Published in final edited form as:

Bone. 2015 February ; 71: 76–88. doi:10.1016/j.bone.2014.10.005.

Connective Tissue Growth Factor Reporter Mice Label a Subpopulation of Mesenchymal Progenitor Cells that Reside in the Trabecular Bone Region

Wen Wang[#], Sara Strecker[#], Yaling Liu, Liping Wang, Fayekah Assanah, Spenser Smith, and Peter Maye^{*}

Department of Reconstructive Sciences, School of Dental Medicine, University of Connecticut Health Center

Abstract

Few gene markers selectively identify mesenchymal progenitor cells inside the bone marrow. We have investigated a cell population located in the mouse bone marrow labeled by *Connective Tissue Growth Factor* reporter expression (*CTGF*-EGFP). Bone marrow flushed from *CTGF* reporter mice yielded an EGFP⁺ stromal cell population. Interestingly, the percentage of stromal cells retaining *CTGF* reporter expression decreased with age *in vivo* and was half the frequency in females compared to males. In culture, *CTGF* reporter expression and endogenous *CTGF* expression marked the same cell types as those labeled using *Twist2*-Cre and *Osterix*-Cre fate mapping approaches, which previously has been shown to identify mesenchymal progenitors *in vitro*. Consistent with this past work, sorted CTGF⁺ cells displayed an ability to differentiate into osteoblasts, chondrocytes, and adipocytes *in vitro* and into osteoblast, adipocyte, and stromal cell lineages after transplantation into a parietal bone defect. *In vivo* examination of *CTGF* reporter expression in bone tissue sections revealed it marked cells highly localized to the trabecular bone region and was not expressed in the perichondrium or periosteum. Mesenchymal cells retaining high *CTGF* reporter expression were adjacent to, but distinct from mature osteoblasts lining bone surfaces and endothelial cells forming the vascular sinuses. Comparison of *CTGF* and *Osterix* reporter expression in bone tissue sections indicated an inverse correlation between the strength of *CTGF* expression and osteoblast maturation. Down-regulation of *CTGF* reporter expression also occurred during *in vitro* osteogenic differentiation. Collectively, our studies indicate that *CTGF* reporter mice selectively identify a subpopulation of bone marrow mesenchymal progenitor cells that reside in the trabecular bone region.

Keywords

Mesenchymal Stem Cell; Stromal Cell; Connective Tissue; Bone Marrow

^{*}Corresponding Author: Peter Maye, Department of Reconstructive Sciences, MC3705, L7007, School of Dental Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, Phone: 860-679-7347, Fax: 860-679-2910, pmaye@neuron.uhc.edu, Website:<http://regenerativemedicine.uhc.edu/faculty/bios/maye.html>.

[#]Equal Contribution of Authors

Disclaimers: None

Introduction

Work by Friedenstein first showed that a stromal derived cell population obtained from the bone marrow selectively attached to plastic, expanded in culture and, upon transplantation, retained skeletal potential and supported a hematopoietic environment [1–4]. Recognizing the therapeutic value of this cell population, many research groups have contributed to its characterization and have most commonly referred to it as the bone marrow mesenchymal stem cell (BMSC) [5–15]. Much of the characterization of BMSCs has taken place after *in vitro* expansion, while our knowledge of this cell population inside the bone marrow still remains an area of great intrigue.

Some studies have indicated that certain types of perivascular cells may be the endogenous source of *in vitro* expanded adult skeletal progenitor cells. Work carried out on human bone marrow samples was the first to identify CD146+ as a selective cell surface marker for a population of adventitial reticular cells that retained skeletal potential [15]. In mice, *Chemokine C-X-C Motif Ligand 12 (Cxcl12) –EGFP* and *Nestin-EGFP* reporters mark marrow perivascular cells that have been shown to display skeletal potential [11, 16]. Surprisingly, *Osterix*-Cre fate mapping studies have revealed that embryonic skeletal progenitors largely contribute to perivascular cells of the bone marrow stroma, possibly explaining their inherent skeletal potential [17, 18]. At the same time, where mesenchymal progenitor cells reside within a tissue is likely to be indicative of their endogenous physiological role. With regard to the marrow, some studies have provided evidence that mesenchymal progenitors isolated near bone surfaces have greater self-renewal capacity and skeletal potential compared to those isolated from the central marrow region [19, 20]. Therefore, a distinction between *in vivo* role versus *ex vivo* therapeutic application(s) may be necessary.

CTGF encodes a multi-domain, cysteine rich, extracellular matrix protein belonging to the *CCN* gene family. The modes of *CTGF* action are complex, with different protein domains capable of interacting with a broad range of ligands and receptors including TGFβs, BMPs, IGF-1, LRP1, LRP6, and integrins (reviewed in [21]). While *CTGF* is perhaps best known for its pathological role in tissue fibrosis (reviewed in [22]), genetic loss of function studies in mice have revealed its importance in a variety of developmental processes, two of which are patterning of the vasculature and skeletal development. During vascular development, *CTGF* expression in endothelial cells and pericytes contributed to the expression of basement membrane proteins, pericyte adhesion, and blood vessel integrity [23]. During growth plate formation, loss of *CTGF* resulted in reduced chondrocyte proliferation and broader zones of hypertrophy [24]. The role of *CTGF* during osteogenesis is less clear. While global *CTGF* mutant mice showed reduced osteoblast proliferation and formation [25], *Prx1*-Cre generated conditional mutants have a significantly milder bone phenotype [26]. Additionally, transgenic animal models that over-express *CTGF* in a skeleton specific manner exhibit osteopenia [27, 28].

A variety of *in vitro* studies have also investigated the expression and function of *CTGF* in primary BMSCs and different mesenchymal cell lines. Large scale gene expression analyses of cultured bone marrow stromal cells have revealed that *CTGF* is highly expressed in this

cell population [29, 30] and decreases upon differentiation [31, 32]. It also has been speculated that CTGF and possibly other CCN family members may contribute to the multipotency of BMSCs [32]. *In vitro*, CTGF has been shown to be important for stromal cell survival and growth as well as inhibit adipocyte and osteoblast differentiation, possibly by redirecting BMSCs into the fibroblast lineage [33–35]. However, there is also contradictory evidence that CTGF promotes osteogenesis [36]. The lack of clarity with regard to CTGF's role in osteoblast differentiation potentially reveals that alternative mechanisms of CTGF action exist that are context dependent. A possible concern of studies carried out in culture is the serum responsive nature of *CTGF* expression [37]. Here we report on a bone marrow cell population labeled by *CTGF*-EGFP reporter expression. Our studies provide evidence that this animal model selectively labels a progenitor cell population inside the bone marrow that retains multipotent mesenchymal potential. Interestingly, the cell population labeled by *CTGF* reporter expression is located within the trabecular bone region.

Materials & Methods

Animals

Genetically modified mouse lines were obtained from the following sources: *CTGF*-EGFP (MMRRC:011899-UCD, GENESAT Project) *Twist* 2-Cre (generously provided by Dr. David Ornitz, Jackson Laboratories; Stock Number: 008712) *Osterix*-EGFP-Cre (OEC) (generously provided by Dr. Andrew McMahon, Jackson Laboratories; Stock Number: 006361), Ai9 Cre reporter mice (generously provided by Dr. Hongkui Zeng, Jackson Laboratories; Stock Number: 007909), NIHIII Nude mice (Charles River; Strain Codes 201 and 202). *Osterix*-Cherry reporter mice were generated as previously described [38]. All mice were maintained in a pathogen free barrier facility and all experiments were carried out in a humane manner after receiving approval from our institutional animal care committee (University of Connecticut Health Center).

Harvesting and Cell Culture of BMSCs from Mouse Bone

Mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation. Femurs and tibia were dissected from the surrounding tissues. The epiphyseal growth plates were removed and the bone marrow was collected by flushing with α MEM culture medium containing 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% FCS (Hyclone) with a 27 gauge needle. Single cell suspensions were prepared by gently mixing the cells with a pipette followed by filtration through a 70- μ m strainer. Cells were centrifuged at 350g for 10 min and plated at a density 1.2×10^6 cells/cm². At day 4, cultures underwent a complete media change. On day 5, the cells were sorted and processed.

FACS Analyses and Sorting

Bone marrow cells were harvested from sacrificed mice as described above. Red blood cells were then selectively lysed in suspension using Red Blood Cell Lysing Buffer (Sigma, R7757) as recommended by the manufacturer. Bone marrow cells were then resuspended in a solution containing PBS, 0.5% BSA, 2 mM EDTA, pH 7.2 for FACS analyses. Bone marrow stromal cells cultured for five days or at later time points were washed twice with

PBS then digested using a sterile filtered mixture of 0.1% Collagenase P (Roche), 0.1% Hyaluronidase (Sigma), 2% FBS (Hyclone), 49% OPTI-MEM (Gibco) and 49% PBS (Gibco). Cells were digested for 10 minutes at 37°C, scraped, then digested for an additional 5 minutes at 37°C. Cells were harvested, run through a 40µm cell strainer, and centrifuged at 350g for 10 min. The cell pellet was resuspended in a solution containing 2% FBS, 49% PBS and 49% OPTI-MEM for FACS sorting. Sorted cells were collected in a 1:1 mixture of OPTI-MEM:PBS containing 20% serum. Cell surface profiling was carried out following the manufacturer's recommendations (Miltenyi). In brief, cells were resuspended in 100 µl FACS staining buffer (PBS, 0.5% BSA, 2 mM EDTA, pH 7.2) and 10 µl of the appropriate antibody was added to the cell suspension. The cell-antibody suspension was mixed well and incubated for 10 minutes in the dark at 4 °C. Cells were then washed by the addition of 1–2 ml of buffer and subsequently centrifuged at 300 g for 10 minutes. Supernatant was aspirated and the cells were resuspended in 500 µl of staining buffer containing Sytox Blue (Life Technologies) for FACS analysis. All antibodies used were conjugated to APC to be spectrally distinct from the EGFP and included CD11b (Miltenyi, 130-091-241), CD31 (Miltenyi, 130-097-420), CD45 (Miltenyi, 130-091-811), CD44 (Miltenyi, 130-096-836), CD140b (Miltenyi, 130-096-270) CD105 (Miltenyi, 130-092-930), Anti-Sca1 (Miltenyi, 130-093-223), CD29 (Miltenyi, 130-096-356) and CD90.2 (Miltenyi, 130-091-790). FACS analyses was carried out on a Becton-Dickinson LSR II and FACS sorting was carried out using a FACS Aria II (UCHC Flow Cytometry Core).

In Vitro Differentiation of BMSCs

FACS sorted EGFP+ and EGFP– cells were plated as confluent spots ($\sim 2 \times 10^4$ cells in 20 µl of medium) for osteogenic and adipogenic differentiation and as a micromass (2×10^5 cells in 10 µl of medium) for chondrocyte differentiation. Cells pipetted in these small volumes were allowed ~ 2 hours to attach followed by the addition of medium (α MEM, 100 U/ml Penicillin, 100 µg/ml streptomycin and 10% FCS (Hyclone)). The next morning, cells were switched to differentiation media. For osteogenic differentiation, cells were grown in α MEM medium with 10% FCS, 100 µ/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml ascorbic acid, and 8 mM 2-glycerol phosphate. For stromal cultures grown to be analyzed by FACS analyses, 2-glycerol phosphate was omitted, which allows differentiation, but not mineralization. For adipogenic differentiation, cells were cultured in 10% FCS in α MEM medium containing 1.0 µM insulin and 0.5 µM rosiglitazone. For chondrogenic differentiation, cells were cultured in high-glucose DMEM supplemented with ITS + 1, 50 µg/ml ascorbic acid, 100 µg/ml sodium pyruvate, 0.1 µM dexamethasone, 100 units/100 µg penicillin/streptomycin, 40 µg/ml L-proline and 10 ng/ml TGF- β 3.

Quantitative RT-PCR

RNA was purified from tissue culture cells using a Nucleospin kit (Macherey-Nagel). RNA was transiently treated with DNase to remove any possible genomic DNA contaminants followed by cDNA synthesis using the SuperScript II Reverse Transcriptase (Life Technologies). Quantitative PCR was carried out using a QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. Samples were run in a ABI 7900HT (Applied Biosystems) real time PCR machine under the following conditions: 95 °C–15 min; 95 °C–30 s, 55 °C–30 s, 72 °C–30 s for 40 cycles. The specificity of amplification was

verified by the presence of a single dissociation peak. PCR primer sequences for gene expression analyses were: *Osteocalcin (Bglap1)* (sense) 5'-TCCAAGCAGGAGGGCAATAAG-3', (antisense) 5'-GCGTTTGTAGGCGGTCTTCAAG-3'; *Binding Sialoprotein (Bsp)*, (sense) 5'-CGCCACACTTTCCACACTCTC-3', (antisense) 5'-CTTCCTCGTCGCTTTCCTTCAC-3'; *Dentin Matrix Protein 1 (Dmp1)* (sense) 5'-CGCATCCCAATATGAAGACTG-3', (antisense) 5'-GCTTGACTTTCTTCTGATGACTCA-3'; *Adiponectin (Adipoq)* (sense) 5'-CGTGATGGCAGAGATGGCACT-3', (antisense) 5'-GCGAATGGGTACATTGGGAACAG-3'; *Perilipin (Plin)* (PrimerBank ID: 28316726a2) (sense) 5'-AGATCCCCGGCTCTTCAATACC-3', (antisense) 5'-AGAACCTTGTCAGAGGTGCTT-3'; *Fabp4* (sense) 5'-GGGAACCTGGAAGCTTGTCTC-3', (antisense) 5'-CTGCGGTGATTTTCATCGAATTCCAC-3'; *Aggrecan (Acan)* (sense) 5'-CTATGAGGATGGCTTCCACCAGT-3', (antisense) 5'-CCATCTCCTCAGCGAAGCAGT-3'; *Collagen type 2 alpha 1 (Col2a1)* (sense) 5'-TCGCACTTGCCAAGACCTGAA-3', (antisense) 5'-GGTCTCTCCAAACCAGATGTG-3'; *Collagen type 10 alpha 1 (Col10a1)* (sense) 5'-GCTGCCTCAAATACCCTTTCTG-3', (antisense) 5'-GGACCAGGAATGCCTTGTCT-3'; *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* (sense) 5'-AGGTCGGTGTGAACGGATTG-3'; (antisense) 5'-TGTAGACCATGTAGTTGAGGTCA-3'; *Hypoxanthine guanine phosphoribosyltransferase (HPRT)* (sense) 5'-CACAGGACTAGAACACCTGC-3'; (antisense) 5'-GCTGGTGAAAAGGACCTCT';

Dissection, Embedding, and Cryohistology

Tissues were dissected and fixed in 10% formalin buffered in PBS for 4 days at 4°C. Bone tissues from two week old or older mice were decalcified in 14% EDTA for 4–7 days, depending on animal age. Tissues were then placed in 30% sucrose overnight and embedded in Cryomedia (Thermo Scientific) as previously described [39]. Frozen 7µm sections were collected using Cryofilm type II tape transfer system (Section-Lab) using a Leica Cryostat. Sections were mounted using 50% glycerol buffered with PBS for imaging.

Immunostaining of Tissue Sections

For immunocytochemistry of tissue, sections were allowed to air dry for 30 minutes at room temperature to prevent sections from detaching from the tape during subsequent antibody incubations. Sections were then rehydrated in PBS for fifteen minutes. In some cases tissue sections were permeabilized with 0.5% Triton X-100 in PBS for thirty minutes at room temperature. After permeabilization, the sample was washed three times, at five minutes per wash, in PBS. Sections were incubated in 10% blocking serum (goat or donkey derived depending on secondary antibody used) in PBS for sixty minutes at room temperature. Primary antibodies were diluted in 10% blocking serum (anti-Fibronectin; diluted 1:500, ab23750 Abcam, anti-CD31; diluted 1:20, AF3628, R&D Systems) and incubated either overnight at 4°C or for two hours at room temperature. After incubation, sections were gently washed three times, fifteen minutes per wash, in PBS. Secondary antibodies were then diluted in blocking serum (goat anti-rabbit Alexa Fluor 594, diluted 1:500, Life

Technologies, bovine anti-goat Dylight 549, diluted 1:300, Jackson Immunochemicals) and incubated on tissue sections for two hours at room temperature. Finally, tissue sections were washed three times, for fifteen minutes each, in PBS, mounted with 50% glycerol in PBS and imaged with a fluorescence microscope (Zeiss, Observer Z.1).

Hematoxylin staining

After fluorescent imaging, coverslips were removed and tissue sections were briefly washed in water, followed by staining with Mayer's modified hematoxylin (Poly Scientific) for 30 seconds to 1 minute. Sections were then briefly rinsed in water and incubated in bluing reagent (Thermo Scientific) until the tissue sections became visibly blue. Finally, tissue sections were rinsed water and mounted with 50% glycerol in PBS for imaging.

Transplantation Studies

NIHIII mice were anesthetized with a ketamine/xylazine mixture (135mg/kg ketamine and 15 mg/kg xylazine). A 3mm mid-diaphyseal bone segment was removed from the femur using a bone saw. A plastic triangular shaped pin was inserted within the intramedullary space to provide internal support, while an external fixator was attached to the outer periosteal bone surface of the bone with resorbable sutures (Vicryl 6-0, Ethicon; polyglactin; Owens & Minor). The critical size defect was loaded with a Healos scaffold (DuPuy Spine) containing a basement membrane extract (Cultrex) - BMSC mixture. The scaffold contained 1×10^6 cells derived from *CTGF*-EGFP mice that were cultured for 5 days and FACS isolated prior to transplantation. Finally, muscle and skin were sutured to their original positions. After 8 weeks, tissue was harvested, imaged by X-Ray (Faxitron, Bioptics), fixed, decalcified, embedded, and sectioned for histological imaging.

Microscopy and Imaging

Cells in culture and tissue sections were imaged using a Zeiss Observer Z.1 inverted microscope. Fluorescence was detected using the following filter sets (Chroma Technology): (ET500/20x, ET535/30m, T515LP for EGFP) (ET577/20x, ET640/40m, Q595lp for Tomato fluorescent protein, Alexa Fluor 594, and Dylight 549). Images were captured using an Axiocam MRc digital camera and Axiovision software (Zeiss).

Results

The *CTGF*-EGFP Reporter is Expressed in Bone Marrow Stromal Cells

Bone marrow stromal cultures prepared from 5–6 week old *CTGF*-EGFP reporter mice showed EGFP fluorescence in an adherent cell population displaying a fibroblast-like morphology during the first few days of the culture period (Fig.1A). FACS analyses indicated that the percentage of EGFP+ cells more than doubled from ~17% to ~45% between days 4 and 7 of culture (Fig.1B). Unfortunately, efforts to maintain viable FACS isolated EGFP+ cells obtained directly from the bone marrow in culture failed. However, early tracking of cultures from days 2 to 3 by time lapse photography suggested that the increase in EGFP+ cells was a result of existing EGFP+ cells undergoing expansion and not an EGFP– cell turning on the reporter (Fig.1C). At the same time, this result seemed to vary depending on the age and possibly the gender of the animal from which the stromal cultures

were derived. Time lapse photography of stromal cultures derived from 12 week old mice suggested that fewer EGFP positive bone marrow cells were initially present and increased not only from existing *CTGF* reporter labeled cells, but also from cells that did not initially express the reporter (data not shown).

To more carefully examine the decrease in *CTGF* reporter expressing cells with age, FACS analysis was used to quantify the percentage of EGFP+ cells within flushed bone marrow from 2, 5, and 11 week old male (n=3/time point) and female mice (n=3/time point). For these studies, bone marrow samples were individually harvested from femurs and tibias. FACS analyses showed that male and female mice had a considerable reduction in the number of EGFP+ cells with aging (Fig.1D). In males, the percentage of EGFP+ cells decreased ~9 fold in tibias and ~17 fold in femurs from 2 to 11 weeks of age. In females, the percentage of EGFP+ cells decreased ~22 fold in tibias and ~40 fold in femurs from 2 to 11 weeks of age. Male mice had roughly twice as many *CTGF* reporter expressing cells as female mice at every time point examined. Examination of *CTGF*-EGFP reporter expression in bone tissue sections with increasing age and in different genders qualitatively supported these FACS results (data not shown).

To further characterize bone marrow cell types retaining *CTGF* reporter expression, cell surface profiling using different mesenchymal, hematopoietic, and endothelial markers was carried out. Collectively, these profiling studies indicated that the bone marrow cell population retaining *CTGF* reporter expression was heterogeneous. Greater than 90% of CTGF+ were positive for CD44 and CD29. Surprisingly, almost 80% of CTGF+ cells expressed low levels of CD45. Roughly 40% of CTGF+ cells retained the mesenchymal marker CD105, the endothelial marker CD31, and expressed low levels of the myeloid marker CD11b. Also, 20% of CTGF+ cells or less were positive for Sca1, CD140b (PDGFR β), and CD90.2. Interpreting cell profiling data has become increasingly problematic as a variety of groups have reported on markers traditionally thought to identify hematopoietic cells that may also be expressed on bone marrow mesenchymal or even osteoblast cell types [40–42]. It is also conceivable that during the physical dissociation of cells, membrane fragments derived from cells in contact with, in our case CTGF+ cells, stay attached and are potentially misleading. Along these lines, it is noteworthy that while ~40% of CTGF+ cells are positive for CD31 by cell surface profiling during flow cytometry, immunostaining for CD31 in tissue sections indicated that CTGF+ cells are distinct from bone marrow endothelial cells, but are often found in close proximity to them (Fig.5).

CTGF is Expressed in Mesenchymal Progenitor Cells in Vitro

Past studies from our lab have demonstrated the utility of fate mapping strategies with Twist2-Cre mice and, more recently, Osterix-Cre mice to selectively identify mesenchymal progenitor cells *in vitro* [18, 43]. For these studies, Twist-Cre and Osterix-Cre mice were intercrossed with a Cre reporter mouse strain called Ai9, which turns on a tomato fluorescent protein upon Cre recombinase mediated excision of a stop sequence. Based on whether Twist2-Cre or Osterix-Cre mice were used, we have referred to the mesenchymal progenitor population as T2C+ or OC9+ cells, respectively. Immunostaining for CTGF was carried out on stromal cultures derived from Twist2-Cre/Ai9 (Fig. 2A–C) and Osterix-

Cre/Ai9 (Fig. 2E–G) dual transgenic mice to determine how well endogenous CTGF expression correlated with T2C+ and OC9+ cell populations. Cell counts revealed a broad overlap between endogenous CTGF expression and T2C+ and OC9+ cell populations. For the T2C+ cell population, 63.8% and 89.3% of cells retained endogenous CTGF at days 3 and 5 of culture, respectively. For the OC9+ cell population, 52.8% and 93.2% of cells retained endogenous CTGF at days 3 and 5 of culture, respectively. Based on our observations, we believe that the increased correlation from day 3 to 5 was the result of a combination of expansion of the double positive cell population (CTGF+/T2C+ or CTGF+/OC9+) and apoptosis of CTGF– fate marked populations that did not survive the transition into a cell culture environment.

To examine the overlap between cells marked by *CTGF* reporter expression and those marked by the Osterix-Cre/Ai9 approach, stromal cultures were derived from *CTGF*-EGFP/+; *Osterix*-Cre/+; Ai9 triple transgenic mice (Fig. 2G) and reporter expression was quantified by FACS analyses from day 5 cultures (Fig. 2H). OC9+ cells represented a larger cell population (~43%) compared to the cell population retaining *CTGF* reporter expression (~33%) with 67% of OC9+ cells retaining *CTGF* reporter expression. The majority of *CTGF* reporter expressing cells (~89%) were OC9+. While FACS analyses were consistent with our immunostaining data, we noted that endogenous CTGF showed a higher overlap with the OC9+ cell population compared to the *CTGF* reporter (93% versus 67%). Therefore, we compared *CTGF*-EGFP expression to endogenous CTGF at day 5 of culture (Fig. 2K–M). These studies revealed a considerable overlap between reporter expression and endogenous CTGF protein (73.3% double+, 23.8% CTGF+, 2.8% EGFP+). However, a disparity in the levels of reporter expression with the intensity of immunostaining was apparent and, based on cell counts, we estimate that reporter expression represented roughly three quarters of the total stromal cell population expressing endogenous CTGF. Therefore, our data suggest the *CTGF*-EGFP BAC reporter accurately represents a subpopulation of cells that naturally express endogenous CTGF; thus providing an explanation for the moderately lower correlation between OC9 cells and *CTGF* reporter expression.

Bone Marrow Derived CTGF+ Cells Displayed Mesenchymal Multipotency In vitro

To evaluate the multipotent properties of stromal cells marked by *CTGF* reporter expression, EGFP expressing cells were sorted from day 5 stromal cultures, replated, and differentiated along the osteoblast (Fig. 3, A–D), adipocyte (Fig. 3, E–H), and chondrocyte (Fig. 3, I–L) cell lineages. Differentiation was evaluated by visualizing cells under DIC optics (Fig. 3, B, F, J), carrying out whole mount staining on differentiated cultures (Fig. 3, C, G, K), and analyzing the expression of known lineage specific marker genes at progressive time points (Fig. 3D, H, L). During osteoblast differentiation, CTGF+ cells produced a robust mineralized matrix that could be viewed under DIC optics (Fig. 3B) and stained positively by von Kossa (Fig. 3C). Expression levels of the bone marker genes *Bone Sialoprotein (BSP)*, *Osteocalcin (Bglap1)*, and *Dentin Matrix Protein 1 (DMP1)* assayed at days 0, 6 and 10 all increased dramatically during osteoblast differentiation (Fig. 3D). Sorted CTGF+ cells grown under adipogenic conditions differentiated into lipid vesicle containing cells, which could be easily viewed under DIC optics (Fig. 3F) and also could be detected by Oil Red O staining (Fig. 3G). The expression levels for the adipocytic gene markers, *Fatty Acid Binding Protein 4*

(*FABP4*), *Adiponectin* (*Adipoq*) and *Perilipin* (*Plin*) were all substantially up-regulated during differentiation (Fig.3H). During chondrocyte differentiation, CTGF+ cells formed condensations over the 12 day culture period, with proteoglycans that stained positive by Alcian blue (Fig.3K). Expression levels of chondrocyte differentiation markers *Collagen type 2 alpha 1* (*Col2a1*), *Aggrecan* (*Acan*), and *Collagen type 10 alpha 1* (*Col10a1*) were all substantially up-regulated during differentiation (Fig.3L). Thus, the cell population marked by CTGF reporter expression displayed multipotent properties characteristic of a mesenchymal progenitor cell *in vitro*.

Bone Marrow Derived CTGF+ Cells Displayed Mesenchymal Multipotency upon Transplantation

The multipotent properties of CTGF reporter-labeled stromal cells were further examined by transplantation. To trace the progeny of these cells after transplantation, stromal cultures were derived from CTGF-EGFP/+; Osterix-Cre/+; Ai9 triple transgenic mice as shown in Fig.2(G,H). Activation of the Ai9 Cre reporter (red fluorescence) in the CTGF+ cell population allowed us to trace their progeny in host mice. Sorted CTGF-/OC9- and CTGF+/OC9+ cells were transplanted into a skeletal defect engineered into the skull parietal bone. Tissue was harvested eight weeks after transplantation to characterize the defect area. By X-ray, CTGF-/OC9- donor cells had a minor impact on the repair of bone tissue (negligible radio-opacity in wound; Fig.4A). In tissue sections, a fibrous tissue layer of cells was observed covering the area of the defect with occasional small bone ossicles being present (Fig.4C). In contrast, CTGF+/OC9+ cells resulted in the formation of bone tissue that entirely covered the defect area (strong radio-opacity in wound, Fig. 4B). In tissue sections, bone tissue enclosed a marrow compartment containing host derived hematopoietic cells and donor derived marrow adipocytes (Fig.4, D-H). Over the eight week period, CTGF-EGFP reporter expression did not persist in the donor cell population; however Ai9 Cre reporter expression (Fig.4, shown in red, E, G and white, F) persisted and allowed us to view the progeny of CTGF+ cells, which directly contributed to the repair of bone tissue. The Ai9 reporter labeled cells were broadly observed throughout the repair, being present in bone tissue and the marrow space (Fig.4E). At high magnification (Fig.2,F,G,H), the progeny of CTGF+ cells directly contributed to osteoblasts and osteocytes, stromal cells within the marrow compartment, and marrow adipocytes. Interestingly, imaging of Ai9 reporter expression in conjunction with DAPI staining indicated that newly formed bone tissue was a mixture of donor and host derived cells as evidenced by DAPI stained osteocytes embedded in bone tissue that did not express the Ai9 reporter.

The CTGF Reporter Marks A Cell Population Preferentially Located Around Bone Trabeculae

To develop a better *in vivo* understanding of the mesenchymal progenitor cells retaining CTGF reporter expression, we examined EGFP expression in a variety of bones, including vertebrae (not shown), tibia (not shown), and femur (Fig.5), at different ages. Within the bone marrow, the distribution of CTGF+ cells progressively varied with age. In 1 week old mice, EGFP+ cells were distributed throughout the marrow (not shown). However, gross examination of CTGF reporter expression at four weeks of age revealed a gradient of expression being highest just below the growth plate and decreasing towards the mid-

diaphysis (Fig.5A). Interestingly, CTGF+ cells favored areas around bone trabeculae in the primary spongiosa (Fig.5A,B,B'), secondary spongiosa (not shown) and femoral neck (Fig. 5G,G'). Surprisingly, CTGF reporter expression was not detected in the perichondrium (Fig. 5C,C', white arrows) or periosteum (Fig.5D,D', white arrows) where other populations of skeletal progenitors reside. Regions of marrow closer to trabecular bone did retain CTGF+ cells (Fig.5E,E'), but at the mid-diaphysis few CTGF+ cells were present (Fig.5F,F'). CTGF reporter expression was also observed in maturing chondrocytes and reporter expression was particularly strong in the femoral head (Fig.5H,H') and at the lateral ends of the growth plate (Fig.5C,C'). Finally, while the trabecular location of CTGF+ cells persisted in older animals, the frequency of cells retaining reporter expression decreased with age and gender (data not shown and Fig.1D). As a result, mice up to four weeks of age were used to further characterize the cell populations retaining CTGF reporter expression.

Among bone trabeculae, CTGF+ cells were present in proximity to bone surfaces and vascular sinusoids. To demonstrate the location of CTGF+ cells relative to both of these structures, immunostaining for fibronectin and CD31 was carried out (Fig. 6). Fibronectin is abundant in bone tissue and in the circulation, thus illuminating bone (b) and blood present within the sinusoids (s) of the marrow (Fig.6A–C). Some CTGF+ cells lined the vascular sinusoids (s) and touched bone surfaces (b), but many CTGF+ cells were not necessarily associated with these structures, located instead in regions between these two structures. Additionally, in contrast to cell surface profiling, immunostaining for CD31 (Fig.6D–F) showed that CTGF+ cells were distinct from endothelial cells, despite being located around the vascular sinusoids.

High CTGF Reporter Expression in Cells Around Bone Trabeculae Suggests the Labeling of an Osteoprogenitor

To more carefully scrutinize the cell types retaining CTGF reporter expression in and around trabecular bone, CTGF reporter mice were intercrossed with the previously characterized osteoblast reporter mouse lines, *Osterix*-Cherry (Fig.7) and *Collagen type I alpha 1* (*Col1a1*)-3.6 Cherry (data not shown) [38, 44]. Reporter expression in dual transgenic offspring was evaluated in hind limb tissues sections derived from two and three week old animals. At all ages observed, a consistent outcome was seen in which the level of CTGF reporter expression was inversely correlated with osteoblast maturation. Cells retaining high CTGF reporter expression often retained low *Osterix* reporter expression and were located in a cell layer overlying the mature osteoblast layer (Fig.7, A–C, and A'–C', white arrows). In contrast, mature osteoblasts retaining high *Osterix* reporter expression along the bone surface (white-dotted line) retained weak to no CTGF reporter expression. Also, most osteocytes had no CTGF reporter expression, but expressed high levels of the *Osterix* reporter (Fig.7D–F, encircled trabecular bone). Although with extreme rarity, one or two osteocytes in a long bone tissue section surprisingly did have CTGF reporter expression. Many of the CTGF+ cells located within the marrow spaces between bone trabeculae also retained low expression of the *Osterix* reporter (Fig.7, D–F, white arrows). Overall, the comparison of CTGF reporter expression to different osteoblast reporter mouse lines implicated the labeling of an osteoprogenitor cell population.

CTGF Reporter Expression Decreases during Osteoblast Maturation In Vitro

Comparison of *CTGF* reporter expression with *Osterix*-Cherry and *Col1a1*-3.6 Cherry mice in bone tissue sections indicated that *CTGF* expression decreases during maturation of the osteoblast lineage. To provide further evidence for the inverse correlation of *CTGF* reporter expression with osteoblast maturation, bone marrow stromal cultures derived from *CTGF*-EGFP/+;*Osterix*-Cherry/+ dual transgenic mice were differentiated under osteogenic conditions. Reporter expression was characterized during differentiation by imaging (Fig. 8A) and FACS analysis at days 4, 7, 14, and 21 of differentiation. Detection of *CTGF* and *Osterix* reporter expression at day 4 of differentiation revealed that a high percent of cells expressed both reporters (Fig.8A). This was confirmed by FACS analyses, which showed that ~15.7% of the total cell population retained both *CTGF* and *Osterix* reporter expression (Fig.8B, black bars), while 6.5% (Fig.8B, green bars) and 1.3% (Fig.8B, red bars) of the total cell population separately retained *CTGF* and *Osterix* expression, respectively. Visualization of cultures at day 7 revealed that the number of double positive cells substantially increased, representing 40.7% of the total cell population, whereas single expressing CTGF+ or Osterix+ cells only represented 0.76% and 4.9% of the total cell population, respectively. However, continued differentiation resulted in significant change in cell populations. Visual assessment of cultures showed that as *Osterix* expression started to robustly increase, *CTGF* reporter expression concomitantly decreased. This was also noted by FACS analyses where the percentage of double positive cells (Fig.8B, black bars) dropped from 40.7% to 26.4%, while the percentage of Osterix+ cells increased from 4.9% to 46.5% (Fig.8B, red bars). By day 21 of differentiation, the absence of *CTGF* reporter expression in mature osteoblasts was even more apparent, with nodules retaining high levels of *Osterix* reporter expression and lacking *CTGF* reporter expression, even though *CTGF* reporter expression could be detected in cells adjacent to the developing nodule. FACS analysis at day 21 showed that the number of double positive cells further decreased to 10.6% of the total cell population, whereas the number of Osterix+ cells increased to 70.2% of the total cell population. Thus, similar to what was observed in bone tissue sections, levels of *CTGF* reporter expression were inversely correlated with osteoblast differentiation.

Discussion

The understanding of bone marrow mesenchymal progenitor cells in vivo remains limited, largely in part from the lack of gene markers and reagents that will enable their discrimination from other cell types. In this study, we have investigated a bone marrow mesenchymal progenitor population largely restricted to the trabecular bone region identified by *CTGF*-EGFP reporter expression. The selective expression of the *CTGF* reporter in these cells potentially provides an important tool to further understand their physiological role. Below we discuss the implications of this cell population within the broader context of mesenchymal progenitors located within the bone marrow.

CTGF Reporter Expression Labels a Bone Marrow Mesenchymal Progenitor

The functional assessment of CTGF+ bone marrow derived cells demonstrated their capacity to perform as mesenchymal progenitors. CTGF+ cells were present in the bone marrow flush and proliferated as part of the fibroblast-like stromal fraction. Roughly 89% of *CTGF*-

EGFP cells were *Osterix-Cre/Ai9* positive *in vitro* (Fig.2H) and in bone tissue sections (data not shown). Past studies by us and others have shown that Osterix-Cre fate mapping labels long lived marrow mesenchymal progenitors [18, 45]. Sorted CTGF+ cells differentiated *in vitro* into osteoblasts, chondrocytes, and adipocytes (Fig.3). Additionally, sorted and transplanted CTGF+ cells differentiated into osteoblasts, osteocytes and marrow adipocytes and populated the marrow compartment as stromal cells (Fig.4). This collective set of data provides evidence that within the CTGF+ cell population exists cells with mesenchymal potential. In this regard, it may be tempting to consider CTGF as a mesenchymal stem cell gene marker as has been previously suggested [32]. However, genetic and biochemical studies that have investigated the functionality of *CTGF* do not provide any support for the possibility that it contributes to the “stemness” of bone marrow skeletal progenitors [26, 27, 34, 36].

Despite our evidence for the mesenchymal properties of CTGF+ cells, cell surface profiling directly from the bone marrow has suggested that this cell population is very heterogeneous and possibly a mixture of mesenchymal, hematopoietic, and endothelial cell types or at least a cell population that collectively expresses cell surface markers normally associated with these distinct lineages (Fig.1E). Less than 20% of CTGF+ cells were positive for Sca1, CD90.2, and PDGFR β , while surprisingly 80% of CTGF+ cells were positive for the pan-hematopoietic marker CD45. With this in mind, review of the literature reveals numerous contradictions with the *in vivo* characterization of bone marrow mesenchymal cell types. For example, while Sca-1 has been considered one of the more favored marrow mesenchymal markers [46], the recent characterization of Leptin Receptor+ perivascular cells showed that only ~12% were positive for Sca1 [47]. Also, while most studies deplete the CD45 cell fraction because it largely consists of hematopoietic cells, lineage tracing using *Smooth Muscle Alpha Actin (Sma)*-CreERT2 mice revealed that ~80% of the mesenchymal progenitors labeled in this approach were positive for CD45 [41]. Thus, the cell surface profiling that we have carried out on CTGF+ cells, in itself, is not very informative. Future studies need to be undertaken that couple immunophenotyping with the functional assessment of different subpopulations to determine whether the heterogeneity that exists actually has biological significance.

The Location of CTGF Cells Around Trabecular Bone Implicates a Role as an Osteoprogenitor

The localization and morphology of CTGF+ cells in bone tissue sections was indicative of an osteoprogenitor progenitor. CTGF+ cells were adjacent to, but distinct from mature osteoblasts lining bone surfaces and endothelial cells forming the vascular sinuses (Fig.6,7). While the perivascular stromal localization of CTGF+ cells was consistent with an increasing body of work suggesting that perivascular stromal/reticular cells may be the source of BMSCs [11, 15, 16], the localization of CTGF+ cells was not limited to vascular structures, being also found adjacent to osteoblasts and in the marrow spaces between bone tissue and sinusoids. Past studies have also identified *Nestin*-GFP, *Cxcl12*-GFP, and SCF-GFP reporter mice as animal models that mark BMSCs [11, 16, 47]. However, in contrast to *CTGF* reporter mice, cells labeled by these reporter mice are broadly distributed throughout the bone marrow compartment and are not preferentially localized near bone surfaces [11,

16, 48]. Thus, their location is indicative of their *in vivo* role in supporting hematopoiesis [16, 48–51]. However, similar to *CTGF*⁺ cells, the harvesting and testing of *Nestin*⁺, *Cxcl12*⁺, *SCF*⁺ cells reveals their skeletal potential [11, 16, 47]. Interestingly, the lineage tracing of embryonic skeletal precursors using *Osterix* Cre animal models has revealed their broad contribution to a wide range of cell types within the bone marrow stroma, including Leptin receptor⁺ perivascular cells that are long lived [17, 18]. Therefore, a likely explanation for why perivascular cells located in divergent regions of the bone marrow retain skeletal potential has to do with their common origin with osteoblasts from an embryonic skeletal precursor. However, in spite of the skeletal precursor origin of bone marrow perivascular cells, ultimately their location within the bone marrow is likely to be a determining factor in their *in vivo* role. Recently, we generated *CTGF*-CreERT2 founder mice and hope to determine what cell lineages, if any, *CTGF*⁺ mesenchymal cells naturally contribute to within the bone marrow.

Age and Gender Differences in *CTGF* Reporter Expression

One of the interesting findings from this study relates to the gradual decrease in *CTGF*⁺ cells with age and gender. Certainly, when it comes to maintaining bone mass, age and gender differences are well recognized. Moreover, past work has suggested that differences in bone loss between genders or during aging can be correlated with the abundance and senescence of skeletal progenitors [20, 52, 53]. Associated with our data, *CTGF* conditional loss of function studies have reported variable impacts on bone volume associated with animal age and gender. Mice heterozygous for *CTGF* or *Prx1*-Cre conditional loss of *CTGF* resulted in decreased trabecular bone volume at 1 month of age, but showed no differences in 4 month old animals [26]. Additionally, a certain degree of sexual dimorphism was also observed in *CTGF* conditional knock out studies where male mice showed differences in bone volume whereas female mice did not [26]. It is conceivable that the greater abundance of *CTGF* expressing cells in young mice and male mice contributes to increased bone volume, which upon loss of function is revealed.

Does The Trabecular Bone Region Contain A Novel Population of Mesenchymal Progenitors?

With regard to long bones, mesenchymal progenitors exist in a variety of locations, including the perichondrium, periosteum, endocortical surfaces, and possibly lining the perivascularity throughout the marrow. However, the cells labeled by *CTGF* reporter expression were rarely observed in these regions. In contrast, our studies showed that for a period of postnatal growth there was an abundance of *CTGF*⁺ cells in the trabecular bone region. Based on this region-specific reporter expression, it may seem appealing to considering *CTGF*⁺ mesenchymal cells as a novel cell population. However, we believe the selective labeling of mesenchymal progenitors in this region by the *CTGF* reporter reflects environmental differences that exist in the trabecular bone region compared to cortical bone. Trabecular bone, in contrast to cortical bone, has a critical role in mineral homeostasis, which requires much greater rates of bone turnover, particularly during adolescence when rapid bone growth occurs. Trabecular bone is also positioned directly beneath the growth plate where it is conceivable that signals derived from the above growth plate may directly or indirectly influence *CTGF* reporter expression. With this in mind, it is worth noting that

the level of *CTGF* reporter expression and the abundance of cells expressing the *CTGF* reporter was highest below the growth plate and decreased as the distance from the growth plate increased. However, trabecular bone volume also decreases as the distance from the growth plate increases and the presence of *CTGF*⁺ cells was highly correlated with the presence of trabecular bone. We suspect a possible mechanism of *CTGF* gene regulation may be via bone resorption. There is strong evidence that bone resorption leads to the release of active TGF β 1, which is thought to be important for the recruitment of mesenchymal progenitor cells to the bone surface [54]. This model fits well with our studies in that we have provided evidence that *CTGF* is expressed in a mesenchymal progenitor in proximity to trabecular bone surfaces and *CTGF* is a known target gene of TGF β signaling. Future studies will investigate the possible link between bone resorption, TGF β , and *CTGF* expression.

Acknowledgments

We would like to thank Mark Kronenberg for carefully reviewing this manuscript and David Rowe for generously sharing his equipment. This work was supported by a grant from NIAMS 1R21 AR060899-01A1 and training grant T90DE021989-01 from the NIDCR.

References

1. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet.* 1970; 3(4):393–403. [PubMed: 5523063]
2. Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet.* 1987; 20(3):263–72. [PubMed: 3690622]
3. Friedenstein AJ, et al. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation.* 1968; 6(2):230–47. [PubMed: 5654088]
4. Friedenstein AJ, Piatetzky S II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol.* 1966; 16(3):381–90. [PubMed: 5336210]
5. Bab I, et al. Kinetics and differentiation of marrow stromal cells in diffusion chambers in vivo. *J Cell Sci.* 1986; 84:139–51. [PubMed: 3805151]
6. Baddoo M, et al. Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem.* 2003; 89(6):1235–49. [PubMed: 12898521]
7. Dexter TM, et al. The regulation of hemopoietic cell development by the stromal cell environment and diffusible regulatory molecules. *Prog Clin Biol Res.* 1984; 148:13–33. [PubMed: 6379657]
8. Gronthos S, et al. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood.* 1994; 84(12):4164–73. [PubMed: 7994030]
9. Gronthos S, et al. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci.* 2003; 116(Pt 9):1827–35. [PubMed: 12665563]
10. Mardon HJ, et al. Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. *Cell Tissue Res.* 1987; 250(1):157–65. [PubMed: 3652158]
11. Omatsu Y, et al. The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity.* 2010; 33(3):387–99. [PubMed: 20850355]
12. Phinney DG, et al. Biological activities encoded by the murine mesenchymal stem cell transcriptome provide a basis for their developmental potential and broad therapeutic efficacy. *Stem Cells.* 2006; 24(1):186–98. [PubMed: 16100003]
13. Phinney DG, et al. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J Cell Biochem.* 1999; 72(4):570–85. [PubMed: 10022616]

14. Pittenger MF, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999; 284(5411):143–7. [PubMed: 10102814]
15. Sacchetti B, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell*. 2007; 131(2):324–36. [PubMed: 17956733]
16. Mendez-Ferrer S, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. 2010; 466(7308):829–34. [PubMed: 20703299]
17. Maes C, et al. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev Cell*. 2010; 19(2):329–44. [PubMed: 20708594]
18. Liu Y, et al. Osterix-cre labeled progenitor cells contribute to the formation and maintenance of the bone marrow stroma. *PLoS One*. 2013; 8(8):e71318. [PubMed: 23951132]
19. Short BJ, Brouard N, Simmons PJ. Prospective isolation of mesenchymal stem cells from mouse compact bone. *Methods Mol Biol*. 2009; 482:259–68. [PubMed: 19089361]
20. Siclari VA, et al. Mesenchymal progenitors residing close to the bone surface are functionally distinct from those in the central bone marrow. *Bone*. 2013; 53(2):575–86. [PubMed: 23274348]
21. Jun JI, Lau LF. Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nat Rev Drug Discov*. 2011; 10(12):945–63. [PubMed: 22129992]
22. Lipson KE, et al. CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis Tissue Repair*. 2012; 5(Suppl 1):S24. [PubMed: 23259531]
23. Hall-Glenn F, et al. CCN2/connective tissue growth factor is essential for pericyte adhesion and endothelial basement membrane formation during angiogenesis. *PLoS One*. 2012; 7(2):e30562. [PubMed: 22363445]
24. Ivkovic S, et al. Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development*. 2003; 130(12):2779–91. [PubMed: 12736220]
25. Kawaki H, et al. Functional requirement of CCN2 for intramembranous bone formation in embryonic mice. *Biochem Biophys Res Commun*. 2008; 366(2):450–6. [PubMed: 18067859]
26. Canalis E, et al. Connective tissue growth factor is required for skeletal development and postnatal skeletal homeostasis in male mice. *Endocrinology*. 2010; 151(8):3490–501. [PubMed: 20534727]
27. Smerdel-Ramoya A, et al. Skeletal overexpression of connective tissue growth factor impairs bone formation and causes osteopenia. *Endocrinology*. 2008; 149(9):4374–81. [PubMed: 18535099]
28. Nakanishi T, et al. Overexpression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 decreases bone density in adult mice and induces dwarfism. *Biochem Biophys Res Commun*. 2001; 281(3):678–81. [PubMed: 11237711]
29. Ren J, et al. Global transcriptome analysis of human bone marrow stromal cells (BMSC) reveals proliferative, mobile and interactive cells that produce abundant extracellular matrix proteins, some of which may affect BMSC potency. *Cytherapy*. 2011; 13(6):661–74. [PubMed: 21250865]
30. Igarashi A, et al. Selection of common markers for bone marrow stromal cells from various bones using real-time RT-PCR: effects of passage number and donor age. *Tissue Eng*. 2007; 13(10):2405–17. [PubMed: 17596118]
31. Schutze N, et al. Differential expression of CCN-family members in primary human bone marrow-derived mesenchymal stem cells during osteogenic, chondrogenic and adipogenic differentiation. *Cell Commun Signal*. 2005; 3(1):5. [PubMed: 15773998]
32. Luo Q, et al. Connective tissue growth factor (CTGF) is regulated by Wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells. *J Biol Chem*. 2004; 279(53):55958–68. [PubMed: 15496414]
33. Battula VL, et al. Connective tissue growth factor regulates adipocyte differentiation of mesenchymal stromal cells and facilitates leukemia bone marrow engraftment. *Blood*. 2013; 122(3):357–66. [PubMed: 23741006]
34. Lee CH, et al. CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model. *J Clin Invest*. 2010; 120(9):3340–9. [PubMed: 20679726]

35. Tan JT, et al. Connective tissue growth factor inhibits adipocyte differentiation. *Am J Physiol Cell Physiol.* 2008; 295(3):C740–51. [PubMed: 18596209]
36. Smerdel-Ramoya A, et al. Connective tissue growth factor enhances osteoblastogenesis in vitro. *J Biol Chem.* 2008; 283(33):22690–9. [PubMed: 18583340]
37. Ryseck RP, et al. Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. *Cell Growth Differ.* 1991; 2(5):225–33. [PubMed: 1888698]
38. Strecker S, et al. Generation and characterization of Osterix-Cherry reporter mice. *Genesis.* 2013; 51(4):246–58. [PubMed: 23180553]
39. Jiang X, et al. Histological analysis of GFP expression in murine bone. *J Histochem Cytochem.* 2005; 53(5):593–602. [PubMed: 15872052]
40. Ohishi M, et al. A novel population of cells expressing both hematopoietic and mesenchymal markers is present in the normal adult bone marrow and is augmented in a murine model of marrow fibrosis. *Am J Pathol.* 2012; 180(2):811–8. [PubMed: 22155108]
41. Grcevic D, et al. In vivo fate mapping identifies mesenchymal progenitor cells. *Stem Cells.* 2012; 30(2):187–96. [PubMed: 22083974]
42. Jones EA, et al. Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow. *Cytometry B Clin Cytom.* 2006; 70(6):391–9. [PubMed: 16977637]
43. Liu Y, et al. Isolation of murine bone marrow derived mesenchymal stem cells using Twist2 Cre transgenic mice. *Bone.* 2010; 47(5):916–25. [PubMed: 20673822]
44. Kalajzic I, et al. Use of type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. *J Bone Miner Res.* 2002; 17(1):15–25. [PubMed: 11771662]
45. Mizoguchi T, et al. Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev Cell.* 2014; 29(3):340–9. [PubMed: 24823377]
46. Jurecic R, Van NT, Belmont JW. Enrichment and functional characterization of Sca-1+WGA+, Lin-WGA+, Lin-Sca-1+, and Lin-Sca-1+WGA+ bone marrow cells from mice with an Ly-6a haplotype. *Blood.* 1993; 82(9):2673–83. [PubMed: 8219220]
47. Zhou BO, et al. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell.* 2014; 15(2):154–68. [PubMed: 24953181]
48. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature.* 2013; 495(7440):231–5. [PubMed: 23434755]
49. Pinho S, et al. PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med.* 2013; 210(7):1351–67. [PubMed: 23776077]
50. Greenbaum A, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature.* 2013; 495(7440):227–30. [PubMed: 23434756]
51. Tokoyoda K, et al. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity.* 2004; 20(6):707–18. [PubMed: 15189736]
52. Valyushina MP, Buravkova LB. Age-related differences in rat multipotent mesenchymal stromal bone marrow cells. *Bull Exp Biol Med.* 2013; 155(1):129–33. [PubMed: 23667890]
53. Zaim M, et al. Donor age and long-term culture affect differentiation and proliferation of human bone marrow mesenchymal stem cells. *Ann Hematol.* 2012; 91(8):1175–86. [PubMed: 22395436]
54. Tang Y, et al. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nat Med.* 2009; 15(7):757–65. [PubMed: 19584867]

Figure 1. The CTGF-EGFP Reporter is Expressed in Bone Marrow Mesenchymal Progenitor Cells

(A) Fibroblast-like bone marrow derived stromal cells retained *CTGF*-EGFP reporter expression at day 4 of culture. (B) FACS analyses of bone marrow derived stromal cultures derived from *CTGF*-EGFP reporter mice. The percentage of CTGF⁺ cells increased from 16.8% to 44.5% from days 4 to 7 of culture. (C) Time lapse imaging of CTGF⁺ cells starting at day 2 of culture showing CTGF⁺ cells undergoing cell division over an 18 hour period. (D) FACS analyses of bone marrow flushed CTGF⁺ cells at 2, 5, and 11 weeks of age. The percentage of CTGF⁺ cells decreased during aging and was roughly half the amount in female mice compared to male mice. (E) Cell surface profiling of CTGF⁺ obtained directly from the bone marrow of 4 week old *CTGF*-EGFP reporter mice. Note: CD45_L and CD11b_L represent low expression of these cell surface markers relative to expression levels detected in hematopoietic cells.

Figure 2. CTGF is Expressed in Bone Marrow Mesenchymal Progenitor Cells

Detection of *CTGF* expression in T2C+ (A–C) and OC9+ (D–F) mesenchymal progenitor cells. Immunostaining for CTGF (A,D) overlaps with T2C+ (B) and OC9+ (E) cell populations. (B) T2C+ mesenchymal cells were labeled by intercrosses of Twist2-Cre with Ai9 Cre reporter mice. (E) OC9+ mesenchymal cells were labeled by intercrosses of Osterix-Cre with Ai9 Cre reporter mice. (G,H) *CTGF* reporter expression labeled a subpopulation of the OC9+ cell population. (H) FACS analyses of stromal cultures derived from triple transgenic mice generated as shown in (G). (I–K) Comparison of CTGF protein expression (I) to *CTGF-EGFP* reporter expression (J). Image overlay (K) showed a high correlation between immunostaining and reporter expression, but the level of reporter expression did not always correlate with protein levels.

Figure 3. Sorted CTGF⁺ Cells Display Mesenchymal Progenitor Potential In Vitro

CTGF⁺ cells sorted from day 5 stromal cultures were replated and differentiated into osteoblasts (A–D), adipocytes (E–H), and chondrocytes (I–L). (A,E,I) Detection of CTGF reporter expression. (B,F,J) DIC images of culture showing markedly different cell morphologies. (C,G,K) Histological staining for bone mineral by von Kossa (C), adipocytes with Oil Red O (G), and chondrocytes with Alcian blue (K). Gene expression analyses was also carried out for known gene markers of differentiation including *Bone Sialoprotein* (BSP), *Osteocalcin* (Bglap1), and *Dentin Matrix Protein 1* (DMP1) for osteoblast formation (D), *Fatty Acid Binding Protein 4* (FABP4), *Adiponectin* (Adipoq) and *Perilipin* (Plin) for adipocyte formation (H), and *Collagen type II alpha 1* (Col2a1), *Aggrecan* (Acan), and *Collagen type 10 alpha 1* (Col10a1) for chondrocyte formation (L). Note: For most genes the fold difference in gene expression after differentiation is 100x to 1000X higher than detected in day 5 sorted CTGF⁺ cells.

Figure 4. Sorted CTGF+ Cells Display Mesenchymal Progenitor Potential after Transplantation
 Day 5 stromal cultures were sorted for CTGF⁻;OC9⁻ and CTGF⁺;OC9⁺ cell fractions, which were subsequently loaded into a Healos scaffold and transplanted into a parietal bone defect. The OC9 label allowed for tracing of CTGF⁺ cellular progeny. Assessment of repair 8 weeks after transplantation showed that CTGF⁻;OC9⁻ cells produced minimal bone tissue based on gross examination of the defect site by X-ray (A) and in tissue sections, which largely revealed fibrous tissue covering the defect area (C). In contrast, the CTGF⁺;OC9⁺ cells resulted in bone formation that bridged the defect (B) and retained a large marrow compartment (D). During the 8 week period, *CTGF* reporter expression declined to undetectable levels in donor cells, but the cellular progeny of CTGF⁺ cells, based on OC9 labelling, revealed the persistence of progeny throughout the defect area (E, OC9; red fluorescence, DAPI; blue). (F–H) At higher magnification, the progeny of CTGF⁺ cells were observed to become osteoblasts on the bone surface, osteocytes embedded in bone, marrow adipocytes, perivascular cells lining sinusoids, in addition to stromal cells throughout the marrow space. (abbreviations: b=bone, s=sinusoid, a=adipocyte). Note: DAPI staining also revealed significant host contribution to bone formation. (F) OC9 fluorescence shown in white. (G) OC9 fluorescence shown in red with DAPI staining in blue. (H) Corresponding region stained by hematoxylin.

Figure 5. *CTGF*-EGFP Reporter Expression Labels a Cell Population in the Trabecular Bone Region

(A) *CTGF*-EGFP Reporter Expression in a four week old female femur bone. (B) EGFP expression in the trabecular bone region with corresponding hematoxylin counterstain (B'). EGFP is not detected in the perichondrium (C,C', **white arrow**) or periosteum (D,D', **white arrow**). Some perivascular cells have *CTGF* reporter expression below the trabecular bone region (E,E'). However, the frequency of EGFP+ cells decreases inside the marrow as you move toward the mid-diaphysis (F,F'). *CTGF* reporter expression is expressed in cells around trabecular bone located in the femoral neck (G,G'). *CTGF* reporter expression is also strongly detected in maturing chondrocytes in the femoral head.

Figure 6. CTGF-EGFP+ Cells Are Near the Trabecular Bone Surface and Line Marrow Sinuoids

(A–F) *CTGF* reporter expression (B, E) was present in cells in proximity to trabecular bone (b) and adjacent to sinusoids (s) in between trabecular bone. Bone tissue and blood within the sinusoids could be distinguished from *CTGF* reporter expression by immunostaining for fibronectin (compare A to B, merge C). Additionally, *CTGF*+ cells were distinct from CD31+ endothelial cells that identified marrow sinusoidal structures (compare D to E; merge F).

Figure 7. High *CTGF* Reporter Expression in Cells Near Trabecular Bone Implicates the Labelling of an Osteoprogenitor

CTGF-EGFP reporter expression (**A, A',D; green**) was compared to *Osterix*-Cherry reporter expression (**B,B',E; red**) in the trabecular bone region. Near trabecular bone surfaces (**A–C; A'–C'**) *CTGF* reporter expression was highest in a mesenchymal cell layer that was at least two cell layers away from the bone surface. Many *CTGF*+ cells retained low *Osterix* reporter expression (white arrows). Larger *Osterix* expressing osteoblasts on the bone surface (dotted white line) retained much lower to undetectable levels of *CTGF* reporter expression. *CTGF* reporter expression was rarely if ever observed in osteocytes. (**D–F**) *CTGF*+ cells were also present in the marrow spaces in between bone trabeculae, many of which also retained weak *Osterix* reporter expression. (b=bone, m=marrow)

Figure 8. CTGF Reporter Expression Decreases as Osteoprogenitor Cells Mature

Bone marrow stromal cultures derived from *CTGF*-EGFP;*Osterix*-Cherry mice were differentiated under osteogenic conditions. **(A)** Representative images taken at days 4, 7, 14, and 21 of differentiation for *CTGF* reporter expression (green, top panel) and *Osterix* reporter expression (red, middle panel). **(B)** Graph quantifying the percentage of *CTGF*+ cells (green bars), *Osterix*+ cells (red bars), and cells expressing both reporter (double+, black bars) at days 4, 7, 14, and 21. Cell percentages were determined by FACS analyses. **(A,B)** At days 4 and 7 of differentiation, *CTGF* and *Osterix* reporters are expressed in many of the same cell types. This is apparent by viewings cells in cultures **(A)** and by FACS analyses **(B)**. However, by day 14 and 21 the percentage of cells retaining *Osterix* expression alone markedly increases, while the percentage of cells positive for both *CTGF* and *Osterix* reporters markedly decreases. This is apparent in culture **(A)** where brighter *Osterix*+ cells retain no *CTGF* reporter expression and by FACS analyses **(B)**.