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Bone marrow fibrosis with fibrocytic and immunoregulatory responses induced by β-catenin activation in osteoprogenitors

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ABSTRACT

Wnt/β-catenin signaling has been reported to contribute to the development of bone fibrous dysplasia. However, it remains unclear whether fibrocytes and immune cells are involved in this β-catenin-mediated bone marrow fibrosis. In this study, we showed that constitutive activation of β-catenin by *Col1a1-Cre* (3.6-kb) exhibited bone marrow fibrosis, featured with expanded populations of fibrocytes, myofibroblasts and osteoprogenitors. Lineage tracing and IHC examinations showed that *Col3.6-Cre* display Cre recombinase activity not only in osteoprogenitors, but also in monocyte-derived fibrocytes in the endosteal niches of bones. Additionally, β-catenin stimulated the secretion of cytokines and pro-fibrotic signals in bone marrow, including GM-CSF, TGFβ1 and VEGF. Consequently, the frequency of differentiated immature monocyte-derived dendritic cells and naïve T cells was markedly increased in the mutant bone marrow. These phenotypes were quite different from those following β-catenin activation in mature osteoblasts driven by *Col1a1-Cre* (2.3-kb). Our findings suggested that a conserved pro-fibrotic signal cascade might underlie β-catenin-mediated bone marrow fibrosis, involving TGFβ1-enhanced fibrocyte activation and immunoregulatory responses. This study might shed new light on the understanding and development of a therapeutic strategy for bone fibrous dysplasia.

1. Introduction

Fibrosis frequently accompanies a number of diseases in skin, intestine, lung, liver and bone. Fibrosis is characterized by the accumulation of an extracellular matrix, permanent scarring and organ dysfunction and usually arises from aberrant wound healing caused by trauma, infection, inflammation or tumor-induced reactions [1,2]. The fibrotic process involves the orchestrated action of different cells, including fibrocytes, myofibroblasts and immune cells [3]. It has been illustrated that fibrocytes are derived from monocyte lineage cells because they express the hematopoietic surface markers CD45, CD11b, CD115, and Gr1. On the other hand, fibrocytes also exhibit typical characteristics of mesenchymal cells, such as spindle-shaped morphology and expression of collagen I [4,5]. Interestingly, a novel fibrocyte population lacking hematopoietic surface markers, such as CD45 and CD11b, was identified through cell lineage tracing in a hematopoietic-specific Vav1-cre model [6]. Fibrocytes are increasingly recognized as an effector of chronic inflammation during the fibrotic process, which involves the TGFβ1-mediated activation of myofibroblasts [7].

Wnt signaling contributes to multiple fibrotic disease in lung [8,9], kidney [10], heart [11] and bone [12]. Furthermore, Wnt signaling is required for TGFβ1-mediated fibrosis in skin [13]. Bone marrow (BM) fibrosis has also been detected in a number of malignancies, including myelofibrosis, hyperparathyroidism and fibrous dysplasia (FD). Several murine models of BM fibrosis have been reported. For instance, overexpression of the *PTH* receptor or *VEGF* in osteoblasts display BM fibrosis [14,15]. Gain-of-function (GOF) mutations in the *GNAS* (*Gsα*) gene cause FD and are validated by *in vivo* transplantation [16–18]. Recently, it has been reported that *Gsα* mutations or *VEGF* overexpression potentiates Wnt/β-catenin signaling activity in FD pathogenesis [12,15]. However, whether or how the fibrocytes and immune cells contribute to the β-catenin-mediated BM fibrosis remains unclear.

β-catenin is an obligate mediator for canonical Wnt signaling. Ectopic activation of β-catenin in osteoblast progenitor cells by *Osterix-Cre* resembled the features of BM fibrosis [12]. However, reporter analyses in mice revealed that *Osterix-Cre* labels multiple stromal cells, including perineural cells and α-SMA$^+$ pericytes [19], indicating an increased complexity of cellular mechanisms that underlie β-catenin-mediated BM fibrosis. Collagen I (ColI) is a common marker for both fibrocytes and osteoblasts. The rat *Col1a1* (2.3-kb fragment) promoter has been demonstrated to contain osteoblast and fibrocyte populations in BM [14]. In contrast, the activation of β-catenin in mature osteoblasts by *Col1a1-Cre* (2.3-kb) does not induce BM fibrotic features but exhibits high bone mass with normal osteoblast number [20] or progressive leukemogenesis [21]. In this study, we revisited BM with activated β-catenin expression driven by *Col3.6-Cre* (rat promoter) [22]. Our analysis revealed that rat *Col3.6-Cre* could simultaneously label fibrocytes and osteoblast precursors in BM. Ectopic expression of β-catenin by *Col3.6-Cre* displayed

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typical BM fibrosis. Surprisingly, we observed the enrichment of fibrocytes/myofibroblasts and immune cells as well as the elevated secretion of TGFβ1 and VEGF in mutant BM, revealing that a conserved fibrotic signal network underlies β-catenin-mediated BM fibrosis.

2. Results

2.1. Col3.6-Cre demarcates a small population in myeloid progenitor cells

To trace the *myeloid progenitor cells* found in our *Col3.6-Cre* mice, we conducted a FACS analysis on the bone marrow of *Col3.6-Cre*; *R26YFP* mice. Compared to the *R26YFP* mice, approximately 0.87% of myeloid progenitor cells ($Lin^-Sca1^-c\text{-}Kit^+$) were detected as YFP^+ (Fig. 1A), which was consistent with the previous notion that fibrocytes derive from myeloid lineages [7]. In addition, a previous report also shows that the transgenic mice *Col3.6-GFP* (driven by rat 3.6-kb promoter) could label osteoclast cells [23]. However, we did not identify YFP^+ cells in the osteoclast progenitor cell lineages defined by $B220^-CD3^-CD11b^-CD115^+c\text{-}Kit^+$ (Fig. 1B). Of note, the majority of monocyte ($CD11b^+Gr1^{middle}$) and granulocyte ($CD11b^+Gr1^{high}$) populations were YFP-negative (data not shown), indicating that the YFP^+ cells shared common progenitor cells with myeloid cells, but they developed independently from typical myeloid cells, including monocytes, granulocytes and macrophages.

2.2. Accumulation of osteoprogenitors and myofibroblasts in bone marrow of Col3.6-Cre; $Catnb^{+/ex3}$ mice

To understand the molecular mechanisms underlying β-catenin-mediated BM fibrosis, we investigated the phenotypes of β-catenin gain-of-function (GOF, $Catnb^{+/ex3}$) mutant mice driven by our *Col3.6-*

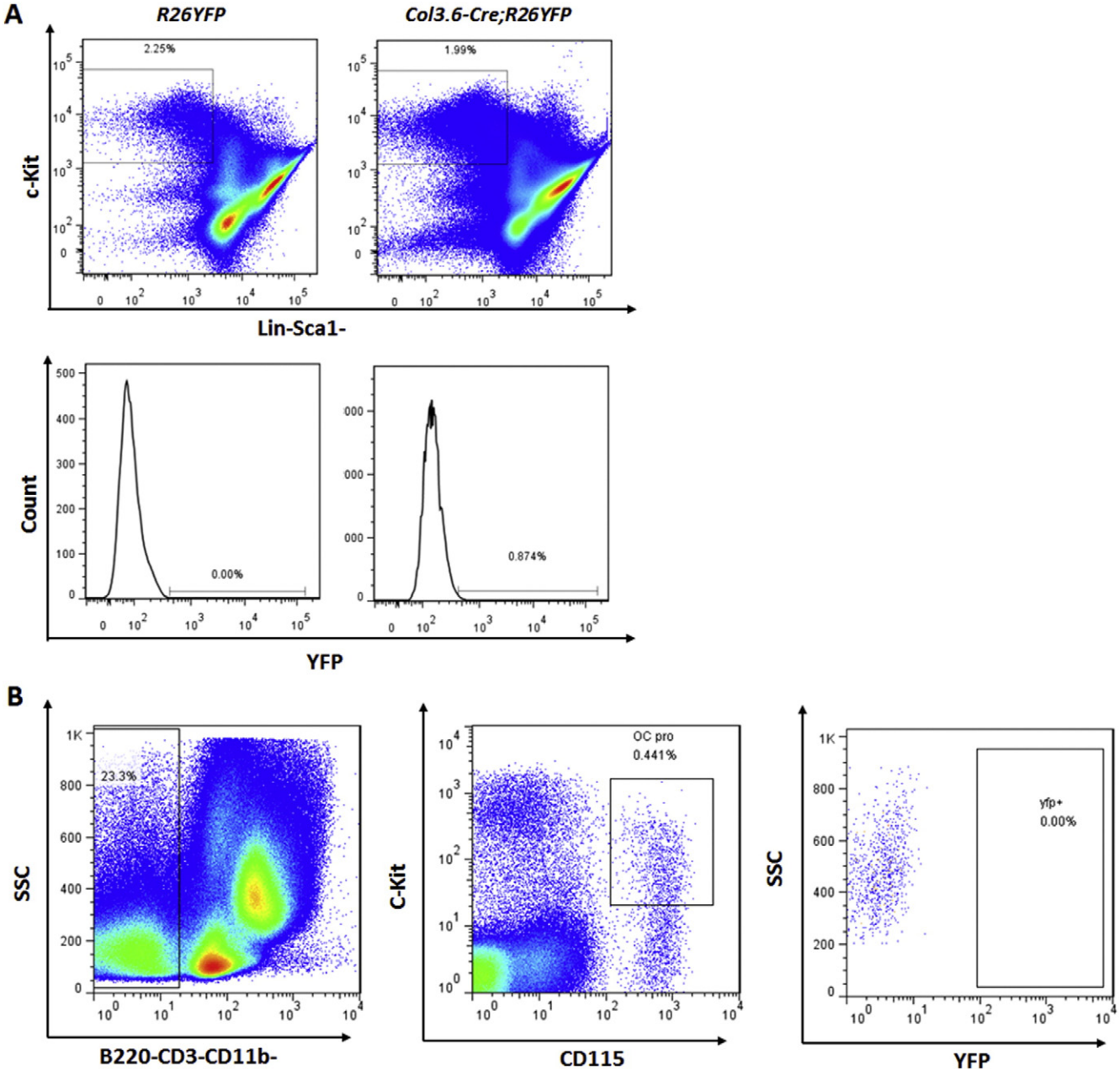

Fig. 1. Lineage tracing of myeloid progenitor cells within bone marrow of *Col3.6-Cre*; *R26YFP*. A. FACS analysis for the frequency of YFP^+ cells in myeloid progenitor (MP) cells from the BM of *Col3.6-Cre*; *R26YFP* mice. The dot plot depicts the MP cells (upper panel, $Lin^-c\text{-}Kit^+Sca1^-$). Histogram analysis shows that YFP^+ cells account for 0.87% of MP cells. *R26YFP* mice were used as controls. Bone marrow, BM. B. The dot plot shows that YFP + cells were not detected in osteoclast progenitor cells ($B220^-CD3^-CD11b^-CD115^+CD117^+$). All of the samples were isolated from BM of mice at P7.

Cre [22]. As previously described [24,25], a rat 3.6-kb *Col1a1* promoter could drive a Cre recombinase in early osteoblasts cells. Trichrome and HE staining showed that the BM of *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mice was filled with fibroblast-like cells (yellow arrows in Fig. 2A–D), as observed in *Osterix-Cre*; *Catnb*$^{+/ex3}$ mice [12]. Additionally, we detected high activation of canonical Wnt signaling in bone marrow cells, as evidenced by anti-LacZ antibody in *Col3.6-Cre*; *Catnb*$^{+/ex3}$; *TopGal* mice (Fig. 2-F′ and Supplemental Fig. S1). IgG was tested as controls for the specificity of anti-LacZ antibody (Supplemental Fig. S2 A–B′). Most of the accumulated YFP^{+} fibroblast-like cells in the *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mice located at the endosteal niches and were Osterix-positive (Fig. 2-H′ and Supplemental Fig. S2-D′). Many YFP^{+} fibroblast-like cells were α-SMA and Vimentin positive (Fig. 2I–L′), indicating that they were likely myofibroblasts. The immunostaining results suggested that the activation of β-catenin led to the accumulation of osteoprogenitors and myofibroblasts in bone marrow, partially resembling the features of bone FD.

2.3. Enrichment of fibrocyte cells in bone marrow of Col3.6-Cre; Catnb$^{+/ex3}$ mice

Furthermore, we investigated the cell surface markers in fibroblast-likes cells in the β-catenin GOF mutant mice. A recent report proposed that fibrocytes contain two subpopulations defined as ColI^{+}CD45^{+}-CD11b^{+} or ColI^{+}CD45^{-}CD11b^{-} [6]. Similarly, FACS analysis indicated that most of the accumulated fibroblast-like cells consisted of two subpopulations in the BM of *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mice. One was defined as ColI^{+}CD45^{+}CD115^{+}, another portion was characterized by ColI^{+}CD45^{-}CD115^{+} (Fig. 3A). We observed that activation of β-catenin simultaneously expanded the two fibrocyte populations by 10-fold, respectively (Fig. 3B, C). Of note, a major part of the enriched YFP^{+} cells were CD11b^{-} (Supplemental Fig. S3) or CD45^{-}CD115^{+} (Fig. 4A, B). Given that myofibroblasts primarily arise from local fibrocytes or fibroblasts, the enriched ColI^{+}CD45^{-}CD11b^{-} cells might also potentially give rise to differentiating fibrocytes CD45^{+}ColI^{+}CD115^{+} or myofibroblasts, which are shown by immunostaining in Fig. 2I–L′.

2.4. Upregulation of pro-fibrotic signals via stimulation by β-catenin

A variety of pro-fibrotic or pro-inflammation signals, such as TGFβ1, VEGF and GM-CSF, contributes to various fibrotic diseases [15,26,27]. To examine whether they were involved in the fibrotic process in β-catenin GOF mutant mice, we examined the expression levels of these signals. First we immunostained sections from the BM of control and mutant mice with antibodies. The distribution of the TGFβ1 and GM-CSF signals was higher in the BM of *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mice than in the controls (Fig. 5A–D′). Meanwhile IgG controls were performed to exclude the background signals in IHC (Supplemental Fig. S2-F′). Then, we isolated osteoblasts from the trabecular bones and conducted quantitative RT-PCR for these signals. The expression of *TGFβ1*, *GM-CSF* and

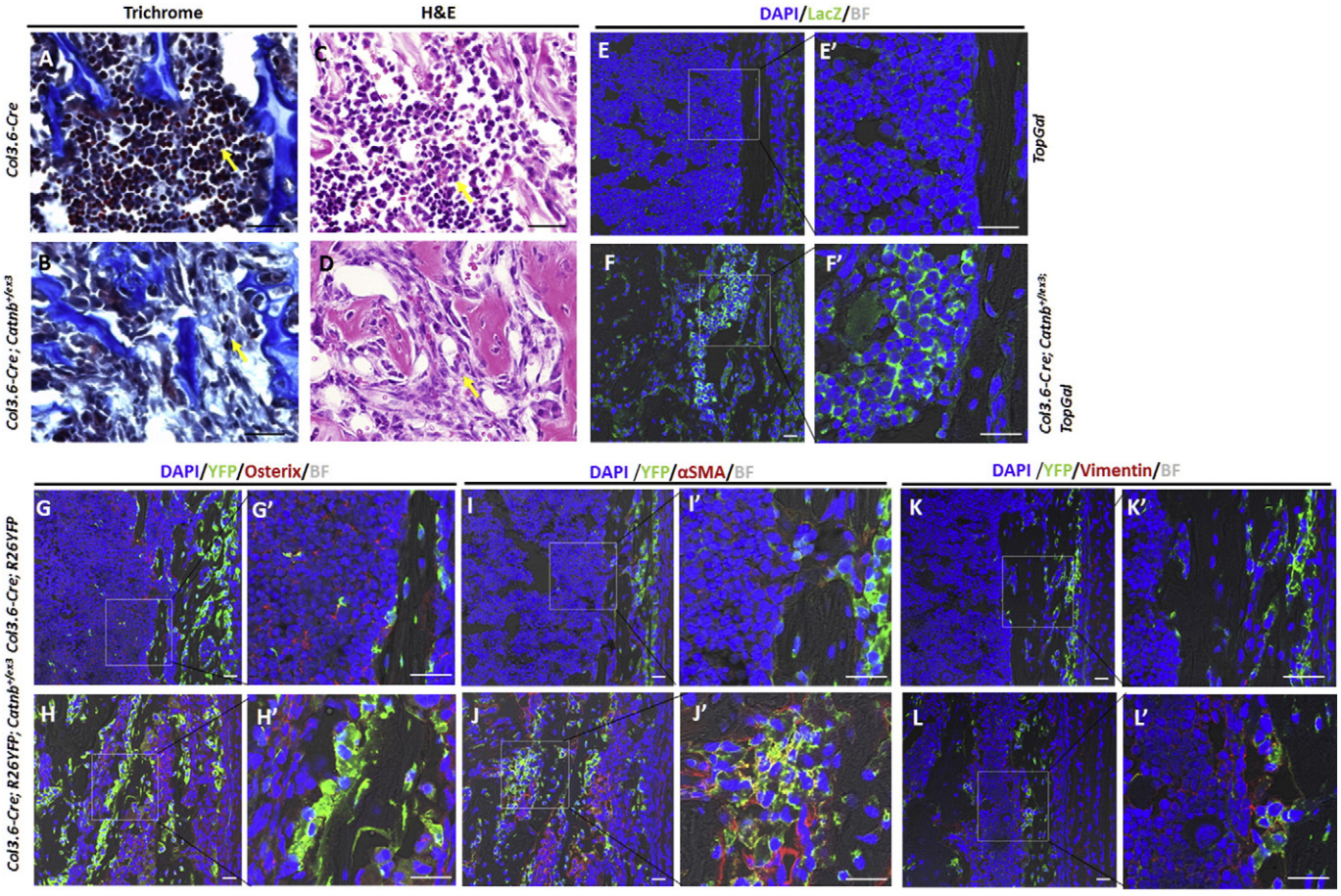

Fig. 2. Activation of β-catenin by *Col3.6-Cre* leads to BM fibrosis. A–D. Trichrome (A, B) and HE (C, D) staining for the BM of *Catnb*$^{+/ex3}$ (A, C) and *Col3.6-Cre*; *Catnb*$^{+/ex3}$ (B, D) mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) E–F′. Immunostaining with anti-LacZ antibody on the sections of BM of *TopGal* (C, C′) and *Col3.6-Cre*; *Catnb*$^{+/ex3}$; *TopGal* (D, D′) mice. G–H′. Immunostaining with anti-Osterix (Osx) antibody. I–J′. Immunostaining with anti-α-SMA antibody. K–L′. Immunostaining with anti-Vimentin antibody. Longitudinal sections from hindlimb BM of *Col13.6-Cre*; *R26YFP* (G, G′, I, I′, K, K′) and *Col3.6-Cre*; *Catnb*$^{+/ex3}$; *R26YFP* (H, H′, J, J′, L, L′) mice at P6-P7. (G′-K′) are a view of (G–K) at higher magnification. Bar, 25 μm. BF, bright field.

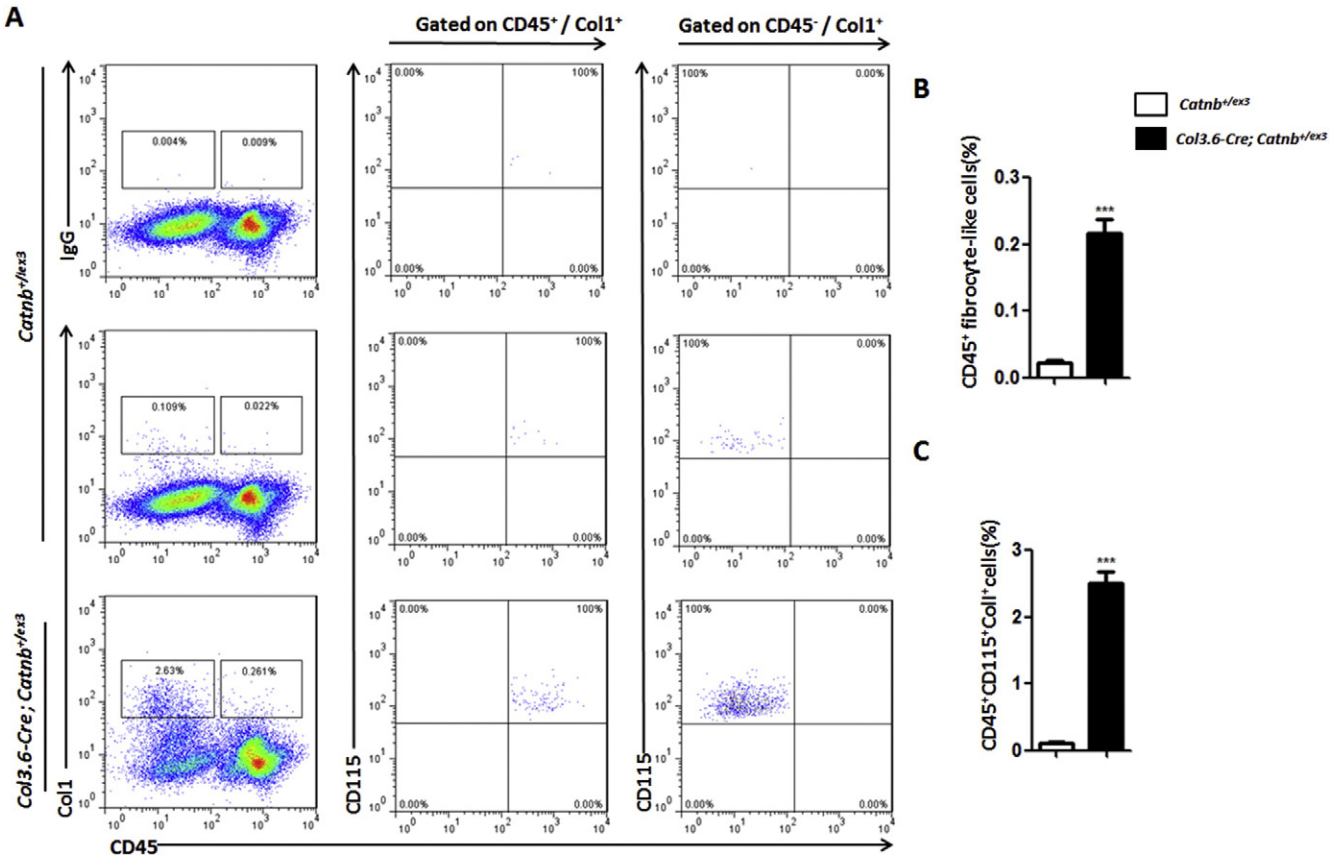

Fig. 3. Activation of β-catenin by *Col3.6-Cre* results in accumulation of fibrocytes in BM. A. A dot plot depicts the $CD45^+Coll^+CD115^+$ and $CD45^-Coll^+CD115^+$ fibrocyte subpopulation in the BM of $Catnb^{+/ex3}$ and *Col3.6-Cre*; $Catnb^{+/ex3}$ mice at P6. B–C. Statistical analysis of the frequency of fibrocytes shown in (A). ***, $p < 0.001$, $n = 3$.

VEGF was increased by approximately 3–5-fold (Fig. 5E, G, I). In line with these results, an ELISA on the BM supernatant showed increased secretion of these signals in similar amounts (Fig. 5F, H, J). Collectively, the expression and secretion of pro-fibrotic signals were increased in BM stimulation by β-catenin, indicating that conserved molecular cascades are involved in BM fibrosis.

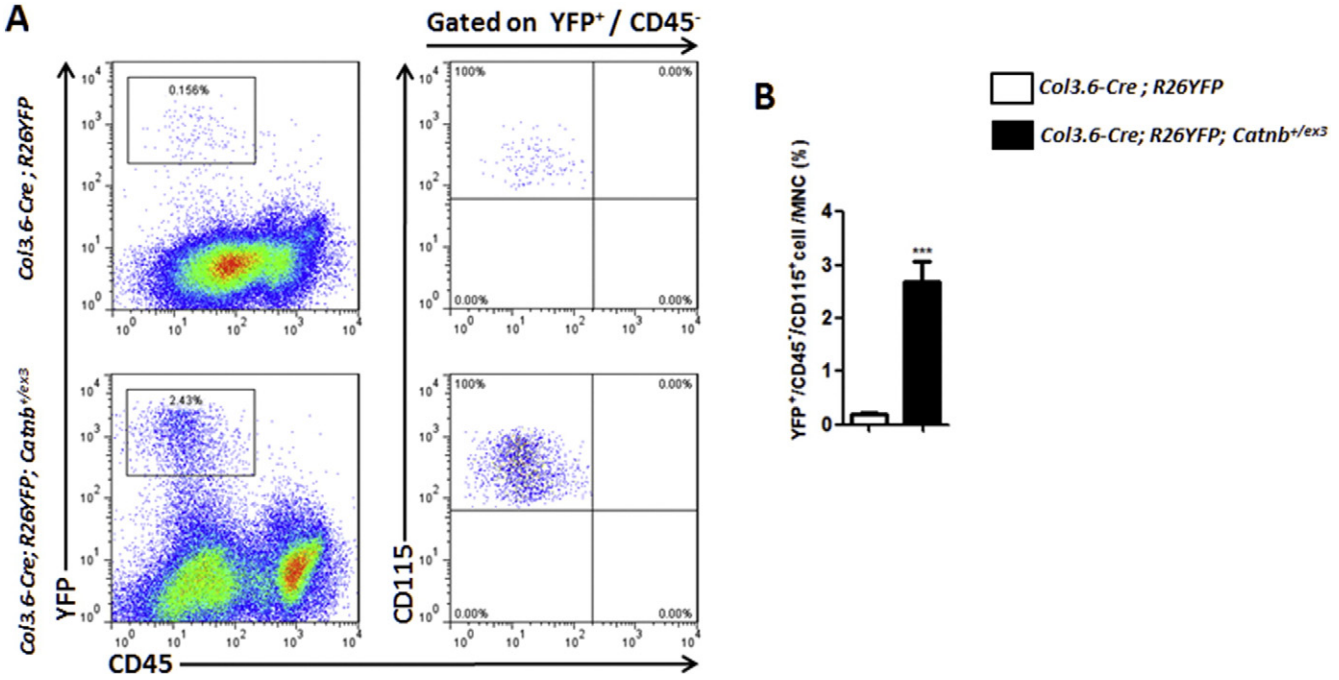

Fig. 4. Inflammatory fibrocytes are expanded in β-catenin GOF mutant BM. A. A dot plot depicts the YFP^+CD45^- fibrocytes are of $CD115^+$. B. Statistical analysis of the frequency of $YFP^+CD45^-CD115^+$ fibrocytes. All of the samples were isolated from the BM of $Catnb^{+/ex3}$ and *Col3.6-Cre*; $Catnb^{+/ex3}$ mice at P6. ***, $p < 0.001$, $n = 3$.

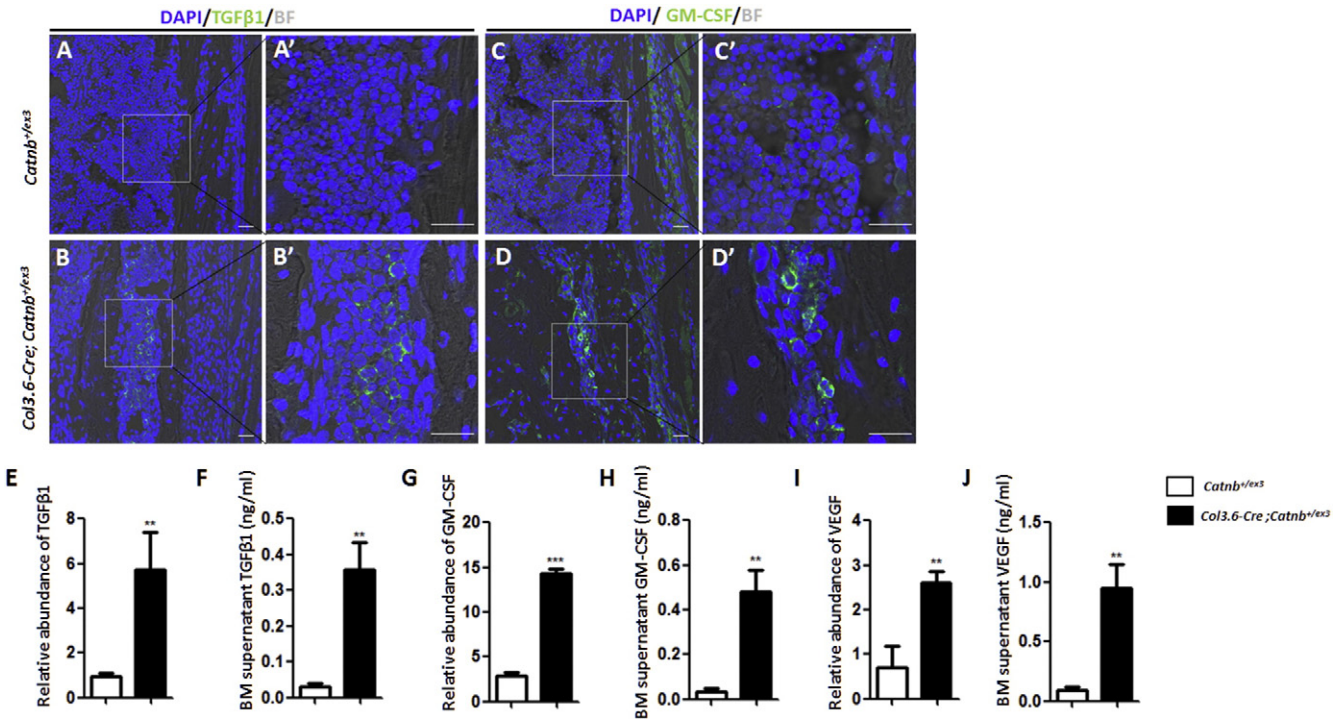

Fig. 5. Upregulation of pro-fibrotic signals by the stimulation of β-catenin in BM. A–D′. Immunostaining with anti-TGFβ1 (A–B′), anti-GM-CSF (C–D′) antibodies on the sections of mice. (A′–D′) are a view of (A–D) at higher magnification. E–F. Relative mRNA level of *TGFβ1* in osteoblasts (E) and TGFβ1 protein level in the BM (F) of mice. G–H. Relative mRNA level of *GM-CSF* in osteoblasts (G) and GM-CSF protein level in the BM (H) of mice. I–J. Relative mRNA level of *VEGF* in osteoblasts (K) and VEGF protein level in the BM (L) of mice. All of the samples are isolated from *Catnb*$^{+/ex3}$ and *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mice at P6. N = 3; **, $p < 0.01$, ***, $p < 0.001$. Bar, 25 μm. BF, bright field.

2.5. Infiltration of myeloid cells and T cells in BM of β-catenin GOF mutant

The enrichment of bone marrow stromal cells and fibrocytes usually accompanies immunoregulatory responses [7,28]. A previous report has shown that β-catenin overexpression in osteoblasts led to AML-like phenotypes characterized by the aberrant accumulation of myeloid progenitor cells in bone marrow [21]. In contrast, we detected distinct reactions of myeloid cells in the BM of *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mice. For instance, we observed an increase of differentiated CD11b^{+}Gr1^{+} myeloid cells (monocytes and granulocytes), coupled with a corresponding decrease of c-Kit^{+}CD11b^{+}Gr1^{+} undifferentiated immature myeloid cells (Fig. 6A, B, C). In addition, the frequency of CD11b^{mid}CD11c^{mid}F4/80^{low} monocyte-derived dendritic cells at early differentiating stage was markedly elevated (Fig. 6D, E). Most of the dendritic cells were also MHC-I$^{low/-}$ and MHC-II$^{low/-}$ (Fig. 6D, F, G), indicating they were immature. Furthermore, we also observed an obvious infiltration of CD4^{+} and CD8^{+} T cells in the *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mutants compared to the controls (Fig. 7A, B). Of note, the frequency of naïve CD62L^{+}CD4^{+} T cells were relatively enriched whereas the frequency of effector memory T-cells (Tem, CD62L^{-}CD44^{+}) was lower in the mutant BM (Fig. 7C, D). Taken together, we observed no increase of myeloid progenitor cells but detected the accumulation of differentiated myeloid cells, including monocytes, granulocytes and dendritic cells, as well as T cells in the mutant BM. These different immunoregulatory responses indicated the time-dependent effect of β-catenin activation on the BM microenvironment.

3. Discussion

3.1. Fibrocytes could be expanded and activated by β-catenin stimulation

Fibrocytes are an elusive cell population that originates from bone marrow and acts as effector cells in a variety of fibrotic diseases [29]. However, bone marrow fibrocytes are difficult to distinguish in transgenic mice. A previous report has shown that transgenic mice *Col1-GFP* generated by a rat *Collagen I* (2.3-kb) promoter contained fibrocytes that expressed CD45 and CD11b [14], as well as maturing osteoblasts [24]. Accordingly, we traced the cell lineages labelled by *Col3.6-Cre* in BM. Analyses of the reporter mice showed labelled fibrocytes that in our *Col3.6-Cre* mice located at the surface of endosteal niches (Fig. 2). They seemed to be components of endosteal niches along with osteoblasts as previously described [30]. The YFP^{+} fibrocytes could be found early in the myeloid progenitor population, consistent with the notion that fibrocytes are of monocyte lineage origin [29]. Additionally, our *Col3.6-Cre* did not label osteoclast progenitor cells, different from a previous analysis in *Col3.6-GFP* mice [23].

Constitutive accumulation of β-catenin in endosteal niches by our *Col3.6-Cre* mice contributed to a higher frequency of BM fibrocytes as well as the accumulation of Osterix^{+} osteoblast progenitor cells and myofibroblasts. Activation of β-catenin stimulated the expansion of two subpopulations of fibrocytes: CD45^{+}Coll^{+}CD115^{+} and CD45^{-}Coll^{+}CD115^{+} (Fig. 3). The latter population could be differentiating myofibroblasts because CD45^{+} fibrocytes would gain CD45^{-} when they underwent transformation into myofibroblasts [31]. Accordingly, the expanded myofibroblasts were characterized by YFP^{+}, α-SMA^{+} and Vimentin^{+} by immunostaining (Fig. 2). Myofibroblasts are also an important mediator of fibrosis [1, 26,32]. Of note, fibrocytes have also been reported to be very efficient antigen-presenting cells and capable of priming of naïve T cells, inducing acquired immune responses [7,33,34]. Therefore the accumulation and activation of fibrocytes and myofibroblasts by the β-catenin stimulation can partially contribute to the immunoregulatory responses in BM. Collectively, the β-catenin-triggered BM fibrosis resulted from the orchestrated action of fibrocyte accumulation, the activation of myofibroblasts and the enrichment of immune cells.

3.2. A conserved pro-fibrotic signal cascade underlies in BM fibrosis

The coordination of Wnt and TGFβ signaling has been implicated in fibrosis in multiple organs. For instance, Wnt signaling is necessary for

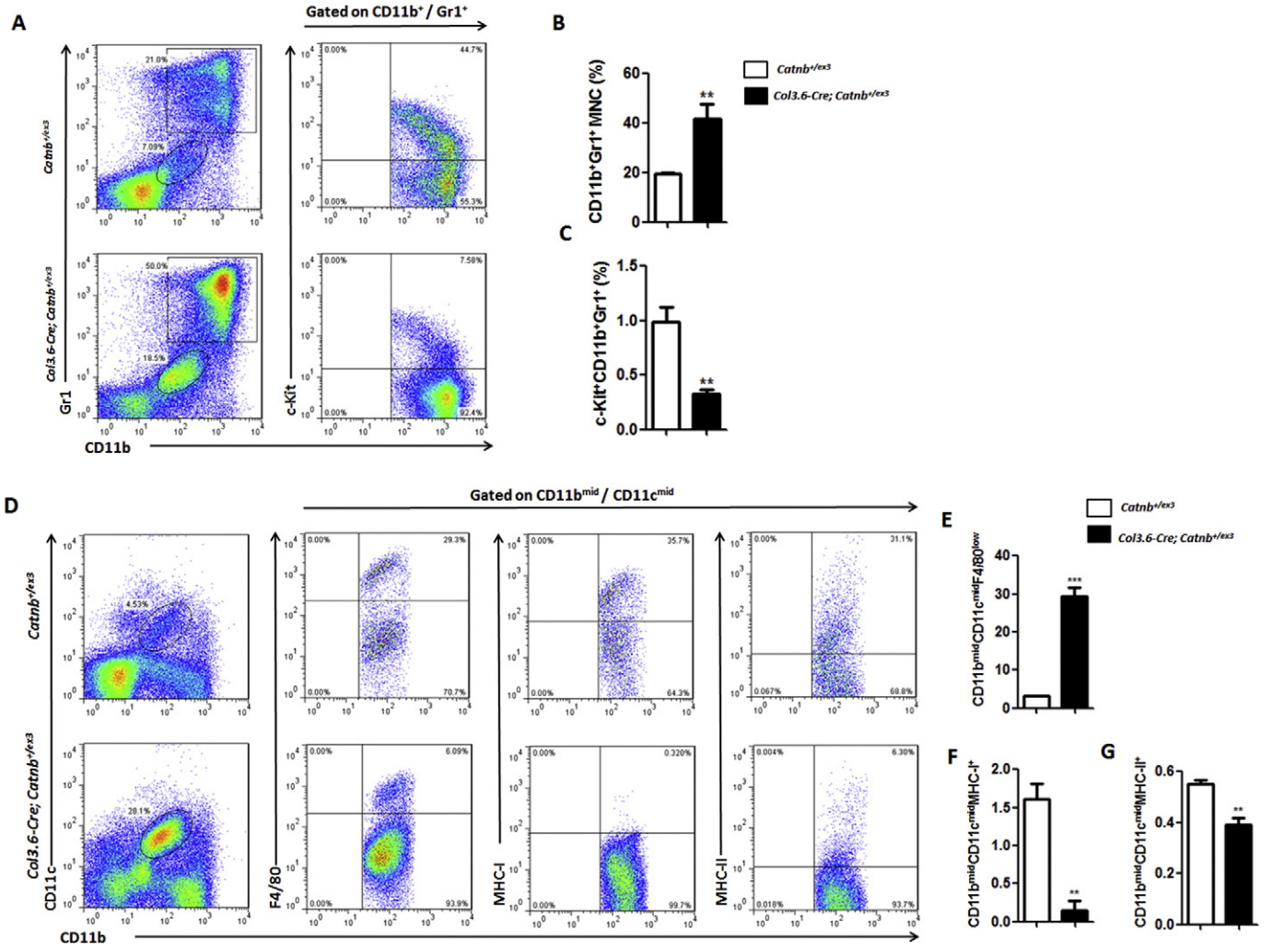

Fig. 6. Enrichment of differentiated myeloid cells in BM with the constitutive activation of β-catenin by *Col3.6-Cre*. A dot plot depicts an increase in differentiated $CD11b^+Gr1^+$ myeloid cells (monocytes and granulocytes) and a decrease in c-$Kit^+CD11b^+Gr1^+$ undifferentiated myeloid progenitor cells. B–C. Statistical analysis of the frequency of differentiated (B) and undifferentiated (C) myeloid cells in (A). D. A dot plot depicts the enrichment of $CD11b^{mid}CD11c^{mid}$ $F4/80^{low}$ monocyte-derived dendritic cells in the BM of *Col3.6-Cre*; $Catnb^{+/ex3}$ compared to $Catnb^{+/ex3}$ mice at P6, which are also MHC-I^{low} and MHC-II^{low}. E–G. Statistical analysis of the frequency of $F4/80^{low}$, MHC-I^+ and MHC-II^+ monocyte-derived dendritic cells dendritic cells. N = 3; **, $p < 0.01$, ***, $p < 0.001$.

TGFβ1-mediated fibrosis in skin as evidenced by the amelioration of skin fibrosis in mice that overexpress DKK1 and show constitutive activation of TGFβ receptor type I [13]. In this study, constitutive activation of β-catenin in osteoprogenitors enhanced the expression of TGFβ1, VEGF and GM-CSF (Fig. 5). Consistent with this, previous reports showed that Wnt/β-catenin signaling induces myofibroblast differentiation by upregulating TGFβ signaling [35]. β-catenin and Smad3 form a complex that induces α-SMA expression during the epithelial-mesenchymal transition and pulmonary fibrosis [36]. In addition, *VEGF* is a downstream target of β-catenin and TGFβ1 [37]. *VEGF* overexpression could induce BM fibrosis by potentiating β-catenin activity [15,38]. So our study results indicate that β-catenin may work with VEGF as a positive feedback to stimulate BM fibrosis. The upregulation of these pro-fibrotic signals accounted for the immunoregulatory responses observed in β-catenin mutant BM. For example, bone marrow stromal cells can also induce differentiating myeloid cells such as monocyte-derived dendritic cells (DCs) by secreting cytokines and paracrine factors such as GM-CSF and TGFβ1 [28,39]. VEGF prevents the dendritic cells from being mature [40]. As antigen-presenting cells, DCs play an important role in inducing T cell immunity and tolerance, depending on their maturation stage, and immature DCs have weak abilities to induce activation of T cells [41]. Therefore, we observed an increase in differentiating myeloid cells and immature dendritic cells in the mutant BM, which were partially consistent with previous work [21]. Collectively, we speculate that a conserved pro-fibrotic signal cascade consisting of β-catenin/TGFβ1/VEGF underlies the development of BM fibrosis.

Another interesting finding is the distinct impact of β-catenin activation on the bone marrow microenvironment at different stages of osteoblast differentiation. For example, a threshold of Wnt/β-catenin signaling activity is precisely controlled during the osteogenic commitment in mesenchymal progenitor cells during skeletal development. The ablation of β-catenin in skeletal or osteoblast progenitor cells impairs osteoblast differentiation [42,43]. In contrast, high level of β-catenin expression in osteoblast precursors leads to FD-like phenotypes with the aberrant accumulation of immature osteoblasts in bone marrow [12]. Furthermore, alterations in the β-catenin level in mature osteoblasts do not influence osteoblast proliferation but couple osteoclast differentiation with OPG secretion [20]. Similarly, a decrease in the β-catenin level in osteocytes perturbs bone homeostasis and anabolic responses to mechanical loading [44,45]. Conversely, an higher β-catenin level in osteocytes increases bone mass but impairs bone mineralization [46]. The constitutive expression of β-catenin in osteoblasts could induce leukemogenesis in BM through enhancing Notch signaling activity [21]. Differently in our study, the activation of β-catenin in osteoblast progenitors increased differentiated myeloid cells as well as

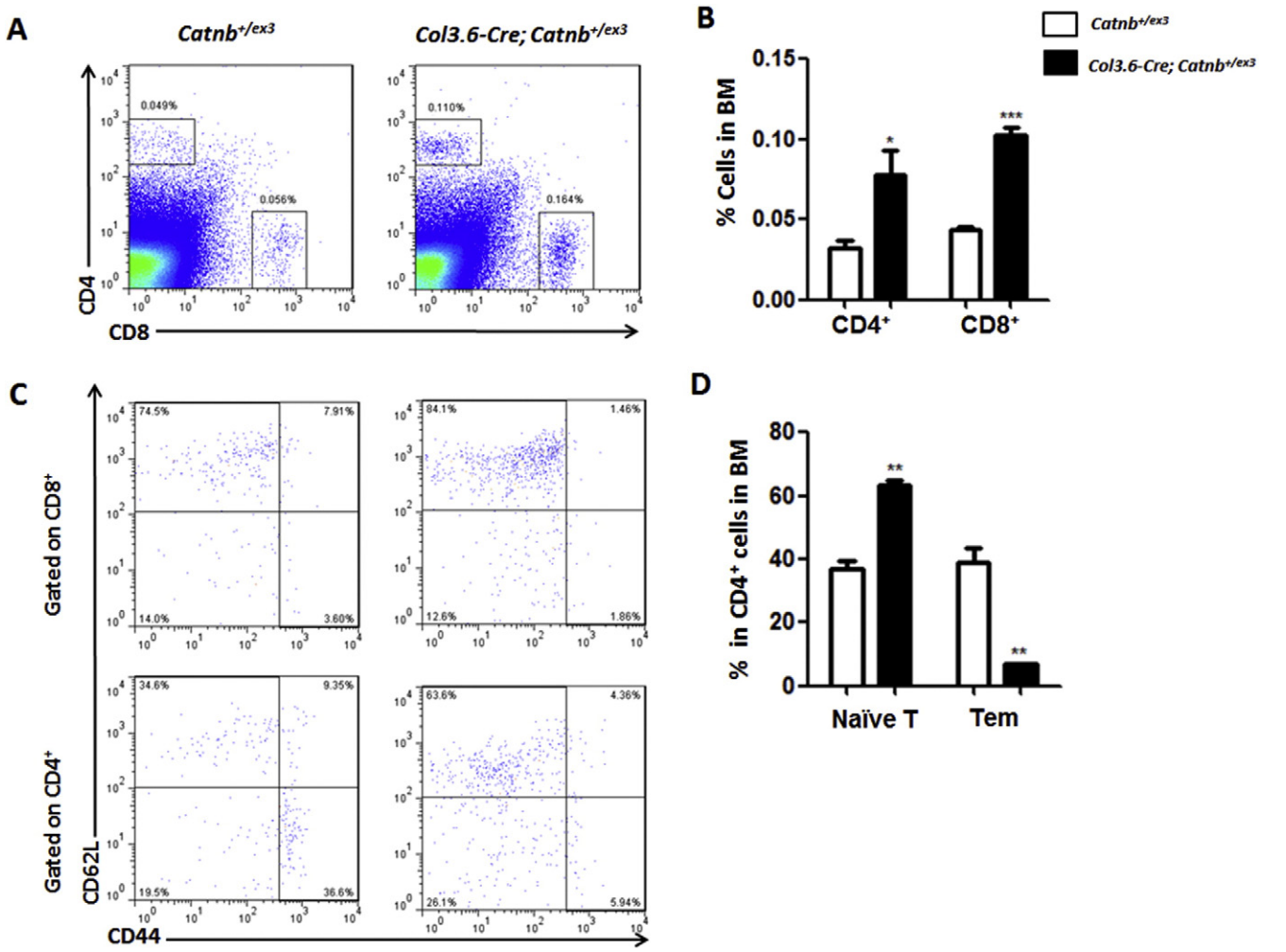

Fig. 7. Infiltration of naïve T cells in the BM of *Col3.6-Cre*; *Catnb*$^{+/ex3}$ mice. A. A dot plot depicts the CD4$^+$ and CD8$^+$ T cells in the bone marrow. B. Statistical analysis of the frequency of T cells in (A). C. A dot plot shows that most of the CD4$^+$ and CD8$^+$ T cells are CD62L$^+$CD44$^-$. D. Statistical analysis of the frequency of naïve CD4$^+$ T cells and effector memory T cells in (C). N = 3; *, $p < 0.05$, ***, $p < 0.001$.

infiltration of naïve CD4$^+$ T cells in bone marrow, which was not observed in the BM with β-catenin activation in mature osteoblasts. Canonical Wnt signaling has a time- and dose-dependent effect on bone development and bone marrow maintenance. Because Wnt/β-catenin signaling contributes to the development of Gsα-mediated bone FD, an investigation of the involvement of TGFβ1-mediated fibrosis and inflammation in the biopsies of FD patients is warranted. Our findings may have widespread physiological and clinical ramifications for the treatment of bone FD.

4. Materials and methods

4.1. Mice

β-catenin activation mutant mice [47], *Col3.6-Cre* (*3.6-kb* promoter) mice [48], *R26YFP* (#006148 in Jax lab) and *TopGal* (#004623 in Jax lab) mice were described previously. Mice with constitutive activation of β-catenin were generated by crossing *Catnb*$^{+/ex3}$ mice with *Col1-Cre* mice expressing Cre under the control of 3.6 kb of the promoter of the mouse pro-α1(I) collagen gene. We also used the Cre-dependent EYFP reporter strain *R26R-EYFP* and canonical Wnt signaling reporter TOPGAL mice to trace cell lineages and the location of the canonical Wnt signal upon constitutive activation of β-catenin. Mice were maintained in a specific pathogen-free environment with free access to food and water and a 12/12 light–dark cycle. All animal experiments were conducted in accordance with guidelines set by Bio-X Institutes in Shanghai Jiao Tong University.

4.2. Histology and immunostaining analyses

The hindlimb bones of P6 were dissected, fixed in 4% paraformaldehyde, decalcified in 10% EDTA and embedded in paraffin or OCT for longitudinal sectioning (10 μm). Masson's trichrome staining and HE staining were performed according to standard procedures to detect fibrosis. For immunohistochemistry, the primary antibodies included rabbit anti-β-galactosidase (Rockland), rabbit anti-Osterix (Abcam), mouse anti-α-SMA (Sigma), goat anti-vimentin (Sigma), rabbit anti-GM-CSF (Abcam), rabbit anti-TGF-β (Abcam), rabbit anti-VEGF (Abcam), rabbit anti-GFP (MBL), and goat anti-GFP (GeneTex). The secondary antibodies used were the Alexa Fluor 488 conjugated and the Alexa Fluor 594 conjugated second antibody (Abcam).

4.3. Cell preparation and FACS

After the mice were killed by standard procedures, whole bone marrow were isolated using 2% FBS (Gibco) in PBS and filtered through 70 μm cell strainers. Cell suspensions were first treated with Fc Block (BD Pharmingen) and then incubated with specific antibodies for surface marker staining. PerCP-Cy5.5-conjugated streptavidin, PE-anti-Rat IgG, Sca1-FITC, c-Kit-APC, CD62L-PE, CD34-PE, B220-PerCP-Cy5.5,

CD19-APC, F4/80-PE, MHC-I-PE, MHC-II-PE, CD115-PE, CD11b-Alexa Fluor 488, Gr-1-PE, CD4-APC, and CD8a-PerCP-Cy5.5 and biotin conjugated markers, including CD11c, B220, Ter-119, CD11b, TCRβ, CD3 and Gr-1, were obtained from eBioscience, CD45-APC; CD44-PE; and CD16/32-APC/Cy7 were obtained from Biolegend. For intracellular staining, the cells were fixed with 1% paraformaldehyde, incubated for 30 min at 4 °C in the dark, washed with PBS, and permeabilized with 0.5% saponin and 1% BSA in PBS. Subsequently, the cells were incubated with rabbit anti-collagen I (Rockland) and rabbit IgG (Santa Cruz), followed by the secondary antibodies goat anti-rabbit IgG-FITC (Proteintech) or goat anti-rabbit IgG-PerCP-Cy5.5 (Santa Cruz). Cells were analyzed on a FACS Calibur instrument, and data were analyzed using FlowJo software.

4.4. RNA extraction and qRT-PCR

The long bones were crushed in liquid nitrogen and homogenized after bone marrow cells were flush away. RNA was extracted using TRIzol reagent (Invitrogen) according to standard procedures. cDNA was synthesized using Oligo dT primers and Superscript II reverse transcriptase (Promega). Real-time PCR was performed on an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using a SYBR Green Kit (Roche). The samples were normalized to β-actin expression. The following primers were used: TGF-β1, sense: 5′-GTGCGGCAGCTG TACATTGACTTT-3′, antisense: 5′-TGTACTGTGTGTCCAGGCTCCAAA-3′; VEGF, sense: 5′-CTTGTTCAGAGCGGAGAAAGC-3′, antisense: 5′-ACAT CTGCAAGTACGTTCGTT-3′; GM-CSF, sense: 5′-CCTTGAACATGACAGCCA GCTA-3′,antisense: 5′-CTATGAAATCCGCATAGGTGG TAA-3′

4.5. ELISA assay

The mice were killed to obtain their long bones. The bone marrow was isolated with 0.5 ml PBS per mouse and centrifuged for 5 min at 300 ×*g*. Then, the supernatant was collected for ELISA assay by centrifuging the cell suspension for 5 min at 300 ×*g*.

4.6. Statistics

Statistical analysis was performed by Student's t test using GraphPad Prism 5 software. Data are represented as the means ± SEM and significance was set at $p \leq 0.05$.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2015.12.003.

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