Heterotopic ossification in mice overexpressing Bmp2 in Tie2+ lineages

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INTRODUCTION

Bone morphogenetic proteins (BMPs) regulate fundamental processes in development and organismal homeostasis [1]. During canonical Bmp signaling, BMPs ligands bind to BMP type I receptors (BMPRIs), or activin-like kinase (ALK) 2, 3, or 6. This complex binds to BMP type II receptor (BMPRII), which phosphorylates BMPRI, which in turn phosphorylates regulatory-Smads (Smad1/5/8). Phosphorylated Smad1/5/8 binds to nuclear Smad4, forming a nuclear complex that accumulates in the nucleus, where it is recruited to transcriptional complexes to mediate BMP-driven gene expression [2]. BMPs were discovered owing to their fundamental role in bone formation and homeostasis [1], and BMP2 is critical for chondrocyte proliferation and endochondral bone maturation, and necessary for bone fracture healing [3, 4].

Heterotopic ossification (HO) is bone formation at extra-skeletal sites, including muscle, tendon, ligament, and other connective tissues, and a complication of injury and surgery [5, 6]. HO occurs through intramembranous and endochondral bone formation, resembling fracture repair processes. Lesions are characterized by immune infiltration in damaged connective tissue, which is eventually replaced by endochondral bone through fibroblast proliferation, mesenchymal condensation, and chondro-osseogen differentiation [6, 7]. Subsequently, the woven bone gives way to the lamellar bone and marrow stroma, with hematopoietic progenitors, adipocytes, osteoblasts and osteoclasts, while capillary-like vessels give rise to bone marrow (BM) sinusoid-type vessels.

Acquired HO is relatively common but its etiology is poorly understood [6]. In contrast, genetic forms, like Fibro dysplasia ossi cans progressiva (FOP; OMIM #135100, ORPHA337) are rare, but provide mechanistic insight [8–10]. FOP patients have progressive spontaneous and injury-induced HO resulting in complete mobility loss. FOP is caused by a mutation in the gene encoding the type I ACVR1/ALK2 BMP receptor [11]. The ACVR1-R206H mutant receptor acquires the ability to respond to the TGFß family ligand Activin A [12, 13], and becomes sensitive to other BMPs [14–16]. ACVR1 mutations alone cannot explain the recurrent “flare-ups” resulting in extra skeletal ossification.
following trauma, muscular fatigue, or other inflammatory insults, which also trigger acquired forms of HO. The innate immune system [17, 18] and local “niche” soft tissue microenvironment [19, 20] need to be further characterized to help clarify this issue. Moreover, Activin A seems to play a significant role in the initial steps of FOP during immune infiltration subsequent to injury. However, once ectopic bone is fused to the normal bone skeleton, additional canonical BMP ligands may be required to sustain ectopic bone development.

The identification of bone osteoprogenitors has generated considerable interest [21, 22]. Skeletal muscle-resident cells including myoblasts [23, 24], satellite cells [25] or fibroangioprogenitors (FAPs) have osteogenic differentiation ability [26, 27]. Hematopoietic progenitors participate in bone formation at sites of tissue inflammation, but are insufficient to initiate this process [28, 29]. Endothelial cells in mice constitutively expressing ACVR1-R206H, transform into mesenchymal cells with progenitor properties, that give rise to ectopic bone [30]. However, lineage tracing using a Tie2Cre driver line and local transplantation of Tie2Cre-derived endothelial cells into skeletal muscle have excluded the endothelium as a source of ectopic bone formation [31]. Rather, non-endothelial Tie2Cre− resident skeletal muscle stem cells including FAP osteoprogenitors appear to be the principal FOP cells-of-origin [31, 32].

In view of ACVR1 receptor activation complexity, the uncertainty of target osteo-progenitors, and modulating “niche” factors, disease modeling is necessary to better understand both acquired and genetic HO. Bmp2 plays fundamental roles in cardiac valves formation and heart chamber patterning [33, 34], but its cardiac overexpression causes lethality [35, 36]. To study Bmp2 vascular gain-of-function postnatally, we generated Tie2Cre;Bmp2tg/tg mice, which overexpress Bmp2 in hematopoietic/endothelial lineages. These mice survive birth, develop pre-calcific valve disease and a systemic bone disorder in skeletal muscle and other connective tissues, resulting in severe skeletal deformities whose nature we have investigated.

RESULTS

Endothelial Bmp2 overexpression results in valve dysfunction

We previously generated a transgenic mouse line in which CAG-driven Bmp2 expression is activated upon Cre-mediated removal of a β-Geo-stop cassette [33] (Supplementary Fig. 1A). We crossed the CAG-Bmp2 allele with Tie2Cre line, which is active in hematopoietic/endothelial lineages from E7.5 onwards [37]. Vascular GFP reporter expression was observed at E9.5, confirming Cre-mediated recombination (Supplementary Fig. 1B).

Ectopic Bmp2 signaling leads to osteogenic differentiation of valve interstitial cells [38]. To determine the effect of increased endothelial Bmp2 expression on valve function, we generated Tie2Cre;Bmp2tg/tg mice. At 16 weeks, circulating Bmp2 levels were almost six-fold higher than in WT animals (Fig. 1A). Tie2Cre;Bmp2tg/tg mice showed shortened pulmonary acceleration time and acceleration to ejection time ratio by ultrasound (Fig. 1B), indicating pulmonary hypertension potentially leading to respiratory insufficiency. Tie2Cre;Bmp2tg/tg mice displayed significantly increased aortic valve mean, peak velocity and pressure gradient (Fig. 1C, D). Three of seven animals displayed chordogenic and lipid droplet islands at the leaflet base (Fig. 1E), indicative of pre-calcific disease. These results indicate that ectopic endothelial and/or hematopoietic Bmp2 expression leads to aortic valve dysfunction compatible with a pre-calcific valve stage.

Hematopoietic/endothelial Bmp2 overexpression causes a HO

During these studies, we found that Tie2Cre;Bmp2tg/tg mice develop severe scoliosis and ankylosis with depressed locomotor behavior and respiratory insufficiency. Extensive HO was diagnosed by PET-CT at 16 weeks of age and confirmed at autopsy (Fig. 2A; Supplementary Fig. 1C; Supplementary Table 1). Localized HO lesions were observed in 95% (19/20) Tie2Cre;Bmp2tg/tg animals by nano PET-CT (Supplementary Table 1, Supplementary Videos 1–5). Only one of 20 mice remained HO-free (Supplementary Table 1). HO was manifested as fused cervical spine and scapula extensions (47.2%) resulting in severe scoliosis (Fig. 2A; Supplementary Videos 1, 2), and as bony plaques adjacent to the chest wall (55%; Supplementary Videos 3). Several animals (65%) displayed bone outgrowths in hindlimbs (Supplementary Table 1; Supplementary Movies 4, 5). At 16 weeks, 70% of animals presented HO in multiple areas, while by 20 weeks, 100% presented severe HO lesions extending to most of the areas described (Supplementary Fig. 1C, Supplementary Table 1 and Supplementary Video 6). Of 7 heterozygous Tie2Cre;Bmp2tg/+ animals analyzed by nano PET-CT at 16 and 32 weeks, only 2 developed mild lesions by 32 weeks (Supplementary Table 1 and Supplementary Fig. 1D). Circulating Bmp2 levels for heterozygous transgenic mice at 24 weeks was 1.5-fold compared with WT (Supplementary Fig. 1E), while Bmp2 levels of homozygous transgenic Tie2Cre;Bmp2tg/tg animals were 5–6 fold, suggesting that HO is highly sensitive to Bmp2 levels.

HO was associated with typical histopathological changes, including mononuclear cell infiltration (Fig. 2B, a and a’), fibroblast accumulation (Fig. 2B, b and b’), and chondro-osteogenic overgrowths (Fig. 2B, c, c’ and d’ and d’) and development of a BM-like stroma (Fig. 2B, d and d’). Chondrogenic tissue organization in the epiphysis and head of the femur and tibia was similar in Tie2Cre;Bmp2tg/tg and WT animals (Fig. 2C, a, a’, b, b’). However, additional cartilage was present in joints of Tie2Cre;Bmp2tg/tg mice, including osteochondral patches surrounding the tibial head and neck (Fig. 2C, b and b’). Ectopic bone was present adjacent to the normal bone in transgenic animals (Fig. 2C), and bone mass was increased in Tie2Cre;Bmp2tg/tg compared to WT animals (Fig. 2C, c, c’, d’ and d’). We measured bone mass density (BMD) in tibia and femur bones in WT and Tie2Cre;Bmp2tg/tg animals [39]. BMD was increased about 20% in Tie2Cre;Bmp2tg/tg mice compared to WT (9226 vs. 7720 Hounsfield Units; Supplementary Fig. 2A, B). Ossification of the meniscus (Fig. 2C, d and d’) would likely result in limb immobilization in transgenic animals. Thus, Tie2Cre;Bmp2tg/tg mice show similar histopathological lesions found in human HO.

Hematopoietic progenitors contribution to HO in Tie2Cre;Bmp2tg/tg mice

Tie2Cre cells are potential osteogenic progenitors in HO [21, 30, 31]. To characterize the hematopoietic contribution to HO in Tie2Cre;Bmp2tg/tg mice, bone marrow (BM) was isolated from normal, and ectopic bone of fore- and hindlimbs, scapulae, hips, and sternum of Tie2Cre;Bmp2tg/tg mice, and processed separately. The combined total BM (normal and ectopic bone) cell number was increased in Tie2Cre;Bmp2tg/tg mice (Fig. 3A, left panel), although there were important variations in the total hematopoietic cell number in ectopic bone (Fig. 3A, right panel) due to uneven HO in individual animals. Granulocyte-monocyte (GM) precursors colony forming-units (CFU) were increased in Tie2Cre;Bmp2tg/tg mice (Fig. 3B), suggesting that enforced Bmp2 expression in Tie2Cre cells drives hematopoietic progenitor expansion. Interestingly, ectopic bone marrow formed CFU-GM, to a lesser extent than normal WT or tg BM (Fig. 3B, right panel). CFU-GM isolated from transgenic spleen and peripheral blood (PB) were unchanged (Supplementary Fig. 1D).

To support the evidence of increased hematopoietic progenitors in Tie2Cre;Bmp2tg/tg BM, we analyzed LinSca1− c-Kit+ (LSK) cells. This population was increased in total BM (normal and ectopic bones) of Tie2Cre;Bmp2tg/tg mice (Fig. 3C, left panel), and present in ectopic BM (Fig. 3C, middle panel). Consistent with widespread Tie2 expression in hematopoietic progenitors [37], more than 93% of the LSK population in tg normal and ectopic BM was GFP+ (Fig. 3C, right panel), reflecting an important contribution of hematopoietic cells to ectopic Bmp2 production and BM formation.
Constitutive endothelial Bmp2 overexpression results in aortic valve dysfunction and pre-calcification. A Circulating Bmp2 levels detected by ELISA in WT and Tie2 \(^{\text{CRE}}\);Bmp2 \(^{\text{tg}}\) adult mice serum. B Quantification of pulmonary acceleration time (PAT, left panel), and PAT-ejection time ratio (PAT/PET, right panel) measured by ultrasound on 16-week-old WT and Tie2 \(^{\text{CRE}}\);Bmp2 \(^{\text{tg}}\) mice. C Quantification of the aortic valve velocity (AoV Mean and Peak Vel), and pressure gradient (AoV Mean and Peak Grad) measured by ultrasound. D Representative images of acquired data of the aortic velocity peaks detected by ultrasound in WT (\(\approx 1000\) mm/s) and tg animals (\(\approx 1200\) mm/s). E Top panels: Masson trichromic staining on consecutive sections of aortic valve from 18-week-old WT and Tie2 \(^{\text{CRE}}\);Bmp2 \(^{\text{tg}}\) mice. Chondrocyte island (arrow) in aortic annulus at the base of the leaflet. Bottom panels: Localization of lipid droplets (arrowheads) identified by Oil Red O staining. Unpaired t test, two tails, mean ± SD *\(P < 0.05\); **\(P < 0.001\); ***\(P < 0.0001\); ns, non-significant. Scale bar 200 µm.
We queried whether inflammation was required for HO in Tie2^{CRE/+};Bmp2^{tg/tg} mice. FACS analysis revealed that the pro-inflammatory CD11b^{+}Gr1^{+} cell population was increased in combined (normal and ectopic) Tie2^{CRE/+};Bmp2^{tg/tg} BM (Fig. 3D, left panel), whereas there was no difference in tg or WT BM (Fig. 3D, right panel). Circulating CD11b^{+}Gr1^{+} cells were marginally increased (p = 0.06; Fig. 3E, left panel), although their numbers increased in PB (Supplementary Fig. 1E, left panel), indicating that a greater proportion of inflammatory cells are mobilized from Tie2^{CRE/+};Bmp2^{tg/tg} BM. We monitored inflammation onset in 4 WT and 4 Tie2^{CRE/+};Bmp2^{tg/tg} mice for 7 weeks (at 13, 19, and 21 weeks; Fig. 3E, right panel), and by the time all the Tie2^{CRE/+};Bmp2^{tg/tg} mice had developed HO, their inflammatory cell numbers had increased in PB (Supplementary Fig. 3E, right panel).

We tested fibroblast (Fb) and osteoblast (Ob) colony-forming potential in Tie2^{CRE/+};Bmp2^{tg/tg} normal and ectopic BM. Fb (Fig. 3F) and Ob (Fig. 3G) CFUs readily formed from WT and transgenic mice BM, from ectopic BM of transgenic animals (Supplementary
Fig. 2  Constitutive Tie2-driven Bmp2 expression causes HO lesions in mice. A Nano PET-computed tomography (CT) images of 16-week-old WT and Tie2 CRE/-;Bmp2 tg/tg mice showing ectopic bone lesions close to ribs, scapulae, and neck (red arrows, ribs, rb; nk, neck; dorsal vertebrae, dv). B H&E staining on sections of skeletal muscle of the hindlimbs of Tie2 CRE/-;Bmp2 tg/tg mice showing histological features typical of HO lesions. a. Evidence of inflammation in HO lesion. a’ Damaged skeletal muscle fibers with central nuclei (arrows), mononuclear infiltration (black arrowheads), and fat cells (white arrowheads). b, b’ Area of massive fibroblast accumulation. c Ectopic bone in skeletal muscle (arrows) next to tibia. c’ Chondro-osteogenic areas with chondrocytes (white arrowhead), and osteoblasts (black arrowheads). d, d’ Mature ectopic bone with colonizing bone marrow cells (arrows), chondrocytes (white arrowhead), and osteoblasts (black arrowhead). c Top panels: Alcian blue staining of sections of WT and Tie2 CRE/-;Bmp2 tg/tg knee joint. a, a’ In WT, chondrocyte tissue (in blue) is located in the epiphysis region, tip of the bone (black arrowheads), and head of the fibula (arrow). b, b’ In Tie2 CRE/-;Bmp2 tg/tg, chondrocyte tissue is located in the epiphysis region at the tip of the bone (black arrowheads), accumulated in connective tissue (arrow) of the meniscus and head of the fibula (white arrowhead) and chondrogenic areas (white arrowhead) inside the skeletal muscle. Bottom panels: Alizarin red staining of WT and Tie2 CRE/-;Bmp2 tg/tg knee joint. c, c’ In WT, osteogenic tissue (in red) is located in the epiphysis region and tip of the bone complementary to chondrogenic areas (white arrowheads). d, d’ Intense staining in the head of Tie2 CRE/-;Bmp2 tg/tg joints (white arrowheads in (d)). Extra ossification inside the skeletal muscle and head of the fibula (arrows in (d)), and the meniscus (black arrowhead). Scale bar 200 μm.

We queried if Tie2-driven Bmp2 expression in Tie2+ cells promotes erythropoiesis in vivo.

Hematopoietic cells overexpressing Bmp2 do not trigger HO in wild-type mice

We studied if Tie2+ Bmp2-expressing BM hematopoietic cells can give rise to HO in wild-type (WT) mice. Eight-week-old lethally irradiated C57BL/6 WT mice were transplanted with BM hematopoietic stem cells (HSCs) from either WT (Bmp22/+) or Tie2 CRE/-;Bmp2tg/+ mice (Supplementary Fig. 3A). Hematopoietic cell (CD45+) engraftment, was examined by FACS five months after BM transplantation and thereafter bimonthly for over a year. WT mice transplanted with WT HSCs engrafted between 95-100% of CD45+ cells. WT mice transplanted with Tie2 CRE/-;Bmp2tg/+ HSCs (Supplementary Fig. 3B) showed almost four-fold after 10 months in WT animals transplanted with Bmp2-overexpressing hematopoietic cells (Supplementary Fig. 3C). Hematopoietic-derived Bmp2 accounted for −50% Bmp2 levels found in Tie2 CRE/-;Bmp2tg/+ mice (Supplementary Fig. 1A and Supplementary Fig. 3B). We analyzed multi-lineage reconstitution in PB (CD11b+, CD3+, and CD45+) by FACS every month for 12 months. Whilst myeloid lineage reconstitution was unaffected, B and T lymphoid cells were decreased at all time points (Supplementary Fig. 3D). Bmp2/4 has been shown to antagonize T-cell lineage differentiation [41, 42]. None of the transplanted animals developed HO by Nano-PET-CT (data not shown), even 12 months after the BM transplant assay. Thus, Bmp2 secreted by hematopoietic Tie2 CRE/-;Bmp2tg/+ cells in WT mice is not sufficient to drive HO. Alternatively, local expression of other Tie2-Cre-targeted cells is crucial to initiate flare-ups and HO.

Transplanting WT BM into Tie2 CRE/-;Bmp2tg/+ mice delays HO onset

We asked whether Bmp2 dosage affected HO formation in Tie2 CRE/-;Bmp2tg/+ mice. Eight-week-old lethally irradiated CD1 mice were used for transplant assays, because Tie2 CRE/-;Bmp2tg/+ mice were in a CD1-enriched mixed background. WT mice were transplanted with 5 million BM nucleated cells from WT (Bmp22/+) (n = 10) or Tie2 CRE/-;Bmp2tg/+ mice (n = 10) (Fig. 4A and Supplementary Fig. 4A). A second group of 8-week-old lethally irradiated Tie2 CRE/-;Bmp2tg/+ mice (n = 7) was transplanted with WT (Bmp22/+) HSCs in CD1-enriched mixed background (Fig. 4A). Three transplanted WT mice died after 19 days, and could not be analyzed further. In four, GFP was either not expressed or expressed in 15–20% of total HSCs (Supplementary Fig. 4C, left panel) and excluded. Three surviving mice expressed GFP (Supplementary Fig. 3C, right panel). Circulating Bmp2 levels in two of these mice were comparable to those found in Tie2 CRE/-;Bmp2tg/+ mice (5.9-fold increased) (Supplementary Fig. 4B). Transgenic mice transplanted with WT HSCs had comparable Bmp2 levels to controls (Fig. 4B), confirming the hematopoietic origin of circulating Bmp2.

To determine lineage contributions to BM reconstitution, blood samples were analyzed by FACS monthly over five months following transplantation. CD45+ cell engraftment in control and WT groups transplanted with Tie2 CRE/-;Bmp2tg/+ HSCs (n = 3) was comparable, reaching 90–98% (Supplementary Fig. 4C). Tie2 CRE/-;Bmp2tg/+ animals transplanted with WT HSCs reached 80–95% engraftment (Fig. 4C). Myeloid CD11b+ cell engraftment was similar between groups (Supplementary Fig. 4D and Fig. 4D, left panel). Lymphoid B220+ cells increased during the first month compared to controls (Supplementary Fig. 4D, right panel, Fig. 4D, right panel). In contrast, CD3+ T lymphoid cells were not reconstituted to WT levels when transplanted with Tie2 CRE/-;Bmp2tg/+ HSCs (Supplementary Fig. 4D, right panel, Fig. 4D, right panel). Thus, B Lymphoid cells were initially depleted in Tie2 CRE/-;Bmp2tg/+ mice transplanted with WT HSC but recovered, while T cells remained depleted for the entire duration of the experiment.

Tie2 CRE/-;Bmp2tg/+ mice transplanted with WT hematopoietic cells eventually develop HO, likely because HO was already taking place at the time of transplantation (8 weeks). Five animals presented typical HO with variable severity, including complete hindlimb immobilization by 16 weeks (n = 1), dorsal vertebrae at 20 weeks (n = 1), and hindlimb and dorsal vertebrae at 22 weeks (n = 2) (Fig. 4E). One mouse presented a fat cyst dorsally but no HO, and another developed HO in dorsal vertebrae at 26 weeks (not shown). One mouse remained asymptomatic 37 weeks after transplantation, thus, 5 out of 7 Tie2 CRE/-;Bmp2tg/+ mice reconstituted with WT hematopoietic cells developed HO, with delayed onset compared to non-transplanted animals. Disease onset in Tie2 CRE/-;Bmp2tg/+ mice occurred at 16–28 weeks in transplanted mice, versus 8–18 weeks in non-transplanted ones (Fig. 4F, left panel). Hence, Tie2 CRE/-;Bmp2tg/+ mice transplanted with WT HSCs survive 10 weeks longer than non-transplanted transgenics (Fig. 4F, right panel). Therefore, Bmp2 expression in non-hematopoietic cells is essential for HO development, and concomitant Bmp2 expression by hematopoietic cells accelerates this process.

Chondro-osteogenic differentiation is associated with BMP signaling activation in FAP cells

Tie2 marks a subset of resident skeletal muscle cells [31], which potentially contribute to HO. Tie2 CRE/-;Bmp2tg/+ cartilage and bone lesions showed GFP+ co-staining with IB4+ (Fig. 5A), co-localizing with Tie2 fibroblast/adipocytic skeletal-muscle cells
GFP+ infiltrating inflammatory cells expressing IB4 were detected in fibroproliferative areas (Supplementary Fig. 5A), and have been previously described [43]. GFP-expressing fat cells were identified based on their peripheral nuclei (Supplementary Fig. 5B, b'). Phospho-Smad1/5-stained nuclei were detected in scattered cells in fibroproliferative areas (Fig. 5A, a' and b' and Supplementary Fig. 5B, a'), as well as among fat cells (Supplementary Fig. 5B, b'). Bmp2 activation may be non-cell autonomous because p-Smad-positive cells did not express GFP (Fig. 5A, a' and b' and Supplementary Fig. 5B, a').

The chondrogenic marker Sox9 was expressed in ectopic bone chondrocyte nuclei (Supplementary Fig. 5C, left panel). Scattered Sox9+ nuclei were also found in GFP-expressing fibroblasts (Supplementary Fig. 5C, d') and damaged skeletal muscle fiber cytoplasm identified by central nuclei (Fig. 5B, c' and d', and Supplementary Fig. 5C, c'). This expression pattern was also observed in areas next to HO, although involving very few nuclei (Supplementary Fig. 5C, c', open arrowheads). The osteogenic marker Osterix, was also detected amongst Sox9-expressing cells in consecutive sections (Fig. 5C, e' and f') and in ectopic bone osteoblasts (Supplementary Fig. 5D, arrowheads).
**Fig. 3** Hematopoietic stem cells contribute to HO in Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice. A. Quantiﬁcation of bone marrow (BM) cellularity. Left, total BM (combined BM of transgenic (tg) and ectopic bone (EB)) cellularity is increased in Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice. Right, cellularity of WT BM, tg BM, and ectopic BM. Ectopic BM cellularity is highly variable. B. Quantiﬁcation of granulocyte, monocyte colony forming units (CFU-GM). Left, total BM (tg + EB) CFU-GM was increased in Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice. Right, ectopic BM can give rise to CFU-GMs. C. Quantiﬁcation of the FACS-isolated Lin<sup>−</sup>Sca1<sup>−</sup>Kit<sup>−</sup> population. Left, the LSK population is increased in Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> BM. Middle, LSK progenitors in ectopic BM are detectable and below normal. Right, the bulk (93.6% in tg and 93.2% in ectopic BM) of the LSK population in Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> BM is GFP<sup>+</sup>. D. Quantiﬁcation of the FACS-isolated pro-inﬂammatory Cd11b<sup>+</sup>Gr1<sup>+</sup> cells from BM. Left, the Cd11b<sup>+</sup>Gr1<sup>+</sup> population is increased in total Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> BM (tg + EB). Right, varying presence of Cd11b<sup>+</sup>Gr1<sup>+</sup> cells in ectopic BM. E. FACS-isolated pro-inﬂammatory Cd11b<sup>+</sup>Gr1<sup>+</sup> cells from PB. Left, Cd11b<sup>+</sup>Gr1<sup>+</sup> cells are marginally increased in Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> PB with HO. Right, Cd11b<sup>+</sup>Gr1<sup>+</sup> population at different time points, 13, 19, and 21 weeks. At 21 weeks, when mice present HO, pro-inﬂammatory cells increase in Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup>. F. Representative eosin staining of CFU-fibroblast (Fb).

**Fig. 4** Transplanting WT BM into transgenic Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice delays HO onset. A. Schematic representation of transplant assays. WT (CD1) and Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice were transplanted with WT BM cells. B. Circulating Bmp2 levels are maintained in each group 3 months after transplant. C. Hematopoietic cell engraftment. FACS-quantiﬁcation of CD45<sup>+</sup> cells from PB. Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> and WT mice show similar HSC engraftment. D. FACS-quantiﬁcation of myeloid (CD11b<sup>+</sup>), B Lymphoid (B220<sup>+</sup>) and T Lymphoid (CD3<sup>+</sup>) cells in the two groups of transplanted animals. E. Nano-PET-CT imaging of transgenic Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice transplanted with WT HSC. These animals develop HO (red arrows) in ribs, hind limbs, and dorsal vertebrae (ribs, h; hind limbs, hl; and dorsal vertebrae, dv) at 8, 12, and 14 weeks after transplant, suggesting that ectopic bone formation started before transplant. F. Representation of the variability of HO onset in non transplanted Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice and Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice transplanted with WT HSCs showing signiﬁcant delay in the onset of HO (8–18 weeks versus 16–28w). Kaplan-Meier curve showing that Tie2<sup>CRE/−;/Bmp2<sup>tg/tg</sup></sup> mice transplanted with WT HSCs survive 10 weeks longer on average non-transplanted transgenic mice. C, D. Two-way ANOVA and Sidak’s correction **P < 0.01; F. unpaired t test, two tails, mean ± SD. t test ****P < 0.0001, ns non-significant. E. Survival curve and survival curve analysis, **P < 0.01.
Fig. 5  Tie2\textsuperscript{Cre/+};Bmp2\textsuperscript{tg/tg} resident skeletal muscle cells are characterized by pSmad1/5 and chondro-osteogenic marker expression, and increased fibro-adipogenic progenitors. Immunodetection of indicated osteo-chondrogenic marker proteins (red), with GFP (green), IB4 (white) and DAPI (blue), on consecutive WT or Tie2\textsuperscript{Cre/+};Bmp2\textsuperscript{tg/tg} hindlimb skeletal muscle sections. A  Nuclear immunostaining of GFP\textsuperscript{+} pSmad 1/5 (arrows) interspersed in muscle interstitium (a' and b'). White arrowheads indicate GFP\textsuperscript{+} adipocytes. B  Cytoplasmic Sox9 immunostaining in cells (arrows in c' and d') surrounded by damaged skeletal muscle fibers identified as central nuclei (open arrowheads in c'). C  Nuclear immunostaining of Osterix (Osx) in cells surrounded by fibers (arrows in e' and f'). Arrowheads indicate strong Osx immunostaining in ectopic bone emerging areas. Arrowheads in f' indicate GFP\textsuperscript{+} adipocytes. D  Top, FACS quantification of the CD45\textsuperscript{+} PDGFR\textalpha\textsuperscript{+} Sca1\textsuperscript{+} fibro-adipogenic (FAP) population in Tie2\textsuperscript{Cre/+};Bmp2\textsuperscript{tg/tg} skeletal muscle. Bottom, FACS quantification showing that 84% of the CD45\textsuperscript{+} GFP\textsuperscript{+} cells are PDGFR\textalpha\textsuperscript{+} Sca1\textsuperscript{+} cells. E  Immunostainings of pSmad1/5/8 and PDGFR\textalpha\textsuperscript{+} skeletal muscle sections showing expression in connective tissue surrounding muscle fibers. Bottom panels are magnifications of areas indicated in top panels. Arrows indicate co-localization of pSmad1/5 and PDGFR\textalpha\textsuperscript{+} cells. Arrowheads indicate co-localization of PDGFR\textalpha\textsuperscript{+} and GFP\textsuperscript{+} cells. Cells expressing the three markers are indicated by arrows and arrowheads (middle bottom panel). Scale bars 200 µm. Unpaired t test, two tails, mean ± SD *P < 0.05; ****P < 0.0001.
Moreover, Osterix staining was observed in larger central nuclei of damaged fibers next to ectopic bone (Supplementary Fig. 5D, e′, and f′).

The expression of chondro-osteogenic markers in damaged skeletal muscle fibers adjacent to ectopic bone, suggests an active repair process. The number of satellite cells labeled by CD45\textsuperscript{−}Sca1\textsuperscript{+}CD34\textsuperscript{−}α\textsuperscript{+}integrin was not significantly different between Tie2\textsuperscript{CRE/−};Bmp2\textsuperscript{tg/tg} and WT hindlimb skeletal muscle (Fig. 5D, left panel). There were no GFP\textsuperscript{+} satellite cells among Tie2\textsuperscript{CRE/−};Bmp2\textsuperscript{tg/tg} skeletal muscle cells (Fig. 5D, right panel), suggesting that satellite cells are not overexpressing Bmp2 and probably not directly implicated in HO in Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice.

Resident FAPs are CD45\textsuperscript{−}Tie2\textsuperscript{Cre/}PDGFR\textsuperscript{α}Sca1\textsuperscript{+} have been proposed as HO cells-of-origin [26]. FAPs were readily increased in Tie2\textsuperscript{Cre/};Bmp2\textsuperscript{tg/tg} adult valve disease pathology [47], and further studies are required.

In fact, among Sox9-or Ostx- expressing damaged muscle identiﬁed in damaged skeletal muscle of CD45\textsuperscript{−}CD34\textsuperscript{−}α\textsuperscript{+}integrin\textsuperscript{−} cells (Fig. 5B, middle panels), and Sox9-expressing cells were GFP\textsuperscript{+} (Supplementary Fig. 5C, c′, d′ and SE right panels). In contrast, Sox9-or Osterix- expressing damaged ﬁbers did not express GFP (Fig. 5B, c′, d′; SC e′, f′ and Supplementary Fig. 5C c′; 5D, e′, f′), suggesting that most FAPs are Tie2\textsuperscript{+} cells, and/or that Tie2 expression is silenced during FAP differentiation.

**DISCUSSION**

Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice develop HO, apparently spontaneously within 4 months. Normal skeletogenesis is not perturbed, with no obvious HO in juvenile mice, while adult mice present a variety of skeletal deformities including scoliosis, and spinal defects resembling lesions found in HO patients. Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice may allow for further studies of therapies designed to mitigate the effects of HO.

Bmp2 is a key pathway activated and causally linked to calcific aortic valve disease in animal models [38, 44–46]. Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice present pulmonary valve hypertension and pre-calcific aortic valve dysfunction by 16–18 weeks, characterized by ﬁbrosis, lipid deposition and chondrogenesis. Bmp2 overexpression promotes ectopic EMT in developing Nkx2.5\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} embryos [35], suggesting that EMT might contribute to pro-calcic disease in adults. However, there is lack of evidence for EMT contributing to adult valve disease pathology [47], and further studies are required. Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice develop systemic HO between 8–18 weeks, precluding a meaningful study of valve changes, but the effect of ageing over a longer-period might be assessed in Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/+} heterozygotes, which are healthy up to 32 weeks.

Our transplant studies indicate that while Bmp2 transgenic progenitors contribute BM stem cell components, the HO chondro-osteogenic stem/progenitor cell is not BM-derived, consistent with previous studies [28, 29]. Otherwise, hematopoietic/endothelial Bmp2 overexpression profoundly affects hematopoiesis. LSK stem cells, myeloid CFU-GM and Cd11b/Gr1\textsuperscript{−} cells, and ﬁbroblast and osteoblast progenitors are all expanded in Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice. Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/+} mice display increased Cd11b/Gr1\textsuperscript{−} cells in BM and PB, and increased erythroid lineage differentiation, consistent with in vitro ﬁndings [40]. Overall increased BM cellularity is consistent with HSC niche enlargement, enhancement of HSC self-renewal and pool size. Within the BM niche, non-canonical BMP signaling regulates intrinsic HSC maintenance in vivo [48–50], and is implicated in determining the HSC fate, by promoting a pro-lymphoid transcriptional program and sustaining lymphoid-biased HSC commitment [48, 49].

The CD45\textsuperscript{−}PDGFR\textsuperscript{α}Sca1\textsuperscript{+} population expansion in skeletal muscle identiﬁes FAPs as potential cells of origin in Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice. Evidence for a soft tissue chondro-osteoprogenitor niche in damaged skeletal muscle of Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice is suggested by the presence of central nuclei, co-immunostaining of isolectin B4 (also a leukocyte marker), pSmad1/5, Sox9, and osterix, in chondro-osteogenic areas of differentiation. Tie2\textsuperscript{−} FAP osteoprogenitors are identiﬁable by PDGFR\textsuperscript{α} and Sca1 cell surface expression [32], bipotent ﬁbro/adipogenic potential [26, 27], and contribution to ectopic cartilage and bone [31, 32]. HO lesions in Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} mice might be attributed to local Bmp2 release through autocrine/paracrine mechanisms. Conversely, resident skeletal muscle satellite cells (CD45\textsuperscript{−}CD34\textsuperscript{−}α\textsuperscript{+}integrin\textsuperscript{−}) that give rise to differentiated myocytes [51] were unchanged, suggesting that muscle regenerative potential is not altered. Further studies are required to characterize other progenitor populations residing in skeletal muscle, ligaments and tendons [31, 52] in the context of Bmp2-driven HO.

Previous BMP overexpression studies using a variety of promoters failed to cause HO (reviewed in [53]), because the relevant progenitor cell type had not been targeted. One exception is the transgenic mouse overexpressing BMP4 under control of neuron-speciﬁc enolase (Nse) promoter [53, 54]. Our Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} model resembles this model, matching a stereotyped spreading pattern of HO formation. Neither the NSE-BMP4 model, nor our Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/tg} model recapitulate any of the congenital phenotypes associated with FOP. Moreover, direct versus indirect Bmp effects on target stem/progenitor cell populations cannot be assessed using these models. Nevertheless, Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/+} mice are viable, and develop HO within weeks. This HO model does not require surgical procedures involving the implantation of a BMP-loaded matrix and Bmp2 dosage can be modulated via copy number gene expression. Tie2\textsuperscript{CRE/};Bmp2\textsuperscript{tg/+} mice are maintained on a heterozygote background, so that a single cross allows for the generation of experimental animals.

**MATERIALS AND METHODS**

**Mouse strains and genotyping**

The following mouse strains were used: male and female mixed background C56BL/6-CD1 R26CAGBmp2tg [35] and Tie2\textsuperscript{CRE/} [37]. For simplicity, R26CAGBmp2tg\textsuperscript{−} and R26CAGBmp2tg\textsuperscript{+} are abbreviated in the text and ﬁgures as Bmp2\textsuperscript{tg/+} and Bmp2\textsuperscript{tg/+}, respectively. Details of genotyping will be provided upon request. Recipient transplanted animals were WT C56BL/6, CD1 or Tie2\textsuperscript{Cre/}Bmp2tg\textsuperscript{−} animals. Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and by the Community of Madrid (Ref. PROEX 83.8/20). Animal procedures conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and scientiﬁc purposes, enforced in Spanish law under Real Decreto 53/2013.

**ELISA**

Blood samples were taken by submandibular vein puncture from different groups: control WT and Tie2\textsuperscript{Cre/}Bmp2tg\textsuperscript{−} mice at 16 weeks of age (n = 10/group); control WT and heterozygous Tie2\textsuperscript{CRE/−};Bmp2\textsuperscript{tg/+} at 24 weeks of age (n = 6/group). 1 year after transplant, WT animals transplanted with WT BMCs, n = 6, and WT animals transplanted with Tie2\textsuperscript{CRE/−};Bmp2\textsuperscript{tg/+} BMCs, n = 8; 3 months after transplant WT animals transplanted with WT BMCs (n = 7) and Tie2\textsuperscript{CRE/−};Bmp2\textsuperscript{tg/+} animals transplanted with WT BMCs (n = 4) were analyzed. Serum was obtained by centrifugation at 4000 rpm for 15 min at RT. Circulating Bmp2 was measured by using the human Bmp2 ELISA construction kit (Antigenix America Inc. RHF913CKC) under the manufacturer’s instructions.

**Ultrasound**

Mice were anaesthetized by inhalation of isoflurane and oxygen (1.25% and 98.75% respectively) and examined by a 30 MHz transthoracic echocardiography probe. Images were obtained with VEVO 2100 (VisualSonics, Toronto, Canada) from Tie2\textsuperscript{CRE/−};Bmp2\textsuperscript{tg/+} (n = 10) and WT (n = 9) littermates. Short axis and long axis, B Mode, and 2D M-Mode views were obtained from the M mode by an expert in ultrasound in a blind fashion as described previously [55]. From these images, left ventricle (LV) function was estimated by fractional shortening (FS) and ejection fraction (EF). For FS measurements a long or short-axis view of the heart was
selected to obtain an M mode registration in a line perpendicular to the LV septum and posterior wall at the level of the mitral chordae tendineae. Pulmonary acceleration time (PAT) and ejection time (PET) were measured in the parasternal short-axis view by pulsed-wave Doppler of pulmonary artery flow [56]. B-mode and color-Doppler guided pulsed-wave Doppler was used to record the maximal transvalvular jet velocity. Specifically, to avoid Doppler misalignment, coaxial interrogation of the aortic flow was ensured by the operator, and all the measurements were obtained using an angle of interrogation <30°. To correct for flow dependence, we computed an EF velocity ratio (EFVR = EF (%)/maximal aortic velocity [m/s]) as an additional indicator of disease severity [57].

Positron emission tomography–computed tomography (PET/CT) imaging
In vivo CT imaging was performed on a nanoPET/CT small animal system (Mediso, Hungary) equipped with a micro-focus X-ray source and a high-resolution radiation-imaging device featuring a 1024 × 512 pixel photodiode array. 16-week-old Tie2Cre+/-;Bmp2tg/+ mice (n = 12) and WT (n = 10), or 20-week-old Tie2Cre+/-;Bmp2tg/+ mice (n = 5); and 16 and 32-week-old Tie2Cre+/-;Bmp2tg/+ (n = 7) and WT (n = 6) or Tie2Cre+/-;Bmp2tg/+ transplanted with WT BMCs (16 (n = 1), 20 (n = 1), 22 (n = 2) and 26 (n = 1) weeks were analyzed. The mice were anesthetized using isoflurane 2% and 1.8 L/min oxygen flow, and positioned in a thermo regulated (38.7 °C) mouse bed with an ophthalmic gel in their eyes to prevent retinal drying. The scan parameters used for the CT measurements were an X-ray beam current of 145 µA and a tube voltage of 55 kVp. Acquisitions and reconstruction were performed by an expert in PET/CT in a blind fashion using proprietary Nucline software (Mediso, Hungary) and analyzed by a whole-body bone 3D volume rendering using Osinix software (Pixonex, Switzerland).

Bone mass spectrometry
Nano-PET-CT acquired and 3D-reconstructed images from 16-week-old WT and Tie2Cre+/-;Bmp2tg/+ animals (n = 3 of each group) were further analyzed for bone mass densitometry. Hind limbs mean attenuation coefficient of X rays expressed in Hounsfield units was measured after segmenting bones using the Multidomarlity Workstation MMWKS [39].

Histology
Skeletal muscles and bone tissue (and ectopic bone in transgenic animals) were fixed in 4% PFA for 24 h at 4 °C, and after decalcification with Immunocal (StatLab, Fisher scientific) embedded in paraffin or sucrose treated to be cryopreserved in OCT. Hematoxylin/eosin (H&E), Masson’s trichromic, Alizarin red, and Alcian blue stainings were performed according to standard protocols on paraffin-embedded 7 µm sections. Oil Red, staining was performed according to standard protocols on 5 µm cryosections.

Immunohistochemistry
Paraffin-embedded 7 µm sections of hind limb tissues were citrate-unmasked and stained with the following primary antibodies: polyclonal P-Smad 1/5 (1:100; (41D10) 9516, Cell Signaling Technology), polyclonal Sox9 (1:100, Santa Cruz Biotechnology, sc-20095), polyclonal Sphysis/OSTERIX (1:100, Abcam ab22552), monoclonal GFP living Colors (1:100 Clontech 632381) or polyclonal GFP (1:200, BD Biosciences) or monoclonal CD140a Abcam ab22552), monoclonal GFP living Colors (1:100 Clontech 632381) or polyclonal GFP (1:200, BD Biosciences), monoclonal CD140a (1:100, Santa Cruz Biotechnology, sc-20095), polyclonal Sp7/Osterix (1:100, Santa Cruz Biotechnology, sc-20095), polyclonal CD45-AV450 (1:200, BD Biosciences) cocktail of antibodies: hematopoietic cells CD45-APC (1:100, BD Biosciences) or CD45-AV450 (1:200, BD Biosciences) cocktail of antibodies: hematopoietic cells CD45-APC (1:100, BD Biosciences) or CD45-AV450 (1:200, BD Biosciences) cocktail of antibodies: hematopoietic cells CD45-APC (1:100, BD Biosciences) or CD45-AV450 (1:200, BD Biosciences) CD71-APC, BD Biosciences 13, Tg, 13; Tg, 13 = mixed background) as donors, (n = 2 per transplantation assay) and two groups of male 8-week-old WT as recipients (n = 7 and 8 per transplantation assay). Briefly, lethality irradiated recipient mice (11 grays) were transplanted with 2 × 10^6 bone marrow cells (BMCs) by tail vein injection. BMCs were obtained as mentioned above in FACS analysis section. The homogenized samples were filtered through a 40-µm mesh to obtain single-cell suspensions, and depleted of red blood cells by lysis. For reciprocal transplantation assays we used 8-week-old WT CD1 or Tie2Cre+/-;Bmp2tg/+ as donors, (n = 7 per transplantation assay) and two groups of 8-week-old WT CD1 (n = 10) or Tie2Cre+/-;Bmp2tg/+ (n = 7), as recipients without HO symptoms. In order to check for engraftment of hematopoietic cells, we bled the animals every month after the transplant, until 4 (WT BMC into Tie2Cre+/-;Bmp2tg/+ or 5 (Tie2Cre+/-;Bmp2tg/+ BMC into WT) months post-transplant.

Isolation of skeletal muscle cells
Skeletal muscle tissues (tibialis anterior, gastrocnemius, quadriceps) were dissected and minced from hind limbs of wet and Tie2Cre+/-;Bmp2tg/+ 9-11-week-old animals. The minced tissue was digested in Collagenase I (0.25%, I and II (0.2%) in DMEM/10% FBS for 1 h min at 37 °C in a shaking bath. Cells were washed with DMEM 2 %FBS, 100- and 70-µm filtered, washed with DMEM/2 %FBS, and centrifuged. The pellet was resuspended in PBS/2 %FBS to determine cell number, centrifuged, and resuspended in staining buffer (PBS/2%FBS).

FACS analysis
For BM FACS analysis, 16-20-week-old WT and Tie2Cre+/-;Bmp2tg/+ animals were sacrificed and bones were dissected as described in CFU assays section. 1 × 10^6 cells were labeled with the following cocktail of antibodies: LSKs (WT, n = 13; Tg, n = 17); biot-Lin (1:100, BD Biosciences 559971)/St-APC/Cy7 (1:100, BD Biosciences 554063), C-kit-PE-Cy7 (1:400, BD Biosciences 558163), Sca-1-PE (1:200, BD Biosciences cat no. 553368); Erythroid lineages (WT, n = 4; Tg, n = 4) CD71-APC, BD Biosciences 567258); Ter-119-PE-Cy7 (1:100, BD Pharmingen 560590); Pro-inflammatory cells (WT, n = 6; Tg, n = 7) CD11b-PE (1:200, BD Biosciences 553311); Gr1-APC (1:100, BD Biosciences 553129) and DAPI for viability. The total number of each BM lineage was calculated referred to the total number of cells obtained from BM. Total number of spleen CFUs (WT, n = 5; Tg, n = 15) and PB-CFUs (WT, n = 11; Tg, n = 13). PB-pro-inflammatory cells (16-20-week-old mice WT, n = 5; Tg, n = 4) or the group at 13, 19 or 21 week-old mice WT n = 3, Tg = 4) was calculated referred to the total number of cells obtained from spleen or white blood cell number determined by hematology. For transplants multi-lineage engraftment FACS analysis peripheral blood cells were labeled with the following cocktail of antibodies: hematopoietic cells CD45-APC (1:100, BD Biosciences 559864), myeloid lineages CD11b-PE (1:200, BD Biosciences 553311), lymphocytes B biot-B220 (1:200, BD Biosciences 559864)/st-APC/Cy7 (1:100), and lymphocytes T CD3-PE-RFCPC-Cy5 (1:200, BD Biosciences 560527). For muscle cells FACS analysis cells were labeled with the following cocktail of antibodies: CD45-AV450 (1:200, BD Biosciences...
560501), Sca1-PE-Cy7 (1:200, AbLab AB10STMW215), CD34-Alexa647 (1:40, BD Pharmigen 560230), CD540a PDGFRα Monoclonal Antibody (AP594A), Biotin (1:200, Thermo Fisher 13-1401-82), streptavidin-APC-Cy7 (1:100, BD Biosciences 559925) for viability. Satellite cells were identified as CD45−Sca1−CD34−α7int and fibro-adipogenic cells as CD45PDGFRα−.

Statistics
Due to the high variability in HO onset and severity, the analysis was made in groups n>10 in some cases. Statistical assessment is indicated in the figure legends. For each experiment comparing two groups, a mean ± SD is represented and a two-tailed t test was performed. For experiments comparing two groups at different time points, a mean ± SD is represented at each timepoint and a two-way ANOVA followed by Sidak’s correction was performed. **P<0.001; ****P<0.0001.

DATA AVAILABILITY
Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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AUTHOR CONTRIBUTIONS
BP and JLdLP performed study concept and design; BP and RdT performed development of methodology. BP and RdT provided acquisition, analysis, and interpretation of data, and statistical analysis; PG-A and TP provided technical assistance and data acquisition; BP and DM drafted the manuscript; BP, RdT, DM, SM-F, PM-C, and JLdLP revised the manuscript; PM-C, SM-F, and JLdLP provided funding and material support. All authors reviewed the manuscript during its preparation and approved the final version.

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ETHICS
Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and by the Community of Madrid (Ref. PROEX 83.8/20). Animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and scientific purposes, enforced in Spanish law under Real Decreto 53/2013.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION

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