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Connective Tissue Growth Factor reporter mice label a subpopulation of mesenchymal progenitor cells that reside in the trabecular bone region

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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 had 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 that 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.

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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

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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 a broader zone 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 skeletal 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*-EGFPCre (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), and 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 αMEM culture medium containing 100 U/ml penicillin, 100 μg/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-μm strainer. Cells were centrifuged at 350 *g* for 10 min and plated at a density 1.2×10^6 cells/cm^2. 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, which had been cultured for at least five days 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 min at 37 °C, scraped, then digested for an additional 5 min at 37 °C. Cells were harvested, run through a 40 μm cell strainer, and centrifuged at 350 *g* 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 min 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 min. 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 were carried out on a Becton–Dickinson LSRII and FACS sorting was carried out using a FacsAria II (UCHC Flow Cytometry Core).

In vitro differentiation of BMSCs

FACS sorted EGFP + and EGFP-cells were plated as confluent spots (~2×10^4 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 to attach for ~2 h followed by the addition of medium (αMEM, 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 αMEM 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 αMEM 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-β3.

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 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, and 72 °C–30 s for 40 cycles. The specificity of amplification was

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