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Tissue-specific expression of a BAC transgene targeted to the *Hprt* locus in mouse embryonic stem cells☆

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Abstract

The hypoxanthine phosphoribosyltransferase (*Hprt*) locus has been shown to have minimal influence on transgene expression when used as a surrogate site in the mouse genome. We have developed a method to transfer bacterial artificial chromosomes (BACs) as a single copy into the partially deleted *Hprt* locus of embryonic stem cells. BACs were modified by Cre/*loxP* recombination to contain the sequences necessary for homologous recombination into and complementation of the partially deleted *Hprt* locus. Modified BACs were shown to undergo homologous recombination into the genome intact, to be stably transmitted through the germ line of transgenic mice, and to be expressed in the proper tissue-specific manner. This technology will facilitate many studies in which correct interpretation of data depends on developmentally appropriate transgene expression in the absence of rearrangements or deletions of endogenous DNA.

Keywords: Gene targeting; Hypoxanthine phosphoribosyltransferase; Transgenes; Chromosomes, artificial, bacterial; Osteocalcin; Boo

The transfer of large DNA fragments to the mouse genome is an important process in the definition of transcription units, the modeling of inherited disease states, and the dissection of candidate regions identified by linkage analysis. The technology exists to transfer large genomic fragments in the form of bacterial (BAC), yeast (YAC), or phage artificial chromosomes into the genome of both mouse embryonic stem cells (ESCs) and fertilized mouse eggs [1–6]. However, as with smaller recombinant transgenes, the transferred sequences are integrated randomly often with accompanying genomic alterations and variable expression of the introduced genes due to the site of integration and/or copy number [1–8].

Various approaches have been taken to minimize the complications of random integration when working with large transgenes. One method introduces fragments of human DNA as *trans*-chromosomes into mouse ESCs using microcell fusion [9,10]. The fragments are not integrated into the mouse genome, thus eliminating the complications caused by random integration; however, *trans*-chromosomes are rarely transmitted through the male germ line and the efficiency of transmission through the female germ line decreases with an increase in fragment size [9,10]. A method implementing Cre/*loxP* recombination has also been used to introduce large genomic fragments site specifically into the mouse genome [11]. While this approach benefits from the efficiency of Cre recombinase, ES cells must first be engineered to contain a *loxP* site, using homologous recombination or random integration, followed by subcloning and screening.

A method of integrating a single copy of a large transgene into the genome at a chosen site by a precise homologous recombination event would greatly improve existing technology. The *Hprt* locus has previously been used as a surrogate site for the integration of small recombinant transgenes [12–20] and provides three major advantages. First the X-chromosome location of the *Hprt* locus, coupled with the potential for random inactivation of transgenes in heterozygous females, can result in mice with mosaic expression of transgenes. In some instances this occurrence can improve the survival of animals with detrimental transgenes or reveal interesting aspects of the transgenic phenotype. Second, homologous recombination integrates the transgene into the partially deleted *Hprt* locus of a male

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ESC line and restores the expression of *Hprt* by the inclusion in the targeting vector of sequences that complement the deletion. Direct forward selection for correctly targeted cells can then be achieved using hypoxanthine–aminopterin–thymidine (HAT) medium [12]. Third, housekeeping promoters that give unpredictable expression when integrated randomly confer broad transgene expression at the *Hprt* locus, perhaps because *Hprt* itself is a housekeeping gene with chromatin organization that permits the accessibility of *cis*-regulatory elements [21,22]. While some tissue-specific promoters give the expected restricted expression when inserted at the *Hprt* locus [16–19], this may depend on the nature of the *cis* sequences that are used. In particular expression of developmentally regulated genes that are dependent on changes in chromatin organization are unlikely to be accurately expressed by recombinant promoter and cDNA combinations even when integrated at the *Hprt* locus. Introducing these genes on a BAC, however, could provide the essential chromatin environment for properly regulated gene expression.

We describe a method to modify any existing BAC for single-copy, site-specific targeting to the *Hprt* locus of ESC lines that contain the naturally occurring E14TG2a deletion or a similar engineered deletion [14,23–27]. We demonstrate that modified BACs recombined intact into the *Hprt* locus at an acceptable frequency, that a BAC transgene was stably maintained in the genome of BAC-transgenic mice, and that genes on integrated BACs were expressed in the predicted tissue-specific manner in vitro and in vivo.

Results

Modification of BACs to contain Hprt homologies

We chose Cre recombinase-mediated *loxP* recombination to introduce into human BAC clones CTD-2338M9 (133 kb) and CTD-2184D3 (100 kb) the sequences necessary for homologous recombination at the *Hprt* locus (Fig. 1A). Because all commercially available BAC libraries are constructed in BAC vectors that contain a wild-type *loxP* site, the same vector can be used to modify any BAC. Cre/*loxP* recombination between the BAC and the modifying fragment duplicates the wild-type *loxP* site and inserts into the BAC backbone the sequences necessary for ampicillin resistance, linearization with the *I-Sce*I homing endonuclease, and directly selectable recombination into the *Hprt* locus.

Following in vitro Cre-mediated recombination, the BAC modification reaction was electroporated into DH10B cells, and dual chloramphenicol- and ampicillin-resistant colonies were screened for properly modified BACs. Following selection, *Eco*RV restriction endonuclease-generated fragments of the modified BACs were compared to those of the wild-type BACs to verify proper modification (Figs. 2A and B). For both modified BACs shown, the expected changes

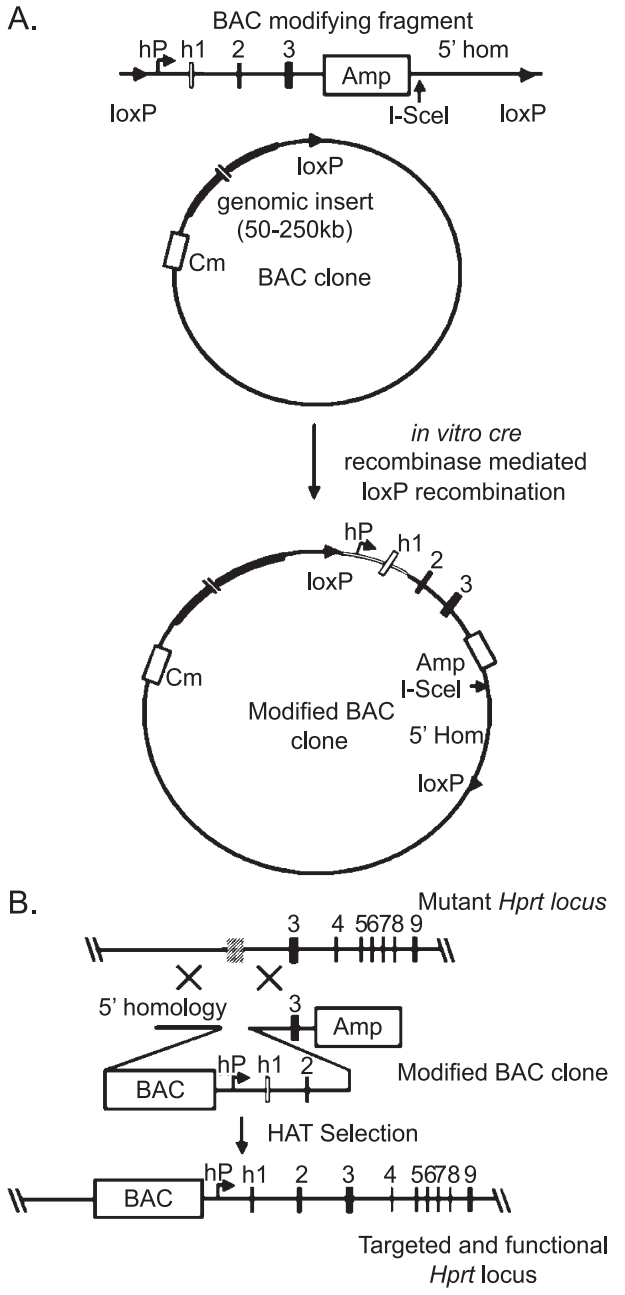

Fig. 1. Modification of a BAC transgene for homologous integration into the *Hprt* locus. (A) In vitro Cre-mediated recombination of the BAC-modifying fragment into a BAC. The human promoter (hP), human exon 1 (h1), and mouse exons are indicated. (B) Targeting of an *I-Sce*I-linearized BAC transgene to the *Hprt* locus by a replacement event. The E14TG2a mutation at the *Hprt* locus (top), a modified BAC linearized with *I-Sce*I (middle), and the corrected *Hprt* locus containing the BAC transgene (bottom) are shown. The hatched box represents the ~36-kb deletion at the mutant *Hprt* locus; "BAC" represents the BAC vector and insert sequences. Drawings not to scale.

in the *Eco*RV fragment patterns were observed, in the absence of other alterations. Southern hybridization with probes that span the sites of *loxP* recombination verified the presence of the expected fragments after modification and the loss of the fragment disrupted by modification (Figs. 2A

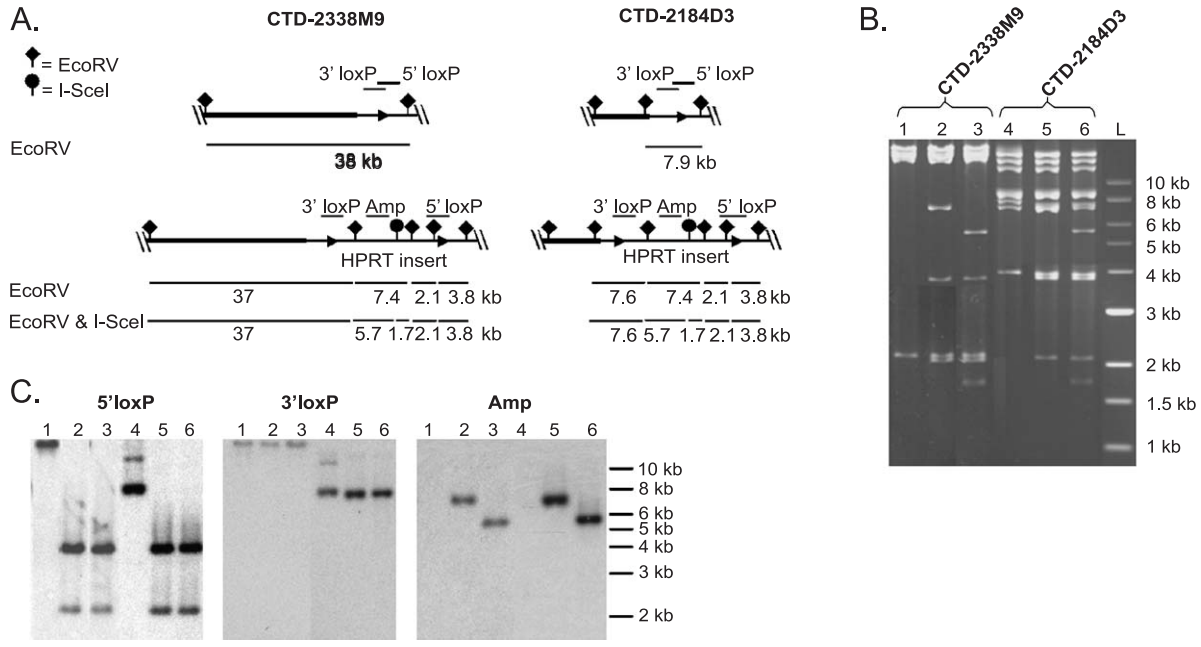

Fig. 2. Verification of properly modified BACs. (A) Schematic diagrams of the regions of the BACs that were altered by recombination. Portions of the wild-type (top) and modified (bottom) BACs are shown. Thick black lines represent genomic sequence (not to scale), thin black lines vector sequence, and arrowheads *loxP* sites. The sizes of the unique fragments generated by *Eco*RV digests and *Eco*RV/*I-Sce*I double digests of the wild-type and modified BACs are indicated. The locations of probes used for Southern analysis are shown. (B) Restriction endonuclease analysis of BACs before and after modification (*Eco*RV digestion of wild-type BACs, lanes 1 and 4; *Eco*RV digestion of modified BACs, lanes 2 and 5; double digestion with *Eco*RV/*I-Sce*I of modified BACs, lanes 3 and 6; ladder, lane L). (C) Southern analysis of digests in (B) to verify proper modification of the BACs. The 5′*loxP* and 3′*loxP* probes span the sites of recombination.

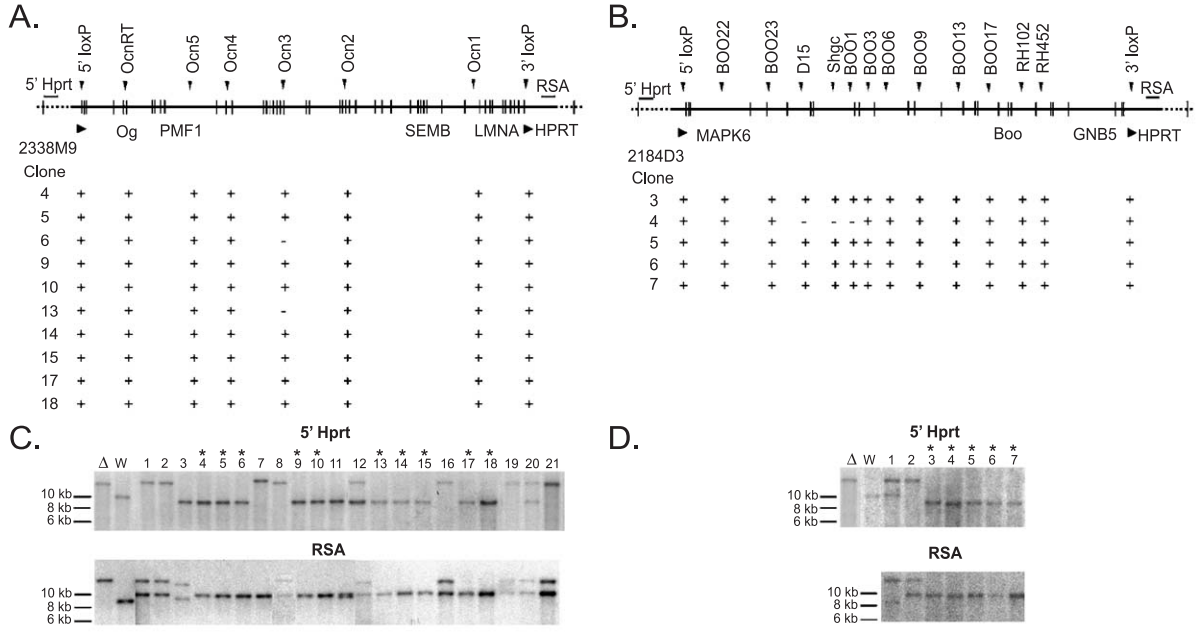

Fig. 3. Recombination of the CTD-2338M9 and CTD-2184D3 BAC transgenes into the *Hprt* locus of HAT-resistant ES cell clones. Physical maps of (A) CTD-2338M9 and (B) CTD-2184D3 targeted into the *Hprt* locus with the localization of PCR markers used for verification of BAC integrity shown. The locations of the 5′Hprt and RSA probes used in (C) and (D) are shown. The locations of known genes and the two *loxP* sites (arrowheads) are indicated. The solid black line represents sequence found on the modified BAC, dashed lines represent genomic sequence, and vertical lines represent *Sac*I sites. STS-PCR amplification results for clones that had both BAC ends properly recombined into the *Hprt* locus are shown. A "+" indicates a positive PCR result. Southern analyses for recombination of the 5′ and 3′ BAC ends of (C) CTD-2338M9 and (D) CTD-2184 into the *Hprt* locus are shown. The expected sizes of the hybridizing fragments using 5′Hprt were 8.5 kb for a properly targeted BAC, 10 kb for a wild-type *Hprt* locus (WT), and 15 kb for the HM-1 ES cell *Hprt* locus containing the deletion (Δ) and using RSA were 10 kb for a properly targeted BAC, 8.5 kb for a wild-type *Hprt* locus, and 15 kb for the parental *Hprt* locus containing the deletion. Clones that have the expected Southern hybridization profile at both BAC ends are marked (*).

and C). Restriction endonuclease fragment analysis with *Hin*dIII confirmed the results of the *Eco*RV analysis (data not shown). Double digests with *Eco*RV and *I-Sce*I and Southern analysis verified that the introduced *I-Sce*I site was the only site in the modified BACs (Fig. 2). Two clones from the RPCI-11 human BAC library, three BACs from the CITB D human BAC library, two mouse BACs, and the pBeloBAC11 vector were successfully modified using this method.

Homologous recombination at the Hprt locus

Properly modified CTD-2338M9 and CTD-2184D3 BACs were linearized with *I-Sce*I and electroporated into the HM-1 ESC line [25]. Upon homologous recombination of a BAC into the partially deleted *Hprt* locus, the expression of *Hprt* is restored, allowing for the exclusive survival of correctly targeted ESCs in HAT medium (Fig. 1B) [12]. For the electroporation of modified CTD-2338M9, 21 colonies survived HAT selection at a frequency of 1 HAT-resistant cell per 1.9×10^6 cells electroporated. Seven ESC colonies survived HAT selection from the electroporation of the modified CTD-2184D3, for a frequency of 1 HAT-resistant cell per 6.3×10^6 cells electroporated. In control experiments, linearized plasmid DNA that contained the same *Hprt* sequences as the modified BACs had frequencies six to seven times higher than that of the BACs (1 per 2.6×10^5 and 1 per 8.8×10^5, respectively). The frequencies observed for the plasmid controls were similar to previous results with recombinant transgenes [12].

We used Southern hybridization with BAC end probes to screen clones for recombination of the BAC into the *Hprt* locus (Figs. 3C and D; Table 1). Ten (48%) of the CTD-2338M9 HAT-resistant ESC clones (2338M9[ES]) and 5 (71%) of the CTD-2184D3 HAT-resistant ESC clones (2184D3[ES]) had the expected Southern hybridization profile for both ends, suggesting proper recombination of the BAC into the *Hprt* locus. Fluorescence in situ hybridization (FISH) was used to verify targeting of the CTD-2338M9 BAC transgene to the *Hprt* locus of two ESC clones (Fig. 4). Both clone 2338M9[ES]-4 and clone 2338M9[ES]-17 were shown to have the BAC transgene on the X chromosome at the relative position of the *Hprt* locus with no detectable signals on other chromosomes.

Table 1
Summary of BAC transgene integration and integrity

BAC	HAT^R clones	Homologous integration			BAC transgene integrity[a]		
		5′ end[b]	3′ end[b]	5′ and 3′ [c]	STS-PCR	*Alu* fingerprint	STS-PCR and *Alu* fingerprint[d]
2338M9	21	12	11	10 (48%)	8	7	7 (33%)
2184D3	7	5	5	5 (71%)	4	4	4 (57%)

[a] Analysis of BAC transgene integrity for HAT-resistant clones that had both BAC ends properly integrated into the *Hprt* locus.
[b] Does not include clones that had, in addition to the expected hybridizing fragment, a second hybridizing fragment of unknown origin.
[c] Percentage of HAT-resistant BAC clones that had both BAC ends properly integrated into the *Hprt* locus.
[d] Percentage of HAT-resistant BAC clones that had both BAC ends properly integrated into the *Hprt* locus with no detectable deletions or rearrangements.

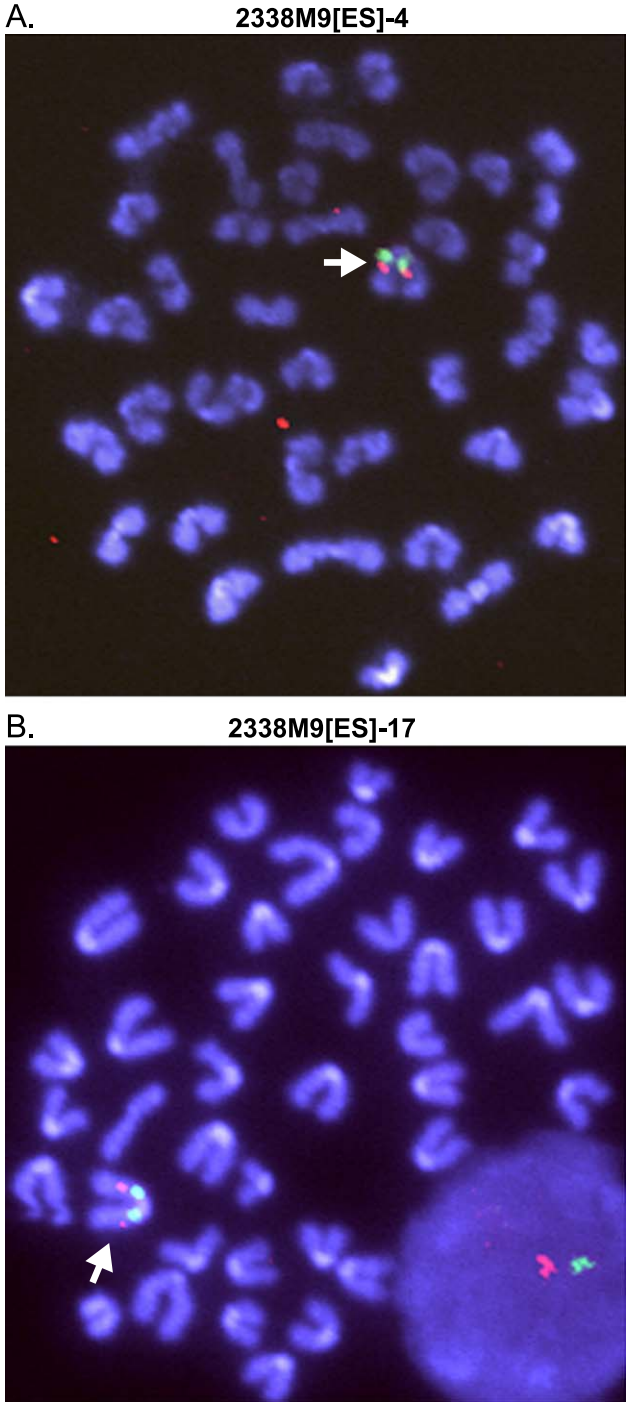

Fig. 4. Localization of the BAC transgene to the X chromosome of clones (A) 2338M9[ES]-4 and (B) 2338M9[ES]-9 by FISH. Metaphase chromosome spreads were hybridized with labeled X-chromosome centromeric repeat probe DXwas70 (green) and labeled modified or unmodified BAC DNA (red). Chromosomes were counterstained with DAPI (blue). Arrows indicate chromosomes with detectable signals from the probes.

The sequence-tagged site (STS) content and *Alu* fingerprints of ESC clones that had both BAC ends properly recombined into the *Hprt* locus were analyzed to verify the integrity of the BAC transgenes. All clones except 2338M9[ES]-6, 2338M9[ES]-13, and 2184D3[ES]-4 were positive for all STSs (Figs. 3A and B; Table 1). The *Alu* fingerprints of the ESC clones verified the STS-PCR data except for clone 2338M9[ES]-15, which was positive for all STS-PCR sites but had an *Alu*-hybridizing fragment that differed from the modified BAC DNA, suggesting that a rearrangement or deletion that was not detected by STS-PCR had occurred (Figs. 5A and B; Table 1). STS-PCR and *Alu* fingerprint analysis of the ESC clones that had one or both BAC ends improperly recombined into the *Hprt* locus verified that most had deletions of various lengths of the 5′ end of the BAC transgene with or without additional internal deletions or rearrangements (data not shown). Seven of the 21 (33%) 2338M9[ES] HAT-resistant clones and 4 of the 7 (57%) 2184D3[ES] HAT-resistant clones were shown to have both BAC ends properly recombined into the *Hprt* locus with no detectable deletions or rearrangements (Table 1).

Cre-mediated excision of a BAC transgene

We utilized Cre/*loxP* recombination to excise the BAC transgene from the ESC genome to demonstrate further that the BAC sequences were integrated into the *Hprt* locus (Fig.

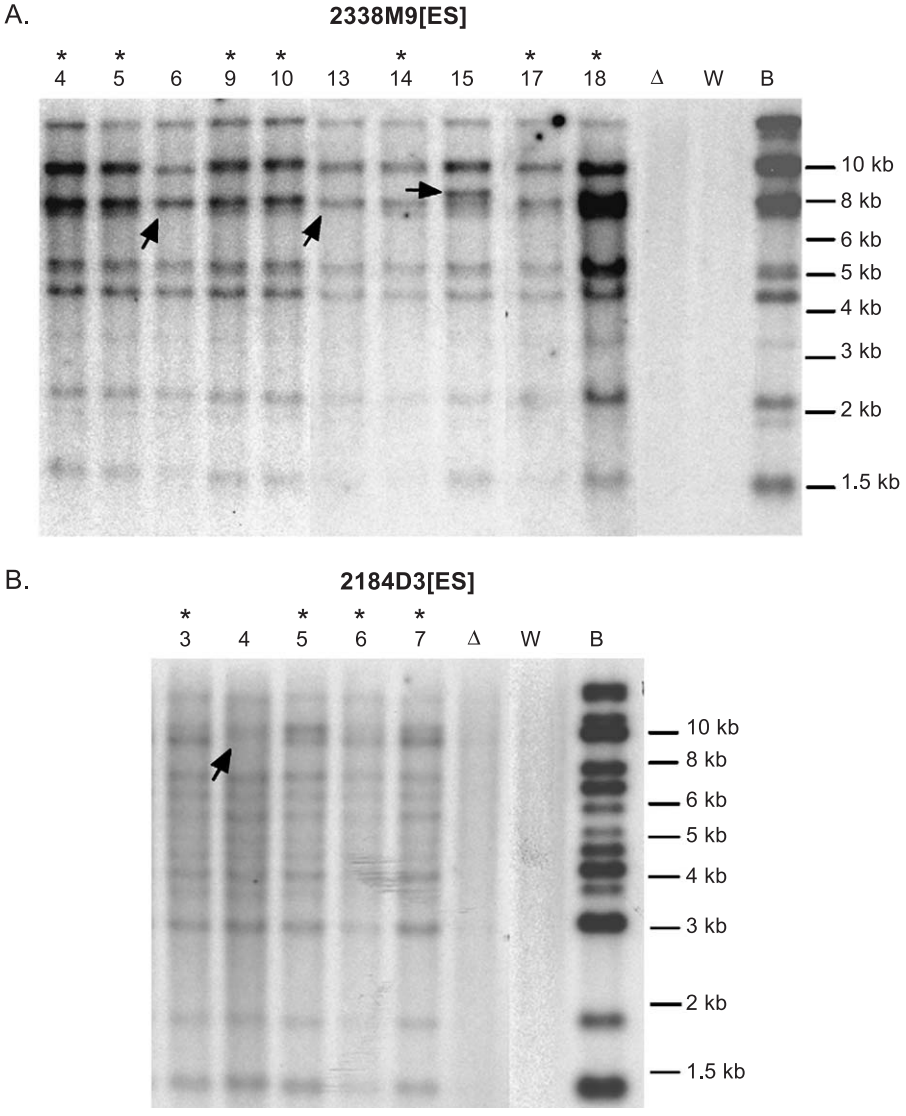

Fig. 5. Determination of BAC transgene integrity in 2338M9[ES] and 2184D3[ES] clones with *Alu* fingerprint analysis. (A) Southern analysis of 2338M9[ES] clones with a human-specific *Alu* repeat probe. (B) Southern analysis of 2184D3[ES] clones using a human-specific *Alu* repeat probe. Digests of wild-type (W) tail DNA and HM-1 ES cell DNA with the *Hprt* deletion (Δ) were used as negative controls. Digests of circular modified CTD-2338M9 or CTD-2184D3 BAC DNA (lanes B) were used as positive controls. The 13.4-kb band detected in the BAC DNA lanes is derived from the BAC vector sequence containing the human *Hprt* promoter. After linearization and recombination into the *Hprt* locus, the DNA fragment containing the human *Hprt* promoter is 10 kb. Clones that have the expected *Alu* fingerprint are indicated (*).

6A). Clone 2184D3[ES]-5 was transiently transfected with a Cre expression plasmid and PCR amplification was utilized to screen ESC subclones for the Cre-mediated excision of the BAC transgene. Because there was no selection for the transient presence of the Cre recombinase expression vector or for the excision of the BAC sequences, it was expected that only a fraction of the subclones analyzed would have the BAC sequences removed from the genome. In 4 of the 17 ESC subclones analyzed, PCR amplification demonstrated that the 3′ BAC end was not present and the *Hprt* sequences flanking the BAC were adjacent to each other (Fig. 6B). Additionally, Southern analysis with the *Alu* probe showed that there were no detectable BAC sequences left in the genome of the four subclones (Fig. 6C). Absence of PCR amplification of STSs from the BAC insert verified the excision of the BAC sequences (data not shown).

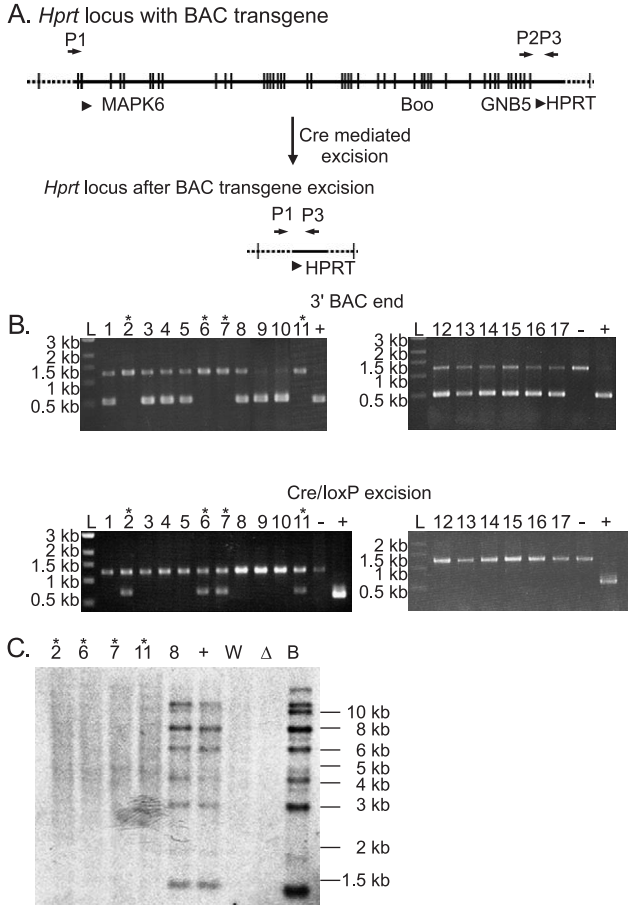

Fig. 6. Cre-mediated excision of a BAC transgene from the ESC genome. (A) Physical map of the CTD-2184D3 transgene at the *Hprt* locus before and after Cre/*loxP* recombination. Vertical lines represent *Sac*I sites and the locations of primers P1, P2, and P3 (Supplemental Table 2) are shown. (B) PCR analysis of ESC subclone candidates for Cre-mediated excision of the BAC transgene. Top: PCR analysis of ESC subclones for the 3′ BAC end using primers P2 and P3 (541 bp). DNA from 2184D3[ES]-5 was used as a positive control (+); DNA from HM-1 ESCs was used as a negative control (−). Bottom: PCR analysis of ESC subclones for Cre-mediated excision of the BAC transgene using primers P1 and P3 (719 bp). Amplification of the *Hprt* targeting vector pSKB1 (629 bp) was used as a positive control (+); DNA from 2184D3[ES]-5 was used as a negative control (−). A nonspecific 1.25-kb product is produced by PCR using primer P3. (C) *Alu* fingerprint analysis of DNA from ESC subclones that tested positive for Cre-mediated excision by PCR. Digests of wild-type (W) mouse DNA and HM-1 ESC DNA (Δ) were used as negative controls. Digests of subclone 8 (8), 2184D3[ES]-5 (+), and circular modified CTD-2338M9 BAC DNA (B) were used as positive controls. Subclones that had the BAC sequences excised from the genome are indicated (*).

Stable germ-line transmission of a BAC transgene

It is well established that BAC transgenes introduced by random integration are stably passed through the germ line of transgenic mice; thus we expected that a BAC targeted to the *Hprt* locus would also pass through the mouse germ line. A chimeric mouse was generated from 2184D3[ES]-6 ESCs and PCR analysis of tail DNA from the chimera verified the presence of all BAC transgene STSs in the genome (Supplemental Fig. 1A). To demonstrate germ-line transmission of the BAC transgene in the mouse genome, the chimera was bred to B6 mice to generate F1 offspring. As expected, STS-PCR analysis of tail DNA verified the transmission of the BAC transgene to F1 progeny (Supplemental Fig. 1B). Southern analysis with the *Alu* probe suggested the presence of the complete BAC insert (data not shown). These results demonstrate that a BAC transgene targeted into the *Hprt* locus can be stably transmitted through the germ line of transgenic mice.

In vitro and in vivo tissue-specific expression of targeted BAC transgenes

To verify that a gene on a BAC targeted to the *Hprt* locus is expressed in the proper tissue-specific manner, we allowed 2338M9[ES]-5 transgenic ESCs to differentiate into bone nodules in vitro [28]. The insert of BAC clone CTD-2338M9 contains the human osteocalcin locus. Osteocalcin is expressed principally by mature osteoblasts, is one of the most abundant noncollagenous proteins in the bone matrix, and is involved in the regulation of bone resorption and remodeling [29–31]. ESCs were allowed to form embryoid bodies (EBs) and after 2 days the EBs were disrupted to yield a mixed population of progenitor cells. When plated in medium containing ascorbic acid and exogenous phosphate a subset of these cells will proliferate and differentiate into mature osteoblasts that secrete and mineralize bone matrix. Osteocalcin gene expression in ESCs, EBs, and bone nodule cultures 14 and 21 days postplating was quantified by quantitative real-time PCR (QRT-PCR) with SYBR green. QRT-PCR demonstrated that the expression of mouse osteocalcin (*Og1*) was minimal in EBs and was 15 to 17 times higher than the expression in ESCs by 21 days of differentiation for both HM-1 and 2338M9[ES]-5 ESC-derived bone nodules (Fig. 7A). The expression of human osteocalcin (*OG*) from 2338M9[ES]-5-derived bone nodules was also minimal in EBs, was 4.5 times higher than the expression in ESCs by 14 days of differentiation, and further increased to 9 times higher than that of ESCs by 21 days in culture (Fig.

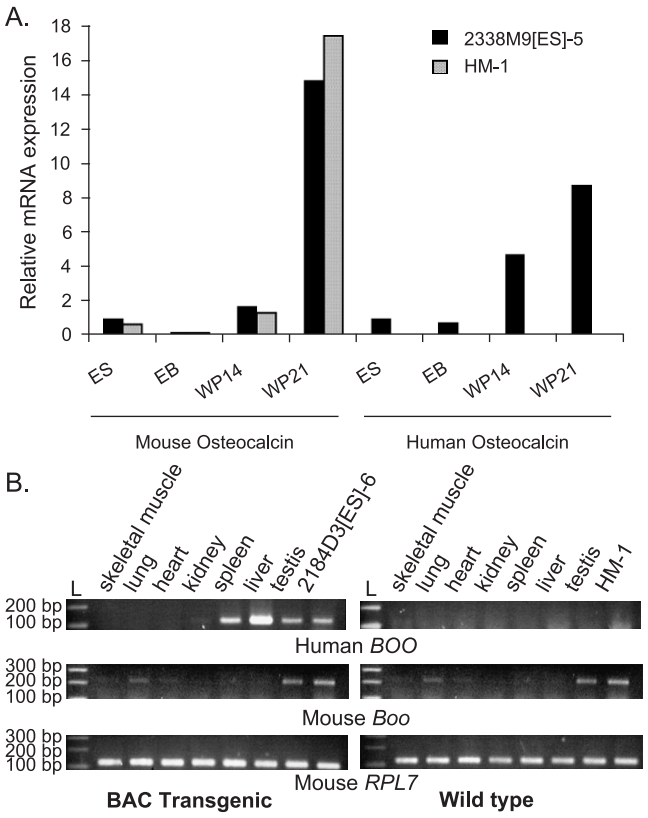

Fig. 7. In vitro and in vivo tissue-specific expression of targeted BAC transgenes. (A) Expression of human osteocalcin from a BAC transgene targeted to the *Hprt* locus. Levels of mRNA in ESCs, day 2 EBs, and cultures after 14 or 21 days, in which ~60% of the colonies were mineralized bone nodules, were quantified by QRT-PCR. Data for human and mouse osteocalcin expression are plotted relative to the expression of human and mouse osteocalcin from 2338M9[ES]-5 ESCs, respectively. The ratio of both human and mouse osteocalcin expression to *Rpl7* expression was approximately 1:1 in ESCs. (B) Expression of the human *BOO* BAC transgene and mouse *Boo* in transgenic and wild-type 12-week-old mice, 2184D3[ES]-6 ESCs, and HM-1 ESCs. First-strand cDNAs made from DNase-treated RNA samples from various tissues were PCR-amplified using human *BOO*- and mouse *Boo*-specific primers. Primers for *Rpl7* were used as a control for amplification.

7A). The slight differences in the level and timing of expression of mouse and human osteocalcin in the bone nodule cultures were most likely due to species-specific variations in the osteocalcin promoter [28,32,33]. Lower expression of human *OG* might also be due to the hemizygous state of the BAC relative to the homozygous state of the endogenous mouse locus.

Additionally, we verified tissue-specific expression of human *BOO* (*BCL2L10*) in F1 mice derived from 2184D3[ES]-6 ESCs. Boo (also called Bcl2l10, Diva, or Bcl-B) is a recently identified member of the Bcl-2 family of apoptosis regulators, whose function as a pro- or antiapoptotic protein remains unclear [34–40]. *Boo* has been shown to be expressed in multiple tissues during mouse embryonic development [39]. However, expression in adult mice becomes highly restricted to granulosa cells of the ovary, the epididymis and spermatids of the male reproductive system, and, at low levels, the spleen [34,38,39]. In humans, *BOO* is expressed at low to undetectable levels in many tissues, including the spleen and kidney, with high levels of expression observed in the liver, ovary, and testis [35–37]. Thus, it was unknown if the expression pattern of human *BOO* from the BAC in transgenic mice would be more similar to that of endogenous human or mouse *Boo*.

As expected mRNA expression of the endogenous mouse gene was restricted to the testis and ESCs with low to undetectable levels in other tissues (Fig. 7B). Interestingly, the expression of human *BOO* from the BAC transgene was easily detectable in mouse liver, as it is in human liver, suggesting that the human promoter contains sequences that are absent in the mouse gene and can drive expression of *BOO* in both human and mouse liver (Fig. 7B). Human *BOO* from the BAC transgene was also expressed in mouse kidney, spleen, and testes, similar to previous reports of expression in humans (Fig. 7B). Therefore the expression pattern of the human transgene seems to resemble more closely that of the endogenous human gene. These results demonstrate proper in vivo tissue-specific expression of a human BAC transgene targeted to the *Hprt* locus.

Discussion

We have shown that it is possible to target efficiently a human BAC as large as 146 kb into the *Hprt* locus of mouse ESCs, that a targeted BAC transgene can be conditionally excised from the genome with Cre recombinase, that a BAC transgene targeted to the *Hprt* locus can be stably maintained in the genome of transgenic mice, and that the expression of genes on targeted BACs showed tissue-specific expression in vitro and in vivo. We had initial concerns that the targeting efficiency of BACs into the *Hprt* locus would be low due to the large size of the BAC relative to the short *Hprt* homologies; however, we observed only a seven- to eightfold decrease in the number of HAT-resistant ESC colonies from electroporations with BAC DNA versus a base vector with identical homology regions.

We have also initiated experiments to target BACs containing mouse genomic DNA inserts to the *Hprt* locus. Our early results suggest that the potential for the BAC insert to mediate homologous recombination into an endogenous locus does not appreciably decrease targeting of the BAC to the *Hprt* locus. While deletions and rearrangements of targeted human BACs were easily detected, the detection of deletions and/or rearrangement of mouse BACs will be more difficult than with human BACs; however, several methods exist to facilitate this analysis. First, the 5′*loxP* and 3′*loxP* probes are diagnostic for the ends of both human and mouse BACs; therefore, deletions of mouse BAC ends can be detected with these probes. In addition, the genomic probes 5′Hprt and RSA can detect deletions of both mouse and human BAC ends in ESCs. Second, most studies use mouse BACs from the C57B6/J libraries employed in the mouse

genome sequencing project. Because the ESC lines are of 129 origin, 286 potential molecular markers exist to detect the presence of BACs in the ESC genome. Similarly, an extensive panel of SNPs exists between the mouse strains and can be utilized to verify the presence of different regions of the BAC in the ESC genome. Third, Southern analysis of long-range restriction digests with rare-cutting restriction endonucleases can also be informative in characterizing the various targeted clones containing both nonisogenic and isogenic BACs.

In several instances, characterization of randomly integrated human or nonisogenic mouse BACs or YACs detected deletions and/or rearrangements of the integrated sequences[1,5–7]. While many deletions are the results of manual manipulation of BAC DNA, internal deletions and rearrangements of BAC transgenes likely occur in either the bacterial host or the ESCs. Although BACs have been shown to be more stable than YACs in their respective hosts, the frequencies of internal deletions and rearrangements for both BAC and YAC transgenes are similar, suggesting that many of the alterations observed occur in the ESC or zygote [5]. Despite these issues, the potential for alteration in transgene content may also provide a useful panel of overlapping clones for complementation of phenotype experiments in physical mapping projects or mapping of chromatin domains in gene expression analysis. In addition, while random integration requires the generation of transgenic mice and subsequent characterization of the independent lines to accumulate such germ-line deletions of BAC transgenes, our ESC-based targeting allows one to identify ESC clones containing fragmented BACs and either include them as a valuable subclone of the original BAC or discard them prior to generating transgenic mice. Finally, the integration of fragmented BAC transgenes into the *Hprt* locus allows for the direct comparison of transgene function in the absence of the variables associated with random integration.

Another useful aspect of our system for introducing BACs into the *Hprt* locus is that the Cre-mediated BAC modification flanks the BAC genomic insert with *loxP* sites and this arrangement is preserved after recombination at the *Hprt* locus. Thus, mice with these conditional BAC transgenes can be bred to mice with spontaneous mutations, chemically induced mutations, or conventional gene disruptions in combination with a tissue-specific Cre transgenic line as an alternative to producing a conditional mutation at the endogenous locus.

Several groups have recently introduced methods of modifying BACs by "recombineering," which allows for the precise introduction of mutations and/or reporter sequences into the genomic BAC insert by homologous recombination in the bacterial host [41–43]. The modified BACs can be used to generate mutations through homologous recombination with their endogenous locus or can be used as a transgene in a near-endogenous context [43–45]. Our method of targeting BAC transgenes to the *Hprt* locus of ESCs by a directly selectable homologous recombination event will facilitate such studies by providing a means of introducing altered BAC transgenic sequences into the mouse genome in which both the copy number and the site of integration have been controlled.

Materials and methods

BAC-modifying vector construction

The *Hprt* targeting vector pSKB1 contains *Sac*I, *Sac*II, *Pme*I, and *Asc*I sites between the *Pvu*I and *Bam*HI sites upstream of the 5′ homology in the targeting vector pMP8SKB [12]. An initial vector, pJDH8A, was generated by ligating (1) an *Eco*RI/*Not*I fragment from pBluescript II SK(+) (Stratagene, La Jolla, CA, USA), which had the *Xho*I site removed; (2) a *Not*I/*Sal*I fragment from pSKB1amp (containing the *Hprt* complementary sequences, 3 kb of the 3′ homology, and an ampicillin resistance gene inserted into a *Sal*I site of pSKB1); (3) an *Xho*I, *I-Sce*I, and *Bam*HI linker; and (4) a *Bam*HI/*Eco*RI fragment from pSKB1, containing 3.8 kb of 5′ homology. To generate the BAC-modifying vector pJDH8/246b, the *Eco*RI site of pBS246 (Life Technologies/Invitrogen) was removed and a linker containing *Eco*RI was inserted into the *Hin*dIII site of pBS246. An *Eco*RI fragment from pJDH8 (containing the *Hprt* sequences, the *I-Sce*I site, and the ampicillin resistance gene) was inserted into the new *Eco*RI site of pBS246.

In vitro Cre/loxP modification of BAC DNA

CITB D library BAC clones (ResGen/Invitrogen, Carlsbad, CA, USA) were purified using BD Bioscience's (Palo Alto, CA, USA) NucleoBond Plasmid Maxi Kit. The BAC-modifying plasmid pJDH8/246b was digested with *Not*I and the 13.2-kb modifying fragment was gel purified. For in vitro Cre/*loxP*-mediated modification of BAC DNA, 200 ng of the modifying fragment and 200 ng of BAC DNA were combined in a reaction with Cre recombinase (BD Bioscience/Clontech, Palo Alto, CA, USA) following the standard protocol for in vitro recombination from the Creator DNA Cloning Kit (BD Bioscience/Clontech). Two microliters of the reaction was electroporated into ElectroMAX DH10B cells (Invitrogen) using a 1-mm cuvette in a BTX Electro Cell Manipulator ECM 600 (BTX, San Diego, CA, USA) set at 1.2 kV, 50 μF, and 129 ohms. Cells were plated on LB–ampicillin (12.5 μg/ml)/chloramphenicol (12.5 μg/ml) overnight at 37°C. Two to twenty colonies resulted from each electroporation with 10 to 100% containing properly modified BACs.

Restriction enzyme digest and Southern hybridization

BAC DNA was digested with *Eco*RV or *Eco*RV and *I-Sce*I and electrophoresed and fragments were visualized by ethidium bromide staining. DNA transferred to nylon membranes was hybridized with the 5′*loxP,* 3′*loxP,* or Amp probe.

Probes were generated by PCR amplification of modified BAC DNA. ESC or mouse tail DNA was digested with *Sac*I and the HM-1 ESC DNA was spiked with modified BAC DNA digested with *Sac*I as a control. DNA transferred to nylon membranes was hybridized to the RSA [12], 5′Hprt, or human *Alu* probe. The 5′Hprt probe was generated by PCR amplification of HM-1 ESC DNA; PCR amplification of BAC DNA generated the human *Alu* probe. See Supplemental Table 1 for a list of primers used to generate probes.

Cell lines and culture

The HM-1 ESC line, derived from HPRT-deficient 129/OlaHsd mice (129), has been described previously [25]. ESCs were grown on murine embryonic fibroblasts in DMEM-H (Life Technologies/Invitrogen) supplemented with 15% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 0.1 mM 2-mercaptoethanol, 2 mM Glutamax, and LIF conditioned supernatants (~1000 U/ml). Following electroporations, homologous recombinants were selected in ESC medium supplemented with HAT (0.016 mg of hypoxanthine/ml, 0.01 mM aminopterin, and 0.0048 mg of thymidine/ml) for 10 to 12 days, at which time individual colonies were picked for expansion and verification of the desired recombination events.

BAC targeting

BAC DNA was purified with Qiagen's Large Construct Kit, resuspended in sterile 1× TE (pH 8), and linearized with *I-Sce*I. Sterile 10× PBS was added for a final concentration of 2 nM linearized BAC DNA in 400 μl of 1× PBS and TE. ESCs (4×10^7) were electroporated in the DNA solution in a 2-mm cuvette with a BTX Electro Cell Manipulator ECM 600 set at 270 V, 50 μF, and 360 ohms. Electroporations with 2 nM pSKB1 (*Bam*HI/*Xho*I fragment) or *I-Sce*I-linearized pJDH8/246b were used as controls for CTD-2338M9 and CTD-2184D3, respectively.

PCR analysis of genomic DNA

After a 5-min incubation at 95°C, samples were amplified for 30 cycles: 30 s at 95°C, 30 s at 55–60°C, and 1 min at 72°C, followed by a final extension at 72°C for 5 min. Samples were electrophoresed on 1–2% agarose gels and stained with ethidium bromide. See Supplemental Tables 2 and 3 for the list of primers used for PCR of genomic DNA. All sequence information for the BAC clones and STS primers was obtained from the UC Santa Cruz Genome Bioinformatics Web site (genome.ucsc.edu).

FISH analysis of CTD-2338M9 ESC clones

Modified or unmodified CTD-2338M9 BAC DNA was biotin labeled and the mouse X-chromosome centromeric repeat probe DXwas70 [46] was labeled directly with Spectrum Green (Vysis, Downers Grove, IL, USA) using the Nick Translation Kit (Roche, Indianapolis, IN, USA). Metaphase chromosome spreads were hybridized with the labeled probes as previously described [47]. The BAC probe signal was detected and amplified using Texas red–avidin and anti-avidin rabbit polyclonal antibody (Vector Labs, Burlingame, CA, USA). Slides were mounted and counterstained in Vectashield antifade containing DAPI.

Cre-mediated excision of a BAC transgene from the ESC genome

The Cre recombinase expression vector pCAGGS-cre has been described previously [48,49] and was used with the following modifications. Clone 2184D3[ES]-5 (2.5×10^7 cells) was electroporated with pCAGGS-cre in a 2-mm cuvette at 270 V, 50 μF, and 360 ohms with a BTX Electro Cell Manipulator ECM 600. ESCs were plated on 10-cm plates for 5 days, trypsinized, and then replated at 1.8×10^3 cells/10-cm plate for 7 days. Individual colonies were picked for expansion and verification of Cre-mediated excision of the BAC transgene from the ESC genome.

ESC differentiation and QRT-PCR

The differentiation of ESCs into osteoblasts has been described previously [28]. RNA was collected from ESCs, day 2 EBs, day 14 whole plates, and day 21 whole plates with TriReagent. RNA, 2 μg, was reverse transcribed with the RETROscript Kit (Ambion, Austin, TX, USA) using an oligo(dT) primer. QRT-PCR was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) and the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). Serial dilutions of 2338M9[ES]-5 ESC cDNA were used to generate standard curves for each primer set. Duplicate dilutions of 1:8 and 1:32 were analyzed for all cDNAs. The amplification program included an initial denaturation step at 95°C for 15 min, followed by 50 cycles of 94°C for 15 s, 53°C for 30 s, and 72°C for 30 s; fluorescence was measured at the end of each extension step. To verify the specificity of the primers, a melting curve for each primer set was generated by heating the product to 95°C for 15 s, cooling it to 60°C, and then slowly heating it at 0.03°C/s to 95°C; fluorescence was measured during the slow heating phase. See Supplemental Table 3 for the list of primers used for QRT-PCR.

RT-PCR analysis

RNA was collected from homogenized transgenic and wild-type F1 mouse tissues, 50–100 mg, with TriReagent. RNA, 1 μg, was DNase (Invitrogen) treated and reverse transcribed with the RETROscript Kit using an oligo(dT) primer. Samples were PCR-amplified for 30 cycles, 30 s at

95°C, 30 s at 60°C (65°C for mouse *Boo*), and 1 min at 72°C, followed by a final extension at 72°C for 5 min. Samples were electrophoresed on 2% agarose gels and stained with ethidium bromide. See Supplemental Table 3 for the list of primers used for RT-PCR.

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