of *Ctgf* would affect the composition of BM stromal cells. We, therefore, isolated BM stromal cells from WT, *Ctgf*$^{+/-}$ and *Ctgf*$^{-/-}$ newborn mice. We found that the absence of *Ctgf* did not affect the cell numbers (*data not shown*) or the proportions of mesenchymal stem cells, CAR cells, endothelial cells, or VCAM-1^{+} reticular cells in neonatal femora and tibiae (*Online Supplementary Figure S3C*). Together, our results demonstrate that BM stromal cells are the major source of *Ctgf* in both adult and neonatal BM and that *Ctgf* is barely detectable in hematopoietic cells.

Connective tissue growth factor potentiates B-cell proliferation in the presence of interleukin-7

To understand how CTGF regulates B lymphopoiesis, we first investigated whether CTGF has an impact on B-cell proliferation. IL-7 plays an essential role in B-cell proliferation and differentiation, and is commonly used to

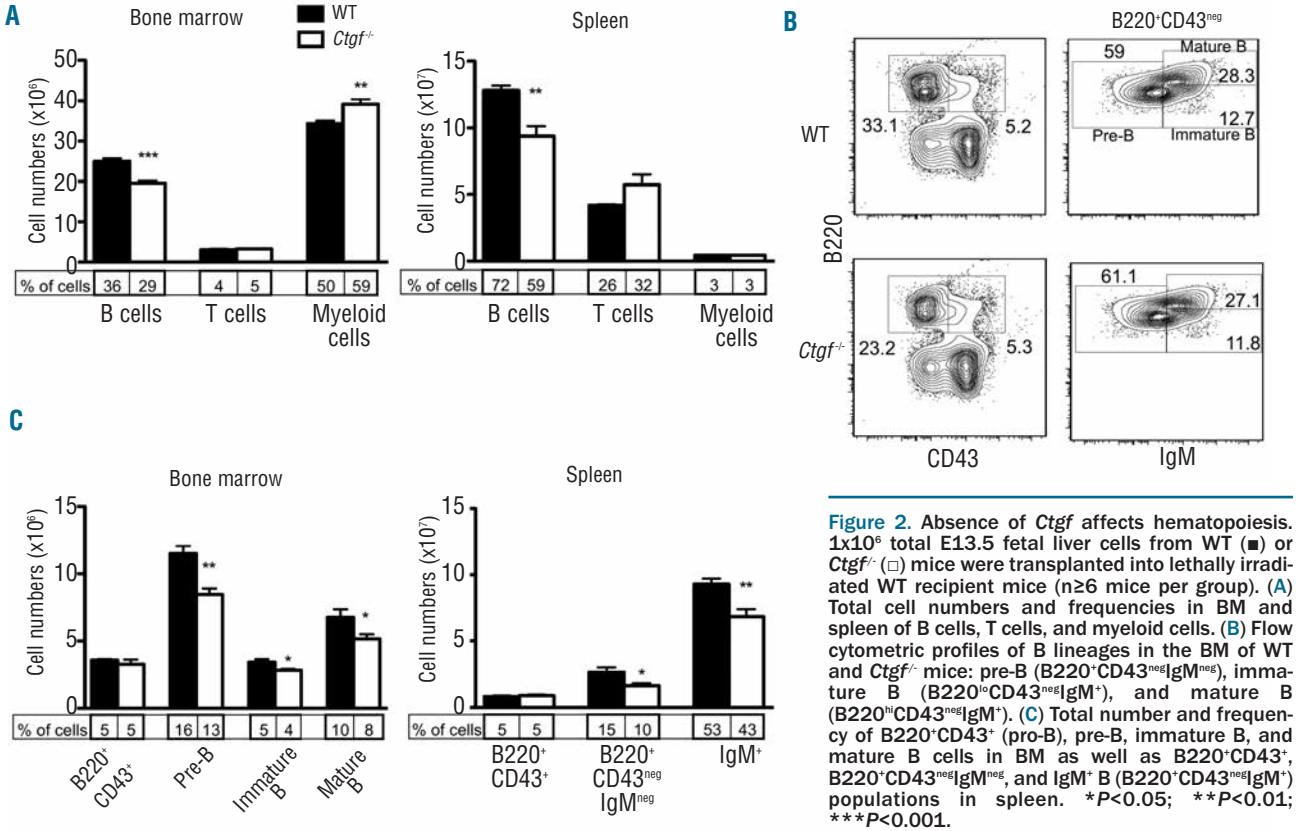

Figure 2. Absence of *Ctgf* affects hematopoiesis. 1x10^{6} total E13.5 fetal liver cells from WT (■) or *Ctgf*$^{-/-}$ (□) mice were transplanted into lethally irradiated WT recipient mice (n≥6 mice per group). (**A**) Total cell numbers and frequencies in BM and spleen of B cells, T cells, and myeloid cells. (**B**) Flow cytometric profiles of B lineages in the BM of WT and *Ctgf*$^{-/-}$ mice: pre-B (B220^{+}CD43^{neg}IgM^{neg}), immature B (B220^{lo}CD43^{neg}IgM^{+}), and mature B (B220^{hi}CD43^{neg}IgM^{+}). (**C**) Total number and frequency of B220^{+}CD43^{+} (pro-B), pre-B, immature B, and mature B cells in BM as well as B220^{+}CD43^{+}, B220^{+}CD43^{neg}IgM^{neg}, and IgM^{+} B (B220^{+}CD43^{neg}IgM^{+}) populations in spleen. *P<0.05; **P<0.01; ***P<0.001.

examine B lineage cells *in vitro*.[35] We cultured B cells ($B220^+$) with recombinant mouse IL-7 and/or recombinant human CTGF for 72 h and determined proliferation by [^{3}H]thymidine incorporation for 24 h prior to harvesting. We confirmed that IL-7 enhances B-cell proliferation approximately 5-fold, and concentrations of more than 1 ng/mL did not increase the level of proliferation (Figure 4A). In contrast, CTGF added to cultures alone showed negligible induction of B-cell proliferation (Figure 4A). IL-7 and CTGF together generated a proliferative response recorded to be: (i) higher than additive and (ii) dependent on the CTGF dose (Figure 4A). To determine which B-cell subpopulations were responsive, BM cells were separated into pro-B ($B220^+CD43^+IgM^{neg}$), pre-B ($B220^+CD43^{neg}IgM^{neg}$) and surface IgM ($sIgM)^+$ B ($B220^+CD43^{neg}IgM^+$) cells and cultured with CTGF and IL-7. In the presence of both IL-7 and CTGF, the highest proliferation index was evident in pro-B cells, followed by pre-B cells, with a 3-fold increase compared to that caused by IL-7 alone (Figure 4B). It is noteworthy that CTGF induced a mild but significant increase in pro-B cell proliferation (Figure 4B). As expected, since $sIgM^+$ B cells do not express IL-7 receptor (IL-7R) and are generally not responsive to IL-7,[36] we did not see any proliferative response in $sIgM^+$ B cells in the presence of IL-7 and/or CTGF (Figure 4B). These data suggest that CTGF potentiates B-lymphoid proliferation only in the presence of IL-7.

Connective tissue growth factor promotes pro-B to pre-B differentiation in the presence of interleukin-7

We next examined whether CTGF affects B-cell maturation in the presence of IL-7. Enriched pro-B cells ($B220^+CD43^+IgM^{neg}$) from adult mouse BM were cultured with CTGF and IL-7 for 48 h in stromal-free conditions.[37] Compared to pro-B cells cultured in IL-7, those cultured with CTGF alone or in combination with IL-7 maintained a higher proportion of $B220^{hi}CD43^+$ cells (Figure 4C, compare gates B). This population is likely to be pro-B cells but plasmacytoid dendritic cells cannot be definitively excluded. Importantly, we found that most of the pro-B cells (Figure 4C, total in gates B+D) differentiated into $B220^{hi}CD43^{neg}$ cells (Figure 4C, gate A) when cultured with CTGF and IL-7, indicating that in the presence of IL-7, CTGF drives pro-B-cell differentiation (Figure 4D). In order to gain more insight into the impact of CTGF on B-cell differentiation, we also examined sIgM expression in $B220^{hi}CD43^{neg}$ and $B220^{lo}CD43^{neg}$ fractions after culture. The combination of CTGF and IL-7 resulted in reduced sIgM-expressing cells in both $B220^{hi}CD43^{neg}$ and $B220^{lo}CD43^{neg}$ fractions compared to those cultured with IL-7 or CTGF alone (Figure 4E and *Online Supplementary Figure S4*). Taken together, we conclude that CTGF in the presence of IL-7 promotes B-cell differentiation from pro-B to pre-B, but not further to $sIgM^+$ B cells.

Connective tissue growth factor did not enhance the phosphorylation of STAT5 in B cells

Lastly, we investigated whether CTGF in the presence of IL-7 can potentiate IL-7R/STAT5 signaling. We incubated adult mouse BM cells for 30 min with IL-7 and/or CTGF, and used flow cytometry to measure the level of phosphorylated STAT5 (pSTAT5) on $B220^+$, pro-B ($B220^+CD43^+$) and pre-B ($B220^{lo}CD43^{neg}$) populations. We found that CTGF did not enhance the proportion of pSTAT5-positive B cells (*Online Supplementary Figure S5*). Although CTGF regulation of B-cell development relied on the presence of IL-7, our results suggest that CTGF acts on an independent mechanism, other than via phosphorylating STAT5 in the IL-7R signaling pathway.

Discussion

The BM is the primary site of hematopoiesis after birth, providing a complex environment in which bone cells, blood cells and immune cells are in close proximity. The concept of a 'stem cell niche' was first proposed by Schofield[38] and extensive studies have since defined its cellular and acellular components.[2,3] Bone cells, ECM, sulfate proteoglycans and stromal cell types coordinate skeletogenesis and the same components play roles in hematopoietic stem cell niches.[39] One of the best-characterized examples is the bone-forming osteoblastic cell and its secreted factors, which closely regulate the properties and numbers of hematopoietic stem cells.[19,20] In this study, we examined an ECM-associated protein, CTGF, which binds strongly to heparin and has a critical role in skeletogenesis.[16,40] Adding to the findings that CTGF is

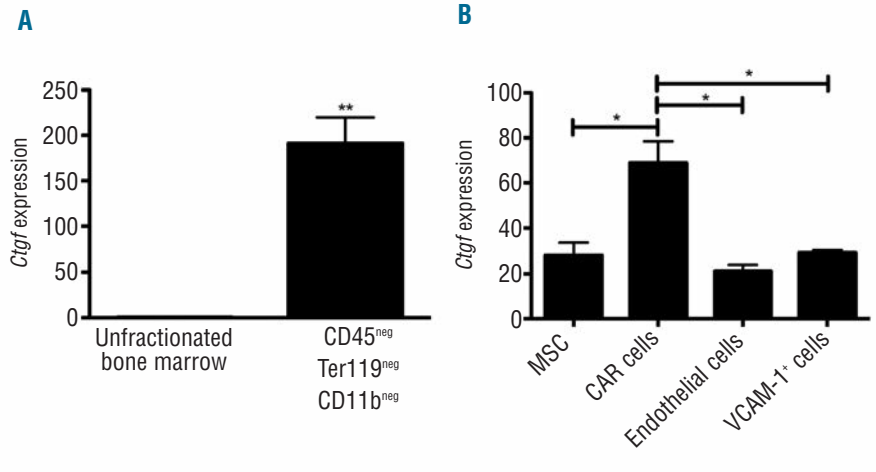

Figure 3. *Ctgf* is highly expressed in bone marrow stromal cells. *Ctgf* expression levels, normalized to the expression of *Eef1a1*, was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in (A) adult and (B) neonatal BM cells from femora and tibiae. Data were collected from two independent experiments and qRT-PCR was performed in duplicate. (A) Unfractionated BM cells and sorted $CD45^{neg}Ter119^{neg}CD11b^{neg}$ cells from adult mice. (B) *Ctgf* expression of BM stromal cells from newborn mice. Hematopoietic cells ($CD45^+Ter119^+CD11b^+$) were first removed. Mesenchymal stem cells (MSC) ($Sca\text{-}1^+PDGFR\alpha^+$), CAR cells ($Sca\text{-}1^{neg}PDGFR\beta^+$), endothelial cells ($Sca\text{-}1^+CD31^+$), and VCAM-$1^+$ reticular cells. Error bars represent SEM *P<0.05; **P<0.0001.

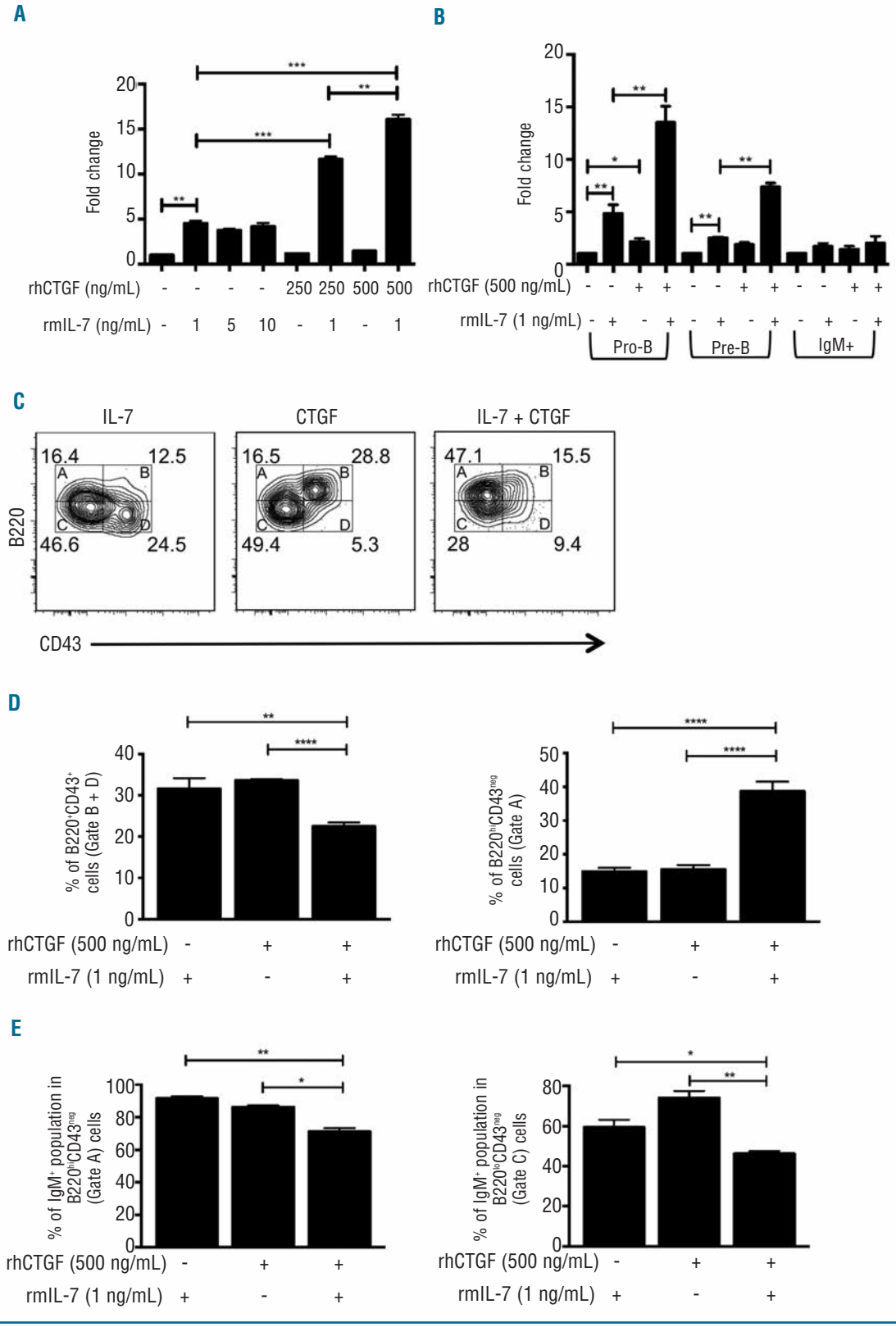

Figure 4. CTGF regulates B-cell proliferation and differentiation in the presence of IL-7. (A) $B220^+$ cells were isolated via cell sorting from BM cells and cultured in the presence of recombinant human (rh) CTGF, recombinant mouse (rm) IL-7, or both. Proliferation at 72 h was measured by [^{3}H]-thymidine incorporation for 24 h. Data were derived from two independent experiments performed in triplicate. (B) Pro-B ($B220^+CD43^+IgM^{neg}$), pre-B ($B220^+CD43^{neg}IgM^{neg}$) and IgM^+ B ($B220^+CD43^{neg}IgM^+$) cells were isolated by cell sorting and stimulated as indicated. Data were derived from three independent experiments performed in duplicate. (C-E) Pro-B cells ($B220^+CD43^+IgM^{neg}$) were isolated by cell sorting and incubated with rhCTGF, rmIL-7, or both. B-cell maturation was measured 48 h after culture. Data were derived from two independent experiments in performed duplicate. (C) Flow cytometric analysis of subpopulations of B cells. (D) Percentage of $B220^+CD43^+$ (gates B+D) and $B220^{hi}CD43^{neg}$ (gate A) populations. (E) Percentage of IgM^+ populations in $B220^{hi}CD43^{neg}$ (gate A) and $B220^{lo}CD43^{neg}$ (gate C) cells. Error bars represent SEM *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

associated with B-lineage ALL[21-26], our studies demonstrate that CTGF exerts a regulatory role on B lymphopoiesis.

The absence of *Ctgf* caused altered hematopoiesis with significantly lower numbers of B cells in BM and spleen detectable in newborn *Ctgf*$^{-/-}$ mice and chimeric mice transplanted with unfractionated *Ctgf*$^{-/-}$ liver cells. Pre-B and later differentiation stages were most affected, while pro-B populations remained unchanged and overall B-cell function was not impaired as measured by B-cell activation upon stimulation with lipopolysaccharide. However, transplantation studies with fractionated hematopoietic stem cells allowed us to conclude that the absence of *Ctgf* in hematopoietic stem cells does not intrinsically affect hematopoiesis or stem cell repopulation capacity.

Since *Ctgf* is not required by hematopoietic stem cells for normal hematopoiesis, we focused on the source of *Ctgf* in the BM. *Ctgf* was not detectable in B cells or unfractionated adult BM cells, where the majority of cells are hematopoietic. However, we showed that *Ctgf* is highly expressed in BM stromal cells from adult and newborn mice, a finding supported by three recently published studies examining mouse and human BM stromal cells as well as human mesenchymal stem cells.[26,41,42] Fractionation of BM stromal cells further indicated a broad distribution of *Ctgf* in the BM microenvironment, with CAR cells displaying the highest level of expression. BM stromal cells play a vital role in microenvironmental niches and, reticular cells form stage-specific cellular niches for early B-cell development.[9-11] These findings are consistent with our hematopoietic stem cell chimeric mouse studies and suggest that *Ctgf*-dependent effects on hematopoiesis are non-cell autonomous.

Given that CTGF is indispensible in skeletogenesis, we examined the BM stromal cell composition of newborn mice and found that *Ctgf* did not alter the proportions of stromal cells compared to WT. These findings further demonstrate a direct role for *Ctgf* in B lymphopoiesis, as reduced numbers of B cells in *Ctgf*$^{-/-}$ mice cannot be attributed to a change in the overall stromal cell compartment.

While IL-7, secreted by BM stromal cells, is essential for B-cell development in the adult mouse,[6,7] a number of other co-factors secreted by BM stromal cells can also regulate B-cell development.[43,44] Here, we examined the role of CTGF in B-cell proliferation and differentiation in the presence and absence of IL-7. CTGF alone induced a proliferative response selectively in pro-B cells, supporting the role of CTGF as a stromal cell co-factor, as early B-cell development is stromal cell-dependent.[31] Additionally, CTGF significantly potentiated IL-7-induced proliferation in both pro-B and pre-B subpopulations, with marked increases in the pro-B cell fraction. CTGF alone increased B220 expression and together with IL-7 induced B-cell differentiation from pro-B to pre-B, resulting in the accumulation of a B220^{hi}CD43^{neg} population. This is consistent with our transplantation studies, in which *Ctgf*$^{-/-}$ chimeras had reduced pre-B cells and suggests that CTGF plays an important role in both pro-B cell proliferation and their differentiation to pre-B cells. However, in the presence of IL-7, CTGF reduced sIgM expression of B220^{lo}CD43^{neg} and B220^{hi}CD43^{neg} populations, highlighting the need to fully elucidate the role of IL-7 and CTGF in late B-cell maturation.[45,46]

Components of the ECM play a role in B lymphopoiesis, and glycosaminoglycans, including heparan sulfate and heparin, are found in abundance in ECM.[39] Both CTGF and IL-7 can bind heparin. Heparin can act as a carrier for IL-7 in the BM ECM,[47] and in an earlier study it was found that IL-7-dependent B lymphopoiesis was mediated by cell surface-associated heparan sulfate in B cells and stromal cells.[48] It is conceivable that CTGF can act via this mechanism although the interaction remains to be further investigated.

We explored the possibility that CTGF regulates B cells via IL-7R/STAT5 since STAT5 signaling is induced by IL-7 and is critical in B-cell development.[49] The IL-7Rα subunit is expressed in B cells from common lymphoid progenitors to pre-B cells and downregulated in sIgM^{+} B cells.[36] Our results indicate that CTGF did not enhance STAT5 phosphorylation beyond the level achieved by IL-7 alone. This suggests that CTGF may act via an independent mechanism and highlights the complex nature of interactions between the two factors in the BM environment.

In conclusion, the present studies establish a novel role for CTGF in B lymphopoiesis, promoting IL-7-induced proliferation and differentiation from pro-B to pre-B cells. Both factors are expressed in BM stromal cells and CTGF is likely a co-factor that acts together with IL-7 to support B-cell development. Our findings provide a better understanding of the functions of CTGF within the BM, showing the dual regulatory role in skeletogenesis and in stage-specific B lymphopoiesis. In depth studies are necessary to elucidate the exact mechanisms, and the relationship between CTGF and leukemogenesis warrants further investigation.

Acknowledgments

We thank Michelle Tourigny for assistance with the flow cytometry facility, Telethon Kids Institute. This work was supported by the Children's Leukaemia and Cancer Research Foundation, Perth, Australia. WSA is supported by a Research Fellowship and Program Grant (1016647) from the National Health and Medical Research Council (NHMRC) of Australia, Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIIS.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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