


# Telomerase expression marks transitional growth-associated skeletal progenitor/stem cells

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## Abstract

Skeletal progenitor/stem cells (SSCs) play a critical role in postnatal bone growth and maintenance. Telomerase (*Tert*) activity prevents cellular senescence and is required for maintenance of stem cells in self-renewing tissues. Here we investigated the role of *mTert*-expressing cells in postnatal mouse long bone and found that *mTert* expression is enriched at the time of adolescent bone growth. *mTert*-GFP<sup>+</sup> cells were identified in regions known to house SSCs, including the metaphyseal stroma, growth plate, and the bone marrow. We also show that *mTert*-expressing cells are a distinct SSC population with enriched colony-forming capacity and contribute to multiple mesenchymal lineages, in vitro. In contrast, in vivo lineage-tracing studies identified *mTert*<sup>+</sup> cells as osteochondral progenitors and contribute to the bone-forming cell pool during endochondral bone growth with a subset persisting into adulthood. Taken together, our results show that *mTert* expression is temporally regulated and marks SSCs during a discrete phase of transitional growth between rapid bone growth and maintenance.

## KEYWORDS

*mTert*, pluripotent, postnatal bone growth, skeletal stem cells, telomerase

## 1 | INTRODUCTION

Postnatal bone formation relies on skeletal progenitor/stem cells (SSCs).<sup>1-3</sup> These cells also play a key role in maintaining the hematopoietic stem cell (HSC) niche.<sup>4-6</sup> Predominantly found in the metaphyseal stroma adjacent to the vasculature,<sup>5,7</sup> SSC populations have also been identified in nonvascular stromal regions as well as in the bone marrow (BM) near/adjacent to bone.<sup>8</sup> In addition, bone

lining cells have been implicated as a major contributor to the osteoblast lineage in response to damage.<sup>9</sup> Furthermore, progenitor/stem cells are present within the periosteum and function in fracture repair. Finally, chondrogenic cells from both the growth plate and the perichondrium contribute to bone formation during rapid bone growth and in response to injury.<sup>10-14</sup>

SSCs were originally defined by their ability (a) to self-renew, using an in vitro colony-forming unit-fibroblast (CFU-F) assay, (b) to

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differentiate into osteoblasts, chondrocytes and adipocytes, the three major cellular components of skeletal tissue, and (c) to form bone and bone marrow following transplantation.<sup>15,16</sup> While these techniques, combined with cell surface marker expression,<sup>17,18</sup> were originally considered the gold standard for defining SSCs, the advent of lineage-tracing has allowed for the identification and functional validation of progenitor/stem cells under physiological conditions, *in vivo*.

Recent lineage-tracing data indicate that SSC populations are temporally and spatially regulated with distinct populations functioning during bone growth and maintenance.<sup>7,13,17,19</sup> In addition, growth-associated SSCs have been suggested as the predecessors to adult SSCs, implying that the SSC population is highly complex, and that a hierarchy<sup>7,13</sup> may exist among the various progenitor/stem cell populations.<sup>7,19</sup> Although there is strong interest in identifying stem cells within the skeleton, and much has been done to understand these cells *in vitro*, these findings are tempered by the fact that the lineage potential of cells *in vitro* can differ from their capacity *in vivo*.<sup>20</sup> Going forward it will be imperative that markers, and the cell populations they identify, are validated *in vivo* before being accepted as a SSC population.

Telomerase (*Tert*) activity prevents cellular senescence and is required for maintenance of stem cells in self-renewing tissues.<sup>21-23</sup> Previously, we showed that *mTert* expression marks embryonic stem (ES) cells, inducible pluripotent stem (iPS) cells, and self-renewing tissue stem cells.<sup>24-26</sup> Prior studies also have shown that telomerase is necessary for SSC self-renewal and differentiation<sup>27</sup> and that a decline in telomerase activity in humans correlates with a decrease in bone homeostasis resulting in osteoporosis.<sup>28</sup> In addition, ectopic expression of telomerase results in increased proliferation, enhanced osteoblast differentiation, and bone formation.<sup>29,30</sup> While these studies indicate that telomerase is important for SSC function and bone homeostasis, it remains unclear whether telomerase expression marks skeletal stem cells.

Here we report that *mTert* is expressed in an age-dependent manner and marks growth-associated osteochondral progenitor cells within the long bone during a discrete time period between rapid bone growth and bone maintenance.

## 2 | MATERIALS AND METHODS

### 2.1 | Mice

*mTert-rtTA* mice were generated using ES cell transgenesis by targeting a single copy of the M2rtTA reverse tetracycline transactivator driven by a 4.4 kb of the *mTert* promoter<sup>25,26,31</sup> to a flippase recombination target (FRT) site targeted into safe-haven chromatin downstream of the *Col1a1* locus.<sup>32</sup> Correctly targeted ES clones were further analyzed using an *otet-GFP* reporter construct to demonstrate reversible doxycycline-inducible activity. A single clone was then used to generate the *mTert-rtTA* mouse line used in this study. For lineage-tracing studies, these mice were crossed with *otet-Cre*<sup>33</sup> and *R26R<sup>fllox</sup>(mT/mG)*<sup>34</sup> mice to generate *mTert-rtTA::otet-Cre::R26R<sup>fllox</sup>(mT/mG)* trigenic mice. To induce recombination, doxycycline (Sigma) was

### Significance statement

A unique population of skeletal stem cells, marked by *mTert* expression and that function during the transitional period between rapid bone growth and bone maintenance, were identified. Given the temporal regulation of this cell population, this presents the opportunity to determine whether alterations in its pattern affect bone formation and to understand the physiological factors that regulate its timing. This is particularly important given that impairment in optimal bone mass accrual during childhood and adolescence has long-lasting effects on bone health, including the development of osteoporosis in the adult.

administered either in a single injection (15 µg/g body weight) or in the drinking water (2 mg/mL in 10 mg/mL sucrose water) for up to 5 or 7 days as indicated. Mice were gamma irradiated (7 Gy) to induce bone marrow adipogenesis. *mTert-GFP*<sup>25</sup> and wild-type mice (Jackson Laboratory) were also used in this study. All procedures were approved by the BCH IACUC.

### 2.2 | Isolation and differentiation of marrow stromal cells

Long bones were collected and the BM was flushed by centrifugation to obtain marrow stromal cells.<sup>35</sup> Following red blood cell (RBC) lysis, 10<sup>5</sup> to 10<sup>6</sup> cells were plated in MesenCult MSC Basal Medium supplemented with MesenCult Mesenchymal Stem Cell Stimulatory Supplement (STEMCELL Technologies), glutamine and antibiotics. Adherent cells were analyzed after 24 hours and colonies were visualized after 2 weeks. To promote differentiation, cells were cultured to confluence and then the media was supplemented with either 50 µg/mL ascorbic acid (Sigma), 7 mM β-glycerophosphate (Sigma), 10<sup>-8</sup> dexamethasone (Sigma) for osteoblastogenesis, 20 ng/mL TGF-β3 (PeproTech), 200 mM ascorbic acid for chondrogenesis or Adipogenic Stimulatory Supplement (Stem Cell Technologies) for adipogenesis. Differentiation media was changed twice a week and cells were cultured up to 4 weeks. Immunofluorescence was performed using rabbit perilipin antibody (Sigma, 5 µg/mL), goat collagen II antibody (Santa Cruz, 1:250) or rabbit osterix antibody (Abcam, 1:600) and Alexa Fluor 350, 633 or 647 secondary antibodies (Molecular Probes, 1:400) and imaged using an inverted Eclipse Ti (Nikon) or LSM 700 laser scanning confocal (Zeiss) microscope. Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) (Sigma, 1:1000).

### 2.3 | Flow cytometry

BM cells were isolated from *mTert-GFP* mice (n = 3). Hematopoietic cells and dead cells were excluded by staining with anti-CD45-APC

(BD-Pharmingen, 1:100) and propidium iodide (eBioscience, 1:500) as previously described.<sup>25</sup> Cells were analyzed by flow cytometry using either a MoFlo cytometer (Dako) or BD FACSAria.

## 2.4 | Immunofluorescence

Isolated long bones were fixed in 4% paraformaldehyde, decalcified using 0.5 M EDTA in phosphate buffered saline (PBS) then vibratome (100-200  $\mu\text{m}$ ) or frozen (20-25  $\mu\text{m}$ ) sectioned. Immunofluorescence was performed using chicken or rabbit GFP antibody (Abcam, 1:1000), rabbit osterix antibody (Abcam, 1:600), rat CD31 antibody (Pharmingen, 1:200), rat CD45 antibody (Pharmingen, 1:100), rabbit perilipin antibody (Sigma, 5  $\mu\text{g}/\text{mL}$ ), rabbit mTert antibody (Millipore, 1:150) or rabbit osteocalcin antibody (Abcam, 10  $\mu\text{g}/\text{mL}$ ) and Alexa Fluor 488, 594, 633, or 647 secondary antibodies (Molecular Probes, 1:400). LipidTOX Deep Red (ThermoFisher, 1:200) was used for lipid staining. Nuclei were stained using DAPI. Sections were imaged using either a 90i Eclipse (Nikon) or LSM 700 laser scanning confocal (Zeiss) microscope.

## 2.5 | Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted from long bones using TRI reagent (Sigma-Aldrich) as per the manufacturer's protocol. cDNA was made using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems) and real time PCR was carried out using TaqMan PCR assays (Applied Biosystems). All the samples were run in triplicate and normalization was carried out using the  $2^{-\Delta\Delta\text{CT}}$  method relative to 18S or GAPDH as indicated in the figure legends.

## 2.6 | Statistics

Two-tailed Student's *t* test was used to compare groups of two- and one-way analysis of variance was used for comparison of groups of three or more and differences among the means were evaluated using Tukey's post hoc test of contrast. Significance was set at  $P < .05$  unless otherwise noted. \* $P < .05$ , \*\* $P \leq .01$ , \*\*\* $P < .001$ .

# 3 | RESULTS

## 3.1 | mTert expression in long bones is age-dependent

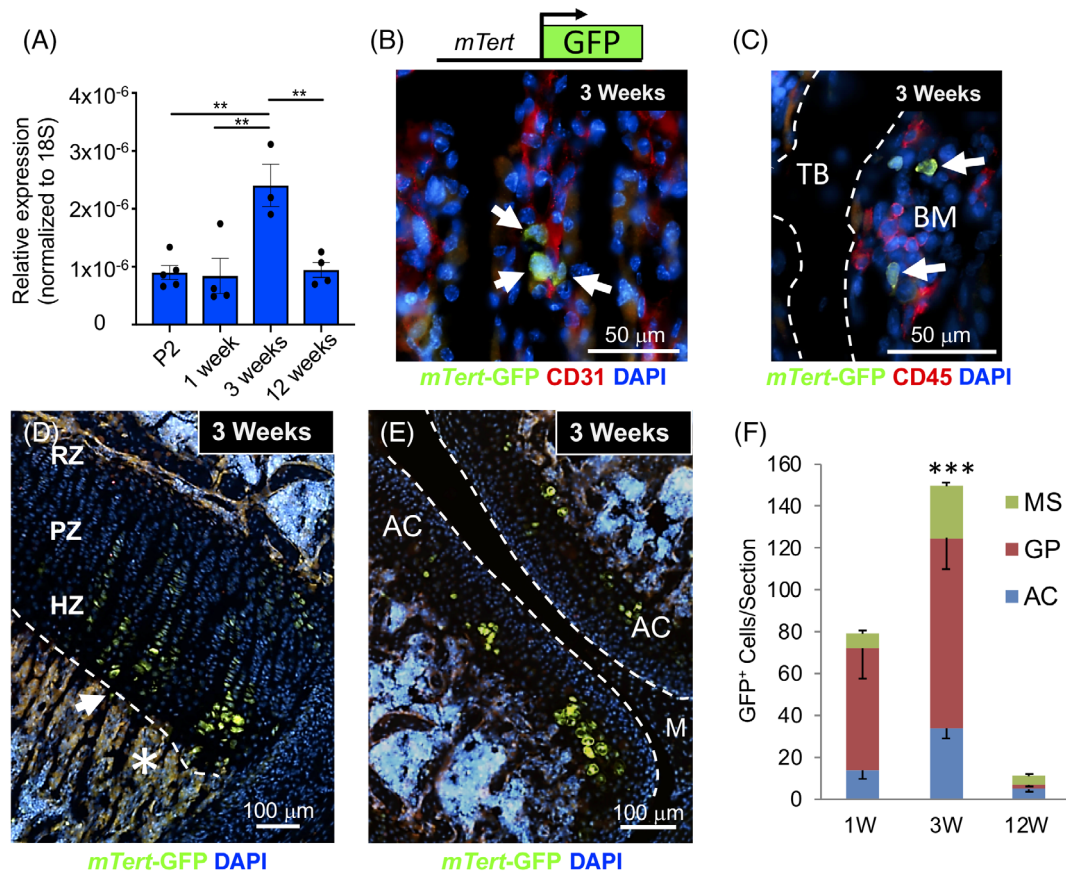
Recent studies indicate that discrete SSC populations function during the specific time periods that correspond to rapid bone growth and bone maintenance.<sup>7,13</sup> To investigate whether *mTert* is expressed during either of these time periods, we examined its expression in long bones at various ages by quantitative (q) RT-PCR analysis (Figure 1A).

Although *mTert* was detected at low levels during multiple time points, it was upregulated at the age of weaning (3-4 weeks) suggesting that *mTert*<sup>+</sup> cells are temporally regulated and mark a discrete time period interposed between rapid bone growth and bone maintenance.

We next investigated the location of *mTert*-expressing cells in 3-week-old long bones using our *mTert*-GFP mouse model.<sup>25</sup> We have previously shown this reporter mouse line faithfully recapitulates endogenous telomerase activity in BM as well as the testis, heart and intestine.<sup>25,26,36</sup> *mTert*-GFP<sup>+</sup> cells were detected within the metaphyseal stroma (Figure 1B), with ~75% of 185 GFP<sup>+</sup> cells analyzed residing adjacent to CD31<sup>+</sup> vascular cells, consistent with the proposed location for SSCs.<sup>1</sup> In addition, a separate subpopulation of GFP<sup>+</sup> CD45<sup>-</sup> BM cells were occasionally detected near the trabecular bone surface (Figure 1C), distinct from the previously described GFP<sup>+</sup> CD45<sup>+</sup> hematopoietic stem and progenitor cells.<sup>25</sup> *mTert*-GFP<sup>+</sup> cells were also present within the hypertrophic zone of both the growth plate and articular cartilage and to a lesser extent in the resting and proliferative zones of the growth plate (Figure 1D,E), consistent with expression of endogenous mTert protein within the growth plate (Figure S1). Interestingly, *mTert*-GFP<sup>+</sup> cells were largely absent from the perichondrium, a site known to house SSCs during rapid bone growth.<sup>8,13</sup> Next, to investigate whether the increase in *mTert* expression at 3 weeks of age (Figure 1A) correlated with enhanced cell number, we quantified GFP<sup>+</sup> cells within the articular cartilage, growth plate, and metaphyseal stroma of long bones at 1, 3, and 12 weeks of age (Figures 1F and S2). Consistent with our *mTert* gene expression data, the total number of GFP<sup>+</sup> cells was highest at 3 weeks of age. In addition, at 1 and 3 weeks of age GFP<sup>+</sup> cells were found within all three areas, with the majority present within the growth plate; in contrast, by 12 weeks of age the number of GFP<sup>+</sup> cell was dramatically decreased, with few found in the growth plate. Similarly, endogenous mTert<sup>+</sup> cells within the growth plate decreased at 12 weeks of age (Figure S1). Taken together, these data confirm that *mTert* expression, cell number, and distribution are temporally regulated in long bones.

## 3.2 | mTert marks a unique colony-forming cell population

To investigate whether *mTert*<sup>+</sup> cells function as SSCs, we performed flow cytometric analysis on BM cells for GFP and CD45, which revealed a small population (<1%) of GFP<sup>+</sup> CD45<sup>-</sup> cells (Figure 2A). To assess whether this population was enriched for SSCs, we isolated GFP<sup>+</sup> CD45<sup>-</sup> cells by fluorescent activated cell sorting (FACS) and measured their capacity to form CFU-Fs, a characteristic of SSCs.<sup>37</sup> Approximately 15% of FACS-isolated GFP<sup>+</sup> CD45<sup>-</sup> cells gave rise to colonies compared with only 0.5% of GFP<sup>-</sup> CD45<sup>-</sup> cells (Figure 2B). This colony formation rate of ~1 out of 6 cells is consistent with a previous report employing human SSCs.<sup>5</sup> As an additional control we analyzed 10 times the number of GFP<sup>-</sup> CD45<sup>-</sup> cells, which yielded a similarly low number of colonies (Figure 2B). These results



**FIGURE 1** *mTert* expression and localization in long bones. A, Quantitative reverse transcription PCR (qRT-PCR) analysis of *mTert* expression in long bones at different postnatal ages. Data are presented as relative expression normalized to 18S. Dots represent individual samples. Three-five mice were used per group. Mean  $\pm$  SEM, \*\* $P < .01$ . B-D, Localization of *mTert*-GFP<sup>+</sup> cells in 3-week-old long bones. GFP<sup>+</sup> cells were identified adjacent to vascular (CD31<sup>+</sup>) cells in the metaphyseal stroma (B, arrows) and within the growth plate (C) and articular cartilage (D). E, Nonhematopoietic (CD45<sup>-</sup>) GFP<sup>+</sup> cells were detected in the bone marrow (BM) near trabecular bone (TB). Representative merged images are shown. Asterisk and arrow in (D) demarcate autofluorescence and GFP<sup>+</sup> cell, respectively. F, GFP<sup>+</sup> cells in the articular cartilage, growth plate, and mesenchymal stroma of long bones were quantitated from 1-, 3-, and 12-week-old *mTert*-GFP mice. Eight-sixteen sections from 2 to 3 mice were analyzed at each time point. Bars represent total GFP<sup>+</sup> cells per section. Mean  $\pm$  SEM, \*\*\* $P < .0001$ . The distribution of GFP<sup>+</sup> cells within the metaphyseal stroma, growth plate, or articular cartilage is indicated within each bar. See also Figures S1 and S2. AC, articular cartilage; HZ, hypertrophic zone; M, meniscus; PZ, proliferative zone; RZ, resting zone

demonstrate that *mTert*-GFP<sup>+</sup> cells exhibit a 30-fold enrichment in their capacity to form CFU-Fs compared with the main population of GFP negative cells and indicate that nearly all of the colony-forming activity in the BM is found within the GFP<sup>+</sup> cell population.

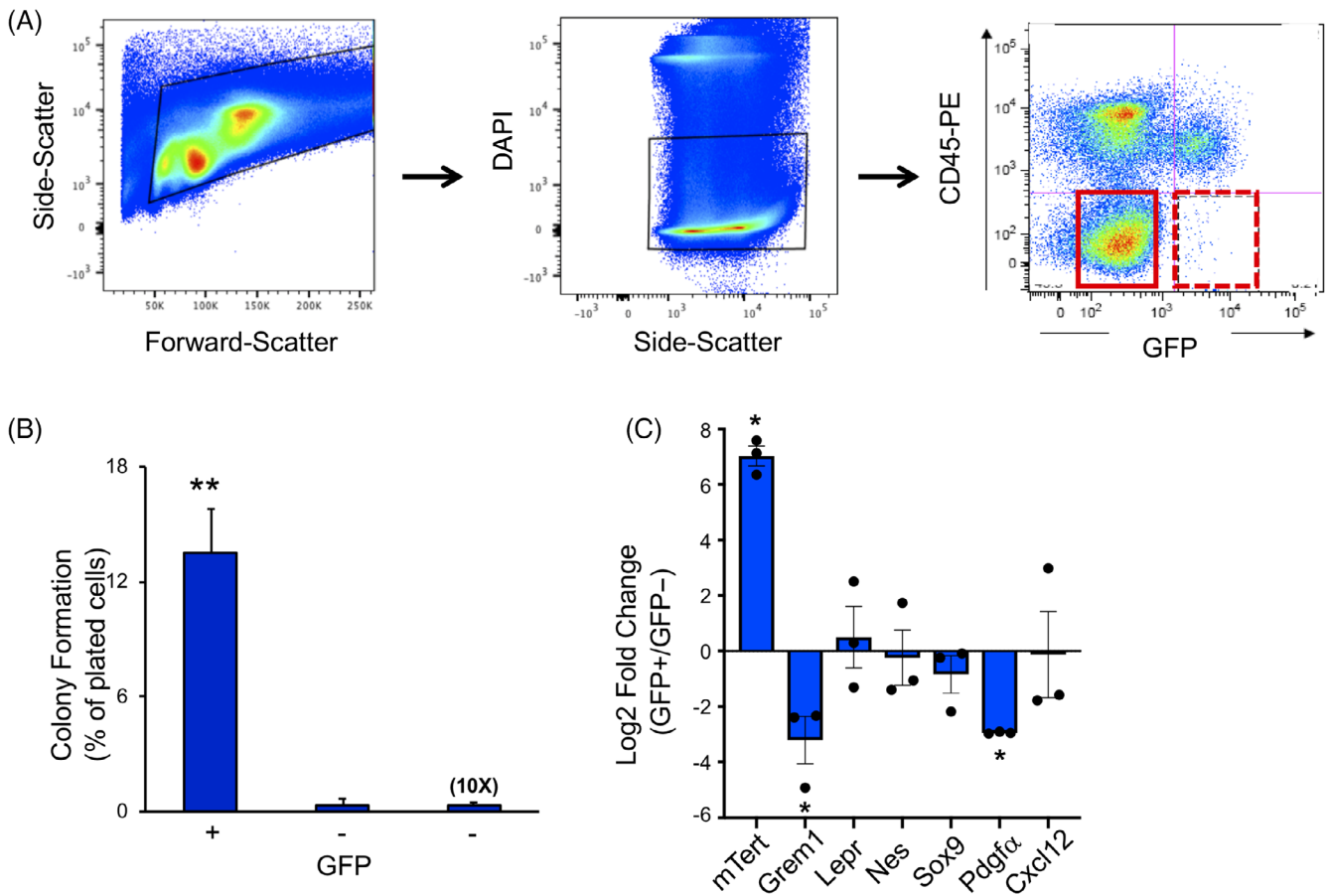
Given the temporal expression pattern of *mTert*<sup>+</sup> cells, we next assessed whether these cells represent a distinct SSC population. For this, we characterized the *mTert*<sup>+</sup> population with respect to other stem cell markers using flow cytometry and qRT-PCR analyses of sorted *mTert*-GFP<sup>+</sup> and GFP<sup>-</sup> populations. Consistent with our findings in other tissues,<sup>25,26</sup> *mTert* expression was enriched in the GFP<sup>+</sup> cells compared to GFP<sup>-</sup> cells (Figure 2C), further confirming that the GFP reporter faithfully marks endogenous *mTert* expression. We failed, however, to detect any enrichment in other known SSC markers which included *Gremlin*, *Leptin receptor (LepR)*, *Nestin*, *Sox9*, *PDGFR $\alpha$*  as well as the perivascular stromal cell marker, *Cxcl12*. In fact, comparison between *mTert*-GFP and the general SSC marker, *PDGFR $\alpha$* , showed that *mTert*<sup>+</sup> cells represent less than 1% ( $0.48\% \pm$

$0.11\%$ ) of *PDGFR $\alpha$* <sup>+</sup> cells (145 of 31,660 *PDGFR $\alpha$* <sup>+</sup> cells analyzed,  $N = 3$  mice). Furthermore, we found that *mTert*-GFP<sup>+</sup> cells do not coexpress the osteoprogenitor cell marker, *Osterix (Osx)* (0 of 144 GFP<sup>+</sup> cells analyzed,  $N = 3$  mice). Taken together, these data confirm that *mTert* expression marks a unique cell population at a distinct time period of bone growth.

### 3.3 | *mTert* expression marks multipotent progenitor/stem cells in vitro

To assess the functional capacity of *mTert*-expressing cells, we generated a doxycycline-inducible mouse model (*mTert*-rtTA) (Figure S3A). These mice were subsequently crossed with otet-Cre and R26R<sup>flox(mT/mG)/+</sup> mice to generate *mTert*-rtTA::otet-Cre::R26R<sup>flox(mT/mG)/+</sup> trigenic mice. We chose the mT/mG Cre-reporter allele because of its high level of constitutive expression of membrane-





**FIGURE 2** Analysis of *mTert*-GFP bone marrow cells. A, Representative fluorescent activated cell sorting (FACS) plot showing live GFP<sup>+</sup> CD45<sup>-</sup> cells in hatched red box (arrow) and GFP<sup>-</sup> cells in solid red box. B, Percentage of colony formation of GFP<sup>+</sup> and GFP<sup>-</sup> sorted cells. N = 3 mice. Mean ± SEM, \*\*P < .005. C, Quantitative reverse transcription PCR (qRT-PCR) analysis of SSC marker gene expression in *mTert*-GFP<sup>+</sup> cells. *GAPDH* was used as the internal control, data were analyzed using the 2<sup>-ddCt</sup> method and expressed as log 2-fold change (GFP<sup>+</sup>/GFP<sup>-</sup>). Symbols represent individual samples. Cells were isolated from three mice. Mean ± SEM, \*P < .05. SSC, skeletal stem cell

targeted Tomato (mTom) protein before Cre-mediated recombination and membrane-targeted GFP (mGFP) after recombination.<sup>34</sup> Analysis of long bones at 3 weeks of age, following a single injection of doxycycline, showed mGFP<sup>+</sup> cells exhibit a distribution pattern similar to *mTert*-GFP (Figure S3B, compare with Figure 1D). Doxycycline-induced labeling was also dramatically decreased in 12-week-old mice (Figure S3C,E) consistent with *mTert* expression. No detectable doxycycline-independent recombination was observed (Figure S3D).

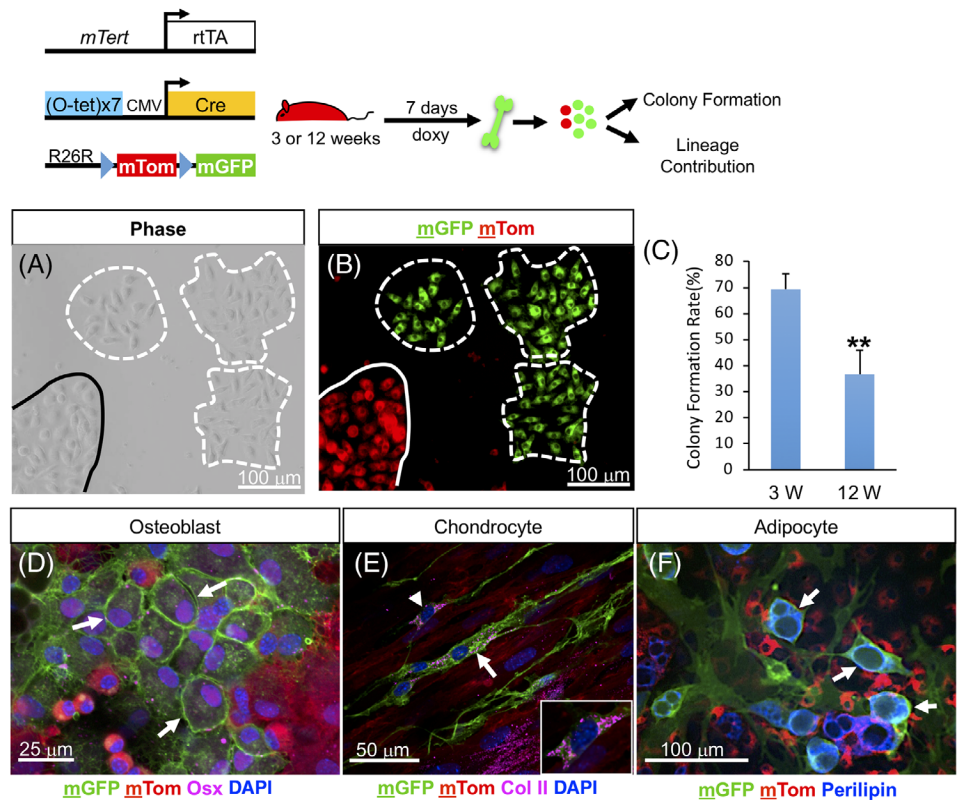
To confirm the self-renewal potential of *mTert*-rtTA-marked cells, and/or their progeny, we next assessed the capacity of lineage-marked mGFP<sup>+</sup> BM cells to give rise to CFU-Fs. Similar to our findings with *mTert*-GFP<sup>+</sup> cells, approximately 70% of mGFP<sup>+</sup> BM cells isolated from 3-week-old mice were able to form CFUs (Figures 3A-C). In contrast, mGFP<sup>+</sup> BM cells from 12-week-old mice showed a marked decrease in the frequency (~35%) of lineage-marked CFU-Fs (Figure 3C), despite no difference in the overall number of mGFP<sup>+</sup> BM cells at the time of isolation (Figure S3F). These results indicate a decline in the progenitor/stem cell activity of mGFP<sup>+</sup> BM cells with age. Finally, in vitro differentiation assays

demonstrated that mGFP<sup>+</sup> cells contribute to multiple mesenchymal lineages, indicating *mTert*-rtTA expression marks SSCs (Figure 3D-F).

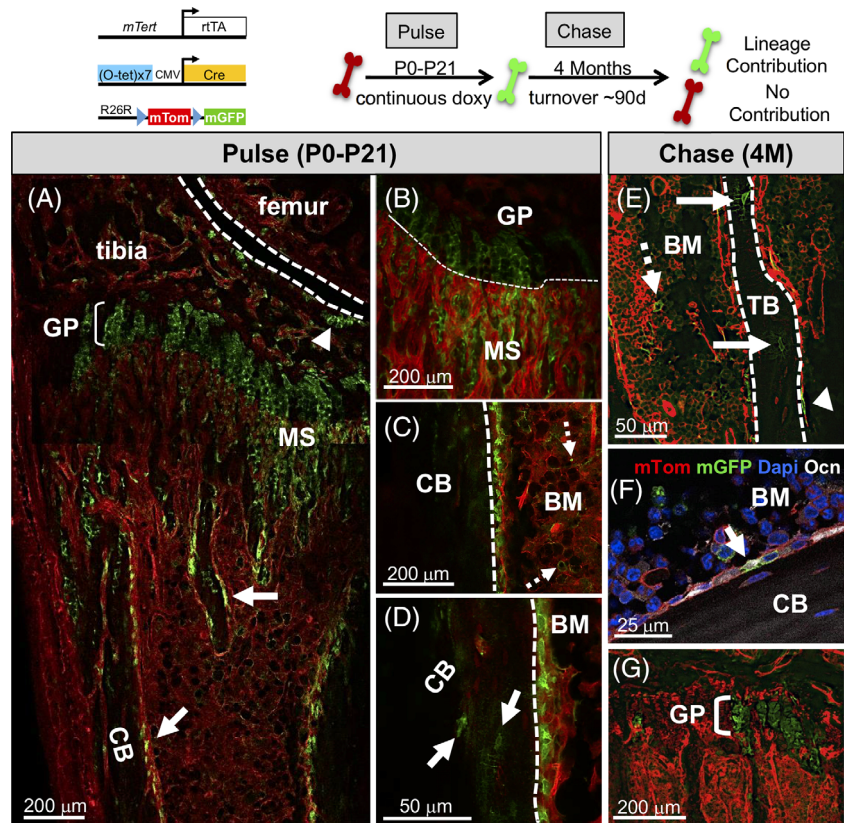
### 3.4 | *mTert* expression marks osteochondroprogenitor cells in vivo

To establish the functional potential of the *mTert*<sup>+</sup> SSC cell population in vivo, lineage-tracing analysis with trigenic mice was performed using a “pulse-chase” paradigm (Figure 4). Analysis of long bones following an extended pulse of doxycycline revealed mGFP<sup>+</sup> cells were present within the metaphyseal stroma (Figure 4A,B), BM (Figure 4C, dashed arrows), articular cartilage and growth plate (Figure 4A, arrowhead and bracket, respectively), consistent with our analysis of *mTert*-GFP mice (Figures 1B-E and S1). A similar pattern of mGFP<sup>+</sup> cell distribution was observed following shorter treatment with doxycycline (Figure S4A). In addition, consistent with SSCs contributing to *Osx*<sup>+</sup> osteoprogenitor cells,<sup>13</sup> we detected mGFP<sup>+</sup> *Osx*<sup>+</sup> cells following

**FIGURE 3** In vitro analysis of *mTert*<sup>+</sup> cells. A,B, Colony formation of BM cells isolated from 3-week-old trigenic mice treated for 7 days. Dashed lines outline mGFP<sup>+</sup> cell derived CFU-Fs; solid line demarcates a non-*mTert* derived colony. C, Percentage of single mGFP<sup>+</sup> BM cells at 3 and 12 weeks of age that formed colonies. N = 5, 3. Mean ± SEM, \*\*P = .01. D-F, In vitro differentiation analysis of mGFP<sup>+</sup> cells isolated from 3-week-old trigenic mice treated for 7 days. Arrows indicate differentiated mGFP<sup>+</sup> cells. The treatment schematic is shown above. See also Figure S3. BM, bone marrow; CFU-F, colony-forming unit-fibroblast

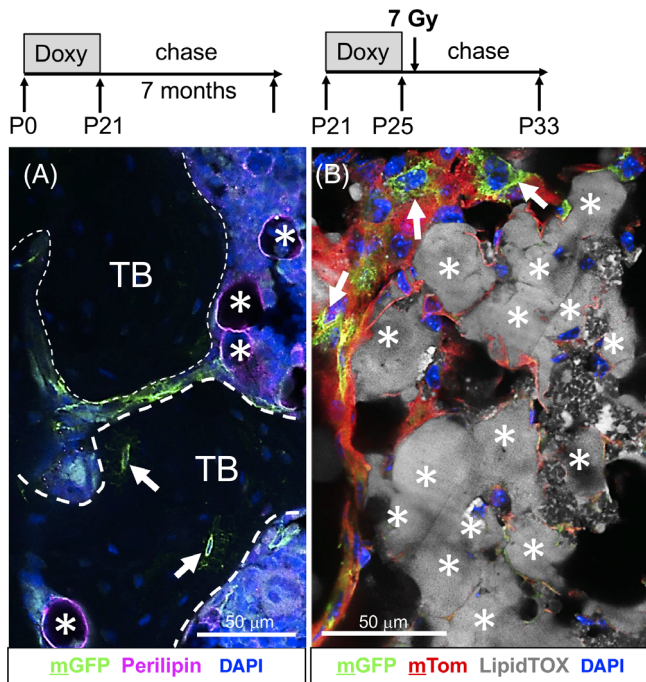


**FIGURE 4** *mTert* expression marks osteochondral progenitor cells in vivo. A-D, Marked cells following an extended pulse of doxycycline. A composite image is shown (A). Arrows indicate mGFP<sup>+</sup> cells lining trabecular and cortical bone (A). Marked metaphyseal stromal (A, B), bone marrow cells (C, dashed arrows) and osteocytes (D, arrows) are shown. Dashed lines demarcate the joint (A), growth plate (B), and cortical bone (C, D). Arrowhead (A) demarcates the articular cartilage. E-G, Marked cells after an extended chase (4 months). mGFP<sup>+</sup> cells lining trabecular (E, arrowhead) and cortical bone (F, arrow), in the BM (E, dashed arrow) and within the growth plate (G, bracket). Solid arrows in (E) indicate marked osteocytes. *Ocn*<sup>+</sup> mGFP<sup>+</sup> cell in (F) confirmed contribution to the osteoblast lineage. The treatment schematic is shown above. See also Figure S4. BM, bone marrow; CB, cortical bone; GP, growth plate; MS, metaphyseal stroma; TB, trabecular bone



doxycycline administration (Figure S4G), indicating that *mTert*-expressing cells can contribute to *Osx*<sup>+</sup> cells. Furthermore, the presence of co-positive cells within 24 hours is likely due to the rapid

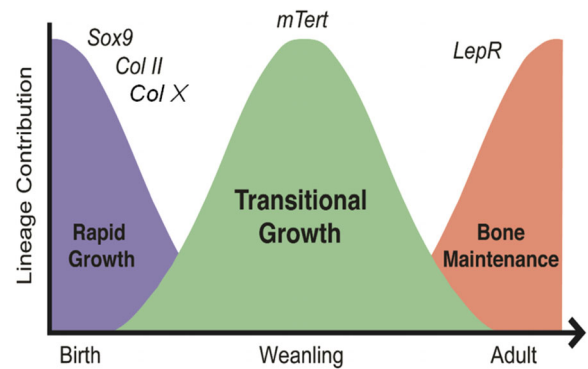
transit of chondrocytes through the growth plate as has been previously shown,<sup>11</sup> although it is possible that the two mouse models do not mark identical cell populations.



**FIGURE 5** *mTert*-rtTA marked cells do not contribute to the adipocyte lineage. A, Immunofluorescent analysis of the adipocytic marker, perilipin, following extended chase. Dashed lines outline trabecular bone; asterisks, perilipin<sup>+</sup> adipocytes; arrows, mGFP<sup>+</sup> osteocytes. Representative confocal image is shown. Treatment schematic is shown above. Briefly, trigenic (*mTert*-rtTA::*otet*-Cre::*R26R*<sup>fllox(mT/mG)/+</sup>) mice were treated with doxycycline for 3 weeks from birth (P0-P21) and then chased for 7 months at which time long bones were isolated and analyzed for lineage contribution. TB, trabecular bone. B, LipidTOX Deep Red staining of adipocytes in long bone sections following irradiation. Arrows demarcate nonadipocyte mGFP<sup>+</sup> cells. Asterisks demarcate LipidTOX<sup>+</sup> cells. A composite confocal image is shown. Treatment schematic is shown above. Briefly, trigenic (*mTert*-rtTA::*otet*-Cre::*R26R*<sup>fllox(mT/mG)/+</sup>) mice were treated with doxycycline for 4 days and irradiated 24 hours later. Long bones were collected 7 days after irradiation. Scale bars = 50 μm

The presence of *Osx*<sup>+</sup> marked cells dramatically declined at 12 weeks suggesting a functional decline in mTert<sup>+</sup> SSCs with age. In addition to increased osteoprogenitor cell marking, we also observed an increase in mGFP<sup>+</sup> cells along both trabecular and cortical bone (Figure 4A, arrows), likely due to the extended pulse time as few cells were noted at these locations following exposure to doxycycline for shorter time periods (Figures S3B and S4A).

To assess the long-term persistence and self-renewal capacity of *mTert*-rtTA marked cells, trigenic mice pulsed with doxycycline were chased for up to 4 months. The analysis revealed lineage-marked cells lining the surfaces of both trabecular (Figure 4E, arrowhead) and cortical bone (Figure 4F, arrow denotes an osteocalcin [Ocn]<sup>+</sup> osteoblast) as well as osteocytes (Figure 4E, arrow). In addition, marked cells were still present within the growth plate (Figure 4G) demonstrating long-term lineage contribution of *mTert*-expressing cells to both bone and cartilage. These results are consistent with the hypertrophic chondrocyte's continual commitment to the osteogenic fate into adulthood. Given bone turnover



**FIGURE 6** *mTert* expression marks a distinct phase of transitional bone growth. Schematic model illustrating the presence of a period of bone growth designated as transitional growth separate from rapid growth and bone maintenance

occurs within ~90 days,<sup>20</sup> the presence of lineage-marked cells after an extended period of chase demonstrates the self-renewal potential and long-term persistence of the *mTert*-expressing cells. Consistent with *mTert* expression marking a subpopulation of HSCs, lineage-marked cells were also observed in the BM (Figure 4E, dashed arrow). Similar findings were observed when mice were pulsed with doxycycline for a shorter period (Figure S4B-F). Taken together, these data argue that *mTert* expression marks osteochondral progenitor cells in long bones.

In contrast to the *in vitro* lineage analysis, we did not observe contribution of *mTert*-expressing cells to the adipogenic lineage in these mice, as no colabeling of mGFP and perilipin<sup>+</sup> adipocytes were detected even following a 7-month chase (Figure 5A). To further confirm that *mTert*-rtTA cells fail to contribute to the adipocyte lineage *in vivo*, we induced BM adipogenesis in 3-week-old trigenic mice using whole-body irradiation, a known stimulus for marrow-derived adipogenesis. Analysis of 624 LipidTOX-stained adipocytes from three trigenic mice failed to detect colabeling with lineage-marked mGFP<sup>+</sup> cells (Figure 5B). Together, these data indicate that *mTert*<sup>+</sup> cells lack the capacity to contribute to adipocytes *in vivo*, consistent with a recent report that metaphyseal progenitor/stem cells are unable to differentiate into adipocytes.<sup>8</sup>

## 4 | DISCUSSION

In summary, we have identified a population of osteochondral progenitor/stem cells, marked by *mTert* expression, that function during adolescent bone formation between the periods of rapid perinatal bone growth and adult bone maintenance (Figure 6). Our data are consistent with recent findings that bone formation is temporally controlled by discrete populations of SSCs. Our findings are unique in that *mTert*-expressing SSCs are distinct and function at a later stage in postnatal bone growth than do cells marked by either Collagen II, Collagen X, or Sox9 but prior to cells marked by LepR. We have designated this time period as the phase of “transitional growth” and propose that it corresponds to the adolescent growth spurt in humans, since patients with defects in telomere length and stability fail to undergo this event.<sup>38-41</sup> Although our lineage-tracing data



demonstrate the functional capacity of *mTert*<sup>+</sup> SSCs, these studies are limited and additional analysis using transplantation assays would further enhance the significance of their role during transitional growth.

#### 4.1 | *mTert* expressing cells are unique and function at a distinct time period

Over the last few years, multiple laboratories have identified SSCs in a variety of locations within long bones. While our findings show that *mTert*-expressing cells mark regions known to house SSCs, their lack of enrichment in any of the other SSC markers indicates that these cells may constitute a unique SSC population, although it is also possible that the lack of SSC marker enrichment we observe in these cells is due to *mTert* expression marking multiple SSC populations or a subset of them. Additional coexpression studies are warranted in order to fully determine the distinct nature of *mTert*<sup>+</sup> cells. It is clear, however, that *mTert*-expressing cells likely do not mark LepR<sup>+</sup> SSCs based on the fact that *mTert* expression and cell number dramatically decline in adult bone (>12 weeks), the lack of enrichment in LepR<sup>+</sup> cell markers, CXCL12 and PDGFR $\alpha$  as well as their inability to give rise to adipocytes, key characteristics of the LepR<sup>+</sup> cells.<sup>7</sup>

Exactly what factors regulate *mTert* expression during adolescent bone growth is not clear. Prior studies have shown that a variety of endocrine signals including growth hormone (GH), thyroid hormone, and sex steroids act on the growth plate to regulate bone growth,<sup>42</sup> and it is possible these factors play a role. In fact, GH has been shown to regulate *mTert* transcription both directly and indirectly,<sup>43,44</sup> and it is tempting to speculate that the increase in *mTert* expression during bone growth is mediated, in part, by GH. Alternatively, estrogen, a key regulator of the pubertal growth spurt, may also play a role in regulating *mTert* expression as it has been shown to transcriptionally upregulate *hTERT* in human cells.<sup>45</sup> Conversely, estrogen may mediate the decrease in *mTert*<sup>+</sup> cell number at 12 weeks of age given previous findings that it accelerates growth plate senescence via depletion of growth plate progenitor cells.<sup>42</sup> It has also been reported that thyroid hormone enhances fibroblast growth factor receptor 3 expression which, in turn, negatively regulates *Tert* expression, telomerase activity, and bone elongation.<sup>46</sup> Based on these prior studies, it will be important to carefully define which factors are responsible for regulating the temporal expression pattern of *mTert*<sup>+</sup> cells in long bones.

Furthermore, given that multiple cell populations within long bones express *mTert*, it is difficult to identify which of these *mTert*<sup>+</sup> cell subpopulations contribute to bone formation. Going forward it will be important to identify unique markers that will allow us to study each subpopulation independently. How *mTert*<sup>+</sup> cells vary from other SSC populations that function during this time also remains unclear. A more complete understanding of the relationship between these various SSC populations is necessary in order to fully decipher their role in bone biology as well as their potential use in the treatment of bone disease.

#### 4.2 | Stem cell hierarchy in long bones

Whether SSCs function at defined time periods during the lifetime of the skeleton or contribute to other populations resident in skeletal tissues remains unknown and is of high importance in the field. Our data, in combination with other reports, indicate that there are waves of stem cell activity in long bones. Exactly how many waves of SSCs exist within long bones, what controls their timing and functional lineage capacity, and their role in response to injury remains to be determined. It has been proposed that a hierarchical relationship exists between the different SSC populations. That is, SSCs present during embryonic or early postnatal life may be the precursors to those required for adult bone maintenance. To this end, lineage-tracing studies in which Collagen II-derived cells were detected well into adulthood<sup>13</sup> have led many to propose that LepR<sup>+</sup> SSCs arise from cells present in embryos and young mice. However, definitive proof for this hypothesis is lacking. Given the heterogeneity of progenitor/stem cells within the BM, it is not likely that all bone cells arise from a common progenitor population. Therefore, going forward, it will be imperative to define the inter-relationship of the various SSC populations.

#### 4.3 | Role of telomerase in chondrogenic cells

In humans, telomerase has been shown to predominantly mark hypertrophic chondrocytes within the growth plate and is required for endochondral bone growth.<sup>47</sup> In addition, telomerase activity declines with age, suggesting a possible role in growth plate closure. Our findings are consistent with this idea, as indicated by the presence of endogenous mTert protein and *mTert*-GFP expression within murine hypertrophic chondrocytes, which declined with age. Of note, while immunohistochemical analysis suggests that mTert is expressed in all hypertrophic chondrocytes, *mTert*-GFP was detected in fewer cells possibly suggesting (a) an artificially lower frequency of mTert<sup>+</sup> cells or (b) discordance between *mTert*-induced GFP and mTert protein stability. This difference is likely not due to the inefficiency of the *mTert*-GFP mouse model given our prior findings<sup>25,26</sup> and Figure 2C. Going forward it will be important to determine the percentage of mTert<sup>+</sup> hypertrophic chondrocytes and to determine what role telomerase plays in hypertrophic chondrocytes. This is especially important given hypertrophic chondrocytes can give rise to osteoblasts, either directly or via an intermediate progenitor/stem cell, during rapid bone growth and following injury,<sup>10-12</sup> raising the intriguing possibility that a subset of hypertrophic cells may represent SSCs. The presence of mTert<sup>+</sup> cells, albeit to a lesser extent, within the resting and proliferative zones of the growth plate suggest an additional role for telomerase in cartilage biology. Whether these cells function as cartilage stem cells is unknown, although the presence of *mTert*-marked cells in the resting zone, a region recently shown to house stem cells,<sup>14</sup> suggests this may be the case. Alternatively,



*mTert* expression within the proliferative zone may function in sustaining chondrocyte proliferation as downregulation of human TERT has been linked with inhibition of chondrocyte proliferation.<sup>47</sup> Going forward it will be important to decipher the role of *mTert* and/or telomerase activity in the different regions of the growth plate. Finally, our data also show that *mTert*<sup>+</sup> cells are present within the articular cartilage, which also has been shown to contain progenitor/stem cells.<sup>48</sup> Whether *mTert* expression marks these cells remains to be determined but prior studies have shown that articular progenitor/stem cells exhibit higher telomere length and telomerase activity<sup>49</sup> warranting further examination.

## 5 | CONCLUSION

Our study has identified a distinct population of osteochondral progenitor cells, marked by *mTert* expression that function during a defined temporal period of bone growth. Future studies focused on understanding if alterations in this cell population during this growth period translate into disorders of linear growth or bone homeostasis (such as osteoporosis) are warranted. In addition, understanding what role physiological factors play in regulating both the temporal pattern of *mTert*<sup>+</sup> SSCs and their influence on the ability of these cells to participate in bone formation will be important. Given that the adolescence growth spurt represents the period of greatest bone mass accrual, understanding the requirement of SSCs during this period of transitional growth has important implications for understanding skeletal homeostasis.

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### CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

### AUTHOR CONTRIBUTIONS

D.L.C., D.T.B.: supervision of research, study design, performed experiments, manuscript preparation; R.D.R.-W.: study design, performed experiments; L.T.D., A.T., L.J., D.M.A., B.E.M., M.S.S.: technical assistance with experiments; C.J.L.: study design, performed experiments; R.J.: supervision of research.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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