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Identification of mouse Duffy Antigen Receptor for Chemokines (*Darc*) as a BMD QTL gene

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It is now well known that bone mineral density (BMD) variance is determined by both genetic and environmental factors. Accordingly, studies in human and animal models have revealed evidence for the presence of several quantitative trait loci (QTL) that contribute to BMD variations. However, the identification of BMD QTL genes remains a big challenge. In the current study, we focused our efforts to identify the BMD candidate gene in chromosome 1 (Chr 1) QTL that was detected from a cross involving high BMD CAST/EiJ (CAST) and low BMD C57BL/6J (B6) mice. To this end, we have combined several approaches including: (1) fine mapping the BMD QTL in Chr 1 of the B6.CAST F₂ female mice using a large number of polymorphic markers; (2) the generation of congenic sublines of mice by repeated backcrossing of CAST with B6 mice and phenotype characterization; (3) expression profiling genes in the QTL region; and (4) SNP analyses to identify the mouse Duffy Antigen Receptor for Chemokines (*Darc*) as a candidate gene for Chr 1 BMD QTL2. We verified the involvement of the *Darc* protein in BMD variation by evaluating the skeletal phenotype of *Darc*-knockout mice and congenic sublines of mice carrying small chromosomal segments from CAST BMD QTL. Based on the findings that *Darc*-antibody blocked formation of multinucleated osteoclasts in vitro and that *Darc* from CAST binds chemokines, known to regulate osteoclast formation, with reduced affinity compared with *Darc* from B6 mice, we conclude that *Darc* regulates BMD negatively by increasing osteoclast formation, and that the genetic association between *Darc* gene polymorphism and BMD variations in humans merits investigation.

[Supplemental material is available online at www.genome.org.]

Osteoporosis is a condition associated with decreased bone strength and an increased fracture risk. Its prevalence is increasing as more individuals with low bone mineral density (BMD), the strongest predictor of fracture risk, are detected. Thus, molecular dissection of the BMD regulatory system may reveal novel therapeutic targets for the treatment of osteoporosis. Several studies performed on both humans and mice have shown that BMD has a strong heritable component, with estimates that genes account for 60%–80% of BMD variance (Morrison et al. 1994; Beamer et al. 1996; Devoto et al. 1998; Klein et al. 1998; Eisman 1999; Niu et al. 1999; Baldock and Eisman 2004). Accordingly, linkage studies in humans and experimental animal models have identified several BMD QTLs. However, the big challenge has been identifying the “QTL gene.” To our knowledge, only a single QTL gene, *Alox15*, has been identified thus far as a BMD QTL gene (Klein et al. 2004).

To screen for BMD candidate genes, we have chosen to dissect the BMD QTL in chromosome 1, because this QTL has been identified in four different intercrosses: C57BL/6J (B6) × Cast/EiJ (CAST) (Beamer et al. 1999), B6 × C3H/HeJ (Beamer et al. 2001), B6 × DBA/2 (Klein et al. 1998), and MRL/MpJ × SJL/J (Masinde et al. 2002). Furthermore, a syntenic region in human chromosome 1q21-q43 carries a previously identified BMD QTL, which has been reported in independent studies using human popula-

tions (Koller et al. 2000; Ralston 2002). In this report, we present data on the identification of Duffy Antigen Receptor for Chemokines (*Darc*) as a BMD QTL gene by utilizing a variety of molecular genetic approaches.

Results

Fine mapping and congenic sublines of mice

In a recently published study (Edderkaoui et al. 2006), we reported the identification of three BMD loci at Chr 1 by superimposing the CAST chromosomal regions carried by each congenic subline. The *Bmd1a* locus was located between *D1mit133* and *D1mit453* (133.2–165.2 Mb), the *Bmd1b* locus was located between *D1mit113* and *D1mit150* (171.86–174.76 Mb), and a *Bmd1c*, which regulates BMD negatively, was located between *D1mit221* and *D1mit511* (184.86–192.06 Mb). In this study, we further fine mapped the Chr 1 QTL region by genotyping 565 B6.CAST F₂ female mice with a large number of polymorphic markers from this region. Interval mapping using MapQTL 4.0 to identify the linkage between polymorphic markers and femur total volumetric BMD (vBMD) within the F₂ female mice showed three peaks/QTL: peak 1 at marker *D1mit453* (*Bmd1a*, at 165.2 Mb), peak 2 at marker *D1mit354* (*Bmd1b*, at 172.98 Mb), and peak 3 at marker *D1mit359* (designated as *BMD1-4* at 177.4 Mb) (Fig. 1), which were significantly associated with femoral vBMD. Map-Maker software also identified three significant linkages at the same positions (Supplemental Table 1), which explained 9.3%, 10.7%, and 9% of the BMD variation within the B6.CAST

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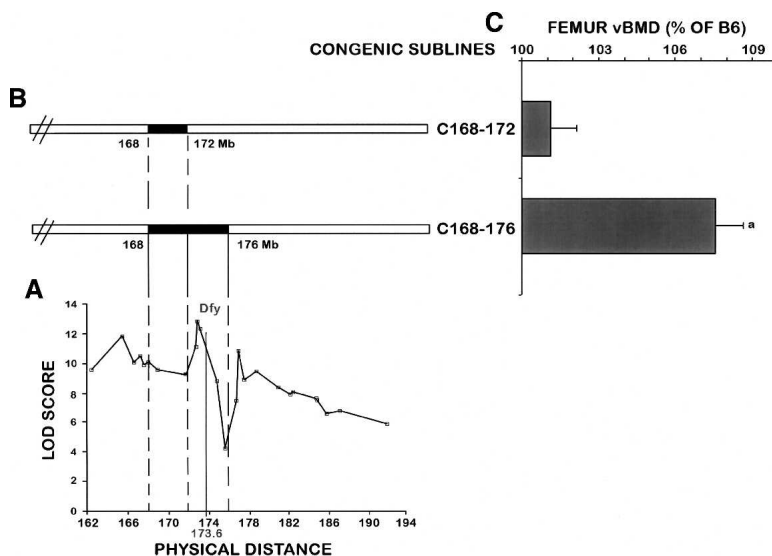


Figure 1. Genetic localization of the *Darc* gene and BMD phenotype of the two congenic sublines C168-172 and C168-176. (A) Fine mapping of BMD QTL in chromosome 1 using 33 polymorphic markers. Linkage analyses showed three peaks suggesting at least three BMD QTLs within Chr 1. The X-axis represents the physical distance beginning with the centromeric side and extending toward the telomeric side of Chr 1, starting from marker *D1mit106* at 162.33 Mb according to the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/>). The Y-axis represents the likelihood values for the presence of a segregating QTL at each marker (LOD score). Genetic localization of the *Darc* gene within the second BMD QTL (*Bmd1b*) is indicated. (B) Solid bars represent the CAST chromosomal regions carried by each congenic sublines. (C) Femoral vBMD at the mid-diaphysis of the two sublines C168-172 and C168-176 compared with B6 control mice. Each congenic subline is presented (C, referring to the cast allele), followed by the position (in megabases) of the two markers flanking the donated CAST segment. We designated the congenic subline B6.CAST-1^{D1mit370-D1mit403} as C168-176, which indicates the approximate proximal and distal megabase limits known for the CAST chromosomal region, and the congenic subline B6.CAST-1^{D1mit370-D1mit113} was designated as C168-172. Data are expressed as a percentage of B6 and are mean $\pm$ SEM. (a) $P < 0.01$ as measured by Student's *t*-test in congenic subline vs. B6 mice. $n = 10$ –14.

F₂ population, respectively. Thus, Chr 1 contains at least four BMD loci, three of which regulate BMD positively and one negatively.

Since the *Bmd1b* QTL showed the highest LOD score, we chose to screen BMD candidate genes within this locus. Therefore, to narrow down the size of the *Bmd1b* QTL region to permit our candidate gene search, we generated additional congenic sublines from the C168-185 subline, as described in Edderkaoui et al. (2006). In the present study, we compared the skeletal phenotype of the congenic subline that carries the CAST chromosomal segment from *D1mit370* to *D1mit403* (168–176 Mb) and the congenic subline that carried the CAST chromosomal region *D1mit370-D1mit113* (168–172 Mb) located between the first and the second peaks/QTL with the B6 control mice (Fig. 1). The congenic subline C168-176 exhibited a significantly greater vBMD compared with the B6 mice (7.5%, $P = 0.01$), while C168-172 did not show any differences compared with the B6 controls. Using this data, we could safely eliminate the genes located at the 168–172 Mb chromosomal region from further analyses (Fig. 1), which narrowed down the size of the *Bmd1b* locus to 172–176 Mb from the centromere in Chr 1. Furthermore, the C168-176 subline exhibited a significantly smaller femur trabecular volume and reduced midshaft endosteal circumference, but no difference in the periosteal circumference (Fig. 2). These changes contributed to an increase in the cortical thickness and correspondingly higher vBMD in the femurs of C168-176 subline compared with the B6 control mice. There was no difference in body weight between C168-176 and B6 control mice (Fig. 2), suggesting that

the variation in BMD phenotype between congenic and B6 mice cannot be explained on the basis of size difference.

Gene expression profiling

In order to screen for potential BMD candidate genes in the 172–176 Mb region, we compared the expression levels of all known genes and ESTs located in this region between the bones of the B6 control mice and the two congenic sublines described above. We chose gene expression change to identify candidate genes, since it is known that SNPs at both the regulatory regions (promoter, 3'-UTR) and coding regions (via feedback loop) could affect the expression of the gene (Schadt et al. 2003; Chesler et al. 2005). In addition, this strategy has been successfully used in a recent study by Klein et al. (2004) to identify a QTL gene that negatively regulates peak bone density. Since C168-172 does not show a BMD phenotype it was used as a negative control, so only genes that showed a difference in the expression between C168-176 and B6, but not between C168-172 and B6, were further analyzed. Among all genes analyzed, only the *Darc* gene, located at 173.6 Mb, showed a sixfold increase of the expression in the C168-176 congenic subline when compared with the control progenitors (Supplemental Table 2), while no difference was found in expression

between the C168-172 and B6 control mice. Since none of the other genes exhibited more than twofold difference that is significant, we focused on *Darc* as a candidate gene.

Skeletal phenotype of *Darc*-KO mice

If the increase of *Darc* gene expression in B6.CAST-1 congenic mice is responsible for the greater femur BMD exhibited by the C168-176 mice, we would expect *Darc* knockout (KO) mice to show a lower femur BMD compared with control mice. To test this hypothesis, we compared the phenotype of *Darc*-KO mice generated in a B6 background with that of age-gender-matched wild-type B6 progenitors. Body weight as well as femur length were similar for the two strains (Fig. 2), while total femur vBMD was increased in the *Darc*-KO mice compared with the wild-type control mice. The trabecular volume was 13% smaller in the *Darc*-KO mice compared with the wild-type mice ($P < 0.01$), the endosteal circumference was decreased by 3.8%, and there was no change in periosteal circumference, which resembles the phenotype of the C168-176 subline and confirms the involvement of the *Darc* gene in BMD variation. Based on these data we considered an alternate possibility that the high expression of *Darc* might be secondary to a loss of the protein function.

SNP analyses

We next sequenced the *Darc* gene to determine the SNPs that might be the cause of the difference in the expression between

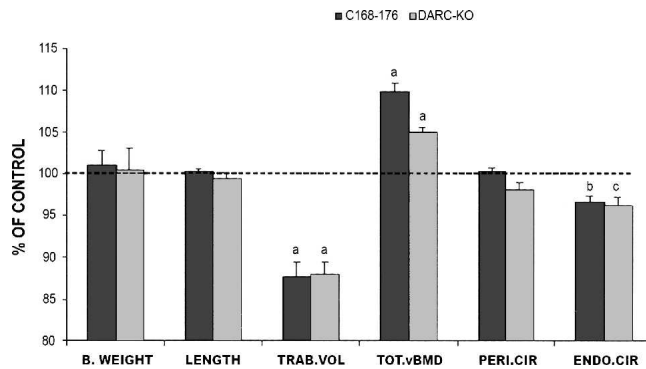


Figure 2. Femoral and body weight data from the C168-176 congenic subline of mice and *Darc*-KO mice. Data are expressed as a percentage of B6 and are mean $\pm$ SEM. (a) $P < 0.01$, (b) $P = 0.01$, (c) $P = 0.05$ as measured by Student's *t*-test, with $n = 10$ –14. (B.WEIGHT) Body weight, (LENGTH) femur length, (TRAB.VOL) total femur trabecular volume, (TOT.vBMD) total volumetric bone mineral density, (PERI.CIR) periosteal circumference, (ENDO.CIR) endosteal circumference.

the B6 mice and the high BMD congenic subline of mice. Using the NCBI database and in-house sequencing, we have identified 28 polymorphisms that distinguish the *Darc* gene in B6 from the CAST strain (Table 1), with three SNPs located at the promoter region, 15 polymorphism sequences located at the intervening sequence, and 10 polymorphisms at the coding region.

Table 1. Summary of sequence variants detected in *Darc* gene

Nucleotide change CAST/C3H/B6	Polymorphism position (bp)	Intron/exon	Amino acid change CAST/C3H/B6	Amino acid position
A/A/C	–456	Promoter		
A/A/C	–325	Promoter		
G/A/G	–234	Promoter		
T/T/G	–169	Promoter		
A/–/T ^a	129	Intron		
GT/GT/Del.	131	Intron		
C/C/T	147	Intron		
C/C/T	163	Intron		
G/A/A	184	Intron		
T/T/C	201	Intron		
C/C/T	285	Intron		
T/T/C	308	Intron		
A/A/T	310	Intron		
A/A/G	372	Intron		
G/G/T	382	Intron		
A/A/T	405	Intron		
G/G/A	408	Intron		
T/T/C	454	Intron		
C/C/T	467	Intron		
C/C/A	493	Exon 2	T/T/N	10
A/A/G	631	Exon 2	D/D/G	56
G/G/T	644	Exon 2	L/L/L	60
G/A/G	690	Exon 2	G/S/G	76
T/C/C	830	Exon 2	H/H/H	122
T/T/C	920	Exon 2	N/N/N	152
T/C/T	997	Exon 2	L/S/L	178
A/A/G	1044	Exon 2	T/T/A	194
CT/CT/TG	1084	Exon 2	T/T/M	207
T/T/C	1126	Exon 2	L/L/P	221
C/C/T	1267	Exon 2	T/T/I	286
T/T/C	1286	Exon 2	C/C/C	292

The positions of the SNPs in the promoter regions were taken according to the sequence reported by Luo et al. (2000), while the polymorphism sequences located within the gene from the start codon to the stop codon were taken according to CAST sequence AB039077. The gene was sequenced from –580 bp to the stop codon.

^a(–) Data unavailable.

Among the polymorphisms in the coding region, only six led to amino acid changes (Table 1). None of the polymorphisms discovered in the promoter region covered the consensus sequences that potentially regulate the transcription process, so it is likely that the difference in the expression of the *Darc* gene is due to the SNPs in the coding region. In order to determine if the SNPs in the *Darc* gene could be responsible for BMD variation, we compared the *Darc* gene sequence of B6 and CAST with another high BMD strain, namely C3H/HeJ (C3H). We found that the six informative SNPs in the coding region were conserved in both CAST and C3H, suggesting that one or more of these SNPs could contribute to the high BMD phenotype exhibited by CAST and C3H mice (Table 1).

Binding assay

The *Darc* gene is an acidic glycoprotein that spans the plasma membrane with seven transmembranous helices, like all members of the G-protein-coupled chemokine receptors (Fig. 3). As the name implies, the Darc protein is known to bind to several chemokines such as CC chemokines, monocyte chemotactic protein-1 (Ccl2), Ccl5 (regulated on activation, normal T cell expressed and secreted), and interleukin-8 (Il8). These cytokines have been shown to regulate formation and activity of osteoclasts via tumor necrosis factor ligand (Tnfsf11)-dependent and -independent mechanisms (Wise et al. 2002; Bendre et al. 2003, 2005; Kim et al. 2005).

Tournamille et al. (1997) have reported that the chemokine binding pocket of the Darc protein is located in the first and fourth extracellular domains, which are brought into close vicinity by a disulfide bridge (Fig. 3). Among the six SNPs found in the coding region, the aspartic acid at position 56 seems to be significant due to its location close to the cysteine at position 49, which is required to form the disulfide bridge between transmembrane helices 1 and 7 in the chemokine binding pocket. The change of a small and neutral amino acid glycine with a negatively charged aspartic acid could change the conformation of the protein as well as the chemical environment of the binding sites, which consequently may affect the binding of the Darc protein to the chemokines and/or the stability of Darc protein. Accordingly, we predict that the Darc protein in CAST binds to chemokines much less efficiently compared with the Darc protein from B6 mice. The decreased protein function could contribute to increased expression as a consequence of loss of the feedback control. To test this hypothesis, we analyzed the binding of chemokines to nonadherent bone marrow cells (NABMC) derived from *Darc* knockout and congenic mice. We chose Ccl2, Ccl5, and Il8 as ligands for the binding studies based on previous findings that these chemokines bound to the Darc protein and all the three chemokines have been shown to regulate osteoclast formation. By using ¹²⁵I-labeled Ccl2, Il8, and Ccl5 for the

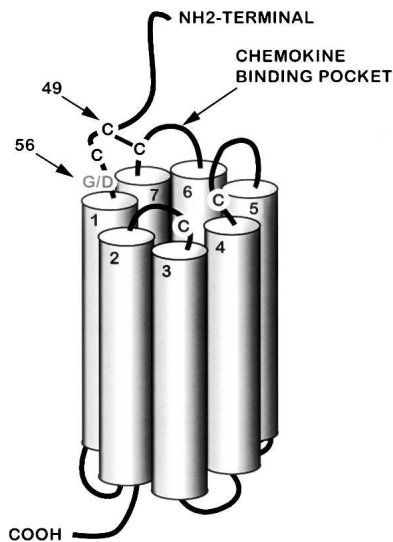


Figure 3. Schematic presentation of the predicted model of Darc protein based on analogy with the IL8 type A receptor (modified from Tournamille et al. 1997). The three cysteine residues that form the chemokine binding site are brought into close proximity by a disulfide bridge between the first and the fourth extracellular domains of the Darc protein. The cysteine residue at the N terminus that forms the bridge is at position 49 in the mouse *Darc* sequence. The position of the SNP G56D is indicated.

binding studies, we found that the specific binding of the three chemokines was significantly reduced in the NABMC derived from both the C168–176 subline and *Darc*-KO mice compared with B6 mice (Fig. 4A).

In vitro osteoclast formation assay

Since Darc protein binds to a number of chemokines that regulate osteoclastogenesis, we next determined if reduced binding of chemokines to Darc in congenic mice is associated with corresponding changes in osteoclast formation.

We treated nonadherent bone marrow cells (NABMC) with Tnfsf11 and macrophage colony stimulating factor (Csf1), the two major cytokines reported to be involved in osteoclast formation (Quinn et al. 1998; Takahashi et al. 1999). The *Darc*-KO mice showed a 50% decrease in the number of TRAP-positive MNC compared with the wild-type mice. Furthermore, despite the increase of *Darc* gene expression in C168–176, this subline showed a 70% decreased formation of TRAP-positive MNC compared with B6 control mice (Fig. 4B). In addition, treatment of NABMC with Darc-antibody (Darc-Ab) in the presence of Tnfsf11 and Csf1 resulted in a significant reduction in the number of TRAP-positive multinucleated cells (Fig. 4C,D). These data together offer strong evidence that Darc protein is involved in osteoclast formation and/or activity, and that the reduced osteoclast formation in the NABMC of CAST mice could be explained on the basis of reduced activity of Darc protein in CAST mice compared with B6 mice.

In vivo histomorphometry study

Consistent with the in vitro data, we found that the TRAP-positive bone resorbing surface was significantly reduced both at the endosteum (47%, $P = 0.002$) and the periosteum (8%, $P = 0.02$) of femurs isolated from *Darc*-KO mice compared with

control mice as determined by histomorphometric analyses (Fig. 5). In contrast, bone formation rate was not significantly different in the femurs of *Darc*-KO mice compared with control mice (Fig. 5). We are in the process of breeding a congenic subline of mice carrying the *Bmd1b* locus for the purpose of performing histomorphometry studies in order to confirm that the increased BMD in congenic mice is also due to reduced bone resorption.

Discussion

Osteoporosis is a common disease characterized by reduced bone mass and increased risk of fragility fractures. Because of the well recognized importance of a genetic component in the pathogenesis of osteoporosis, there is now considerable interest among researchers in the bone field to identify genes that contribute to variation in peak bone mass. To identify osteoporosis genes, several approaches have been employed that include association studies and linkage analysis in humans and in experimental model organisms. While the association studies have identified a number of potential candidate genes that contribute to osteoporosis susceptibility, relatively few candidate gene polymorphisms have been validated by large-scale studies. Linkage studies in humans and various animal models, on the other hand, have revealed evidence in almost every chromosome for the presence of genetic loci that regulate bone mass (Beamer et al. 1999, 2001; Koller et al. 2000; Kammerer et al. 2003; Karasik et al. 2004; Volkman et al. 2004; Peacock et al. 2005; Ralston et al. 2005). Together, the association and linkage studies point to the fact that osteoporosis susceptibility is determined by a large number of genes with a modest or small effect size and not by few genes of major effect as originally thought. Accordingly, our data with a large number of congenic strains carrying overlapping CAST chromosomal segments from Chr 1 BMD QTL have revealed that there are at least four BMD loci in Chr 1, each contributing to 4%–12% of the variation in peak bone mass (this work and Edderkaoui et al. 2006).

The successful cloning of the QTL gene depends on the ability to resolve genetic effects into sufficiently small intervals to make gene identification feasible. In this regard, we were able to utilize strategies involving high-resolution mapping with a large number of markers and congenic sublines of B6 mice containing overlapping segments of CAST Chr 1 in the QTL region to successfully narrow down the size of the *Bmd1b* locus to 4 Mb. Since the 4-Mb regions did not contain any obvious functional candidate gene for peak bone mass, we utilized the alternate approach of identifying candidate genes based on gene expression changes and SNP analyses. This approach led to the identification of the *Darc* gene, which was subsequently confirmed as a BMD QTL gene by functional studies. Our findings along with those of Klein et al. (2004) provide feasibility that the small effect of BMD QTL genes can be identified by an integrated approach involving fine mapping, use of congenic sublines containing the QTL gene, gene expression profiling, SNP analyses, and functional studies.

In the current study, *Darc* was identified as a BMD *QTL1-2* candidate gene based on differential expression and SNP analyses between congenic sublines and B6 mice. The basis for the increased expression of *Darc* in congenic mice can only be speculated at this time. In this regard, it is possible that the loss of protein function caused by changes in key amino acids in the chemokine binding pocket could lead to increased expression due to the loss of negative feedback. Accordingly, we found re-

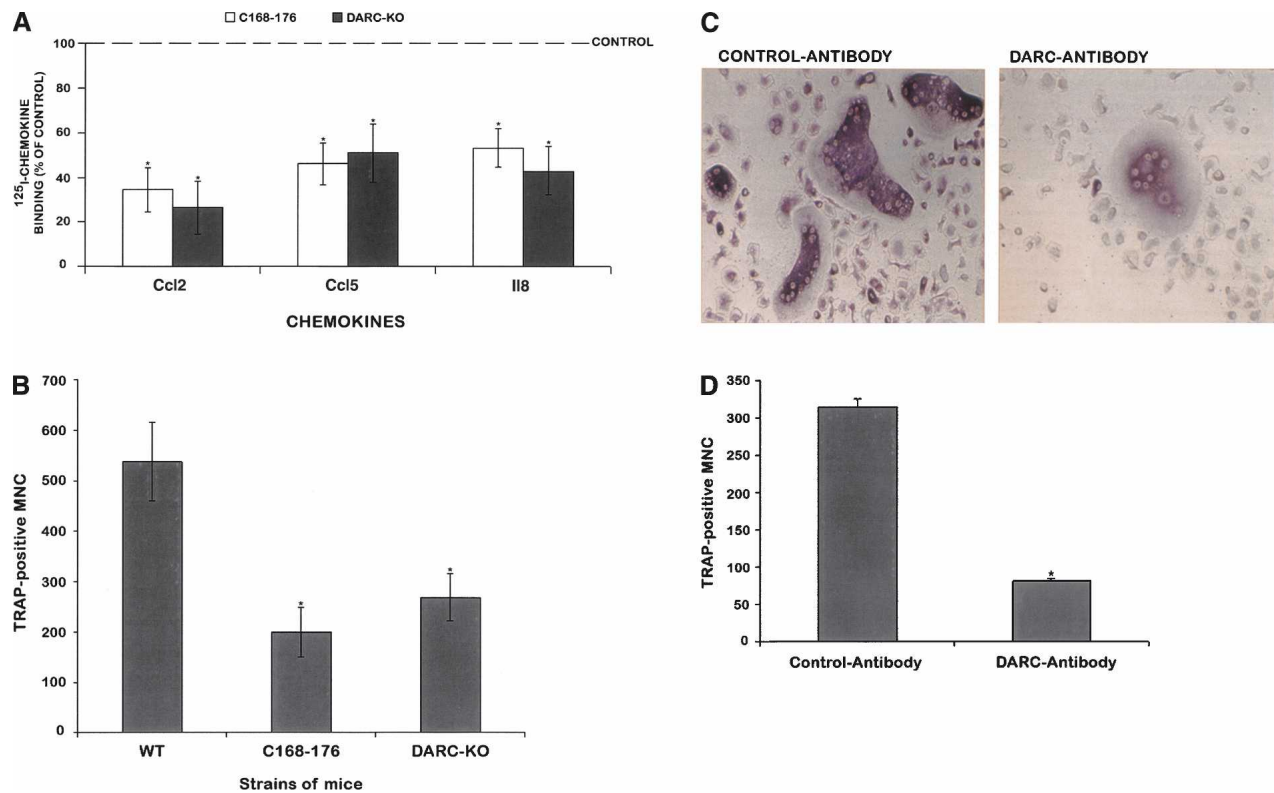


Figure 4. In vitro analysis of nonadherent bone marrow cells (NABMC) from B6, C168-176 congenic subline, *Darc*^{+/+}, and *Darc*^{-/-} mice. (A) Binding of three chemokines to NABMC. NABMC were incubated at 37°C/RT in the presence of either ¹²⁵I-chemokine only or both labeled and unlabeled chemokine. The specific binding was expressed as the CPM value in the absence of cold chemokine subtracted from the CPM value in the presence of cold chemokine. Data are expressed as a percentage of ¹²⁵I-chemokine binding to NABMCs derived from B6 control mice, *n* = 8–10. (B) Osteoclast generation in response to Tnfsf11 (100 ng/mL) and Csf1 (100 ng/mL). After 10 d in culture, cells were stained for TRAP activity, and only cells with more than two nuclei (MNC: multinucleated cells) were counted. Data are expressed as a mean ± SEM. Six to eight mice were used for each strain of mice. (WT) Wild-type mice. Groups were compared with wild-type mice with Student's *t*-test. (*) *P* < 0.05 vs. B6 control mice. (C) Effect of Darc-antibody on TRAP-positive cells derived from NABMC of wild-type B6 mice. After 6 d of treatment with goat polyclonal Darc-Ab in the presence of Tnfsf11 and Csf1 (right panel), the number of TRAP-positive multinucleated cells decreased considerably compared with cells treated with control IgG in presence of Tnfsf11 and Csf1 (left panel). (D) Number of TRAP-positive MNC derived from NABMC of wild-type B6 treated with Tnfsf11 and Csf1 in the presence of either Darc-antibody or control antibody. The data are expressed as mean ± SEM. The experiment was repeated twice with cells derived from four mice each time. (*) *P* < 0.05 vs. control antibody (normal goat IgG).

duced binding of a number of chemokines to Darc from congenic mice compared with B6 mice. Alternatively, the increased expression may be caused by SNPs in the regulatory regions that are

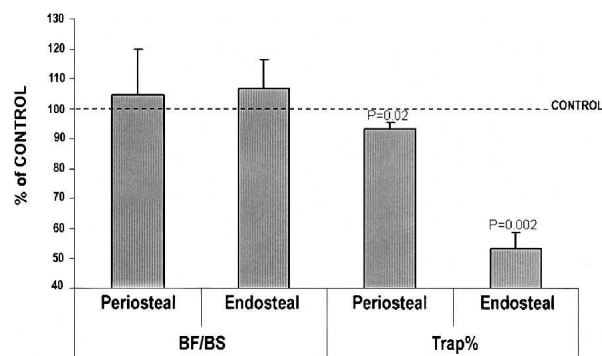


Figure 5. Bone formation and bone resorption rates in femur from *Darc*-KO mice. Bone formation is calculated as bone formation surface (BF) divided by total bone surface (BS). Bone resorption (Trap%) is calculated as (the resorbing surface/bone surface) × 100. The data are expressed as the percentage of wild-type values, *n* = 8–13, (*) *P* < 0.05 vs. wild-type control mice.

involved in transcriptional regulation and/or mRNA stability. In any case, the proof that the *Darc* gene is the BMD *QTL1-2* gene came from in vitro and in vivo functional studies. We have found that *Darc*-knockout mice exhibited a similar skeletal phenotype as that of the congenic subline of mice carrying the *Bmd1b* locus. The higher BMD in the congenic subline of mice carrying the *Bmd1b* locus and *Darc*-knockout mice is contributed by cortical thickness that is caused by reduced endosteal circumference. The reduced bone marrow cavity in the absence of functional *Darc* could be due to reduced resorption and/or increased bone formation at the endosteum. In this regard, our in vitro osteoclast formation assay using NABMC revealed a significant reduction in osteoclast formation in NABMC derived from congenic as well as *Darc*-knockout mice compared with B6 mice. Consistent with these data, bone resorption but not bone formation was significantly reduced in the *Darc*-knockout mice compared with B6 mice. Based on these data, we conclude that *Darc* is a negative regulator of osteoclasts and that bone resorption is reduced in the absence of *Darc*, resulting in increased BMD.

As to how *Darc* regulates osteoclast formation, it is known that *Darc* binds to chemokines that are involved in osteoclast formation. Although *Darc* was first identified as Duffy blood

antigen (Pogo and Chaudhuri 2000), there is now considerable evidence that *Darc* regulates cell trafficking and modulates the bioavailability of several chemokines such as *Ccl2* and *Ccl5* (Chaudhuri et al. 2003; Kashiwazaki et al. 2003), which are chemotactic signals for monocytes and are responsible for the migration of osteoclast precursors to fuse into differentiated osteoclasts (Kim et al. 2005). Therefore, based on our data and the previously published data about *Darc* and the chemokines function, we postulate a model of *Darc* involvement in osteoclast formation. As reported by Kim et al. (2005), the activation of osteoclast precursors by *Tnfsf11* and *Csf1* leads to an increase in the expression of *Ccl2* and *Ccl5*, which are known to bind to *Darc*. Since *Darc* is involved in cell trafficking, and based on our finding that *Darc*-antibody inhibits osteoclast formation, we postulate that *Darc*-chemokine signaling is involved in osteoclast precursors trafficking for subsequent fusion into differentiated osteoclasts. Accordingly, the disruption of Duffy as in *Darc*-KO mice or C168–176 mice would lead to an alteration of osteoclast formation (Fig. 6). Our current studies are focused on testing the possibility that *Darc* is involved in the trafficking of osteoclast precursors for subsequent fusion.

In humans, the Duffy blood group system consists of four alleles, five phenotypes, and five antigens (Pogo and Chaudhuri 2000). Duffy-negative individuals are predominantly Africans and American blacks; they lack the Duffy protein on erythrocytes and are resistant to *Plasmodium vivax* and *Plasmodium knowlesi* infections (Miller et al. 1975, 1976). In addition, it has been well established that African-Americans exhibit significantly higher BMD compared with Caucasians (Yanovski et al. 2000; Cauley et al. 2005). Our finding that total femur vBMD is increased in *Darc*-KO mice compared with B6 mice raises the interesting possibility that BMD variation between African-Americans and Caucasians could be due in part to a difference in the function of *Darc* between the two groups.

Since *Darc*-KO mice were generated in a mixed genetic background involving 129 and B6 strains, it is important to rule out the possibility that another gene contributed by the 129 strain was responsible for the increased BMD in the *Darc*-KO mice. In this regard, *Darc*-KO mice used in this study have been backcrossed with B6 mice for at least eight generations to minimize the genetic contribution of the 129 strain. After eight backcrosses, the genetic contribution of the donor strain is estimated to be <1% (Silver 1995). Accordingly, much of Chr 1 is derived

from B6 mice except a small region surrounding the *Darc* gene that was found to be homozygous for 129 (171–177 Mb), which was not surprising since homologous recombination to disrupt the *Darc* gene was carried out using stem cells derived from 129 mice, and since mice carrying the *Darc*-KO gene from 129 were selected for subsequent backcrosses with B6 mice. Our findings that NABMC derived from both congenic and *Darc*-KO mice exhibit reduced potential to form TRAP-positive multinucleated osteoclasts in vitro and that blockage of *Darc* action using neutralizing antibody to *Darc* reduced osteoclast formation in NABMC derived from B6 mice provided further experimental data for the *Darc* being the Chr 1 BMD QTL2 gene. However, the ultimate proof for *Darc* as a BMD QTL gene will require knock-in of a CAST copy of the *Darc* gene in the B6 background or vice versa and demonstration of the corresponding BMD phenotype in the knock-in mice.

In conclusion, this was the first study, to our knowledge, to provide strong evidence that the *Darc* gene plays an important role in regulating femur BMD via controlling osteoclastogenesis. Investigations into the molecular mechanism by which the Duffy protein regulates osteoclastogenesis could lead to the development of novel drug targets for the diagnosis and/or treatment of osteoporosis.

Methods

Mice

The study used two inbred strains of mice, C57BL/6J (B6) and CAST/EiJ (CAST), which have been previously shown to differ widely in total femur vBMD at 16 wk of age (Beamer et al. 1999). The rationale for selecting these two inbred mouse strains was based on the fact that these strains are highly polymorphic for genetic differences at >95% of their genomes (Dietrich et al. 1996). The B6.CAST F₂ mice were produced and maintained at the animal research facility of The Jackson Laboratory (TJL; Bar Harbor, ME), as previously described (Beamer et al. 1999), while the congenic sublines of mice were generated and maintained at the Jerry L. Pettis VA Medical Center (LL; Loma Linda, CA) under the standard conditions previously described by Edderkaoui et al. (2006). The animal work performed in this study was approved by the institutional animal care and use committees at both TJL and LL. *Darc*-knockout (*Darc*-KO) mice as well as the control mice were generated as previously described by Luo et al. (2000), and were bred and maintained in LL under the same conditions previously described by Edderkaoui et al. (2006).

Fine mapping the B6.CAST F₂ population

Spleens from 565 B6.CAST F₂ female mice were received from Wes Beamer (TJL) to be used for genotyping. Extractions of genomic DNA from the spleens were performed following the Gen-ta PurGene protocol (PUREGENE Tissue kit, part no. D-7000A). In order to fine map the BMD QTL in Chr 1, we used 33 polymorphic markers located within the BMD QTL region that covers 24.6 cM in mouse Chr 1, between *D1mit106* and *D1mit511* as described by Edderkaoui et al. (2006), instead of the seven markers that were previously used to identify the major BMD QTL in this region (Beamer et al. 1999). The number of markers was increased in order to detect and evaluate the effect of more potential recombinations within the major QTL region.

Genotyping data from the F₂ population were analyzed using MapQTL (4.0) software (CPRO-DLO), and Mapmaker software was used to test the reproducibility of MapQTL data. MapQTL interval mapping was used for quantitative trait loci

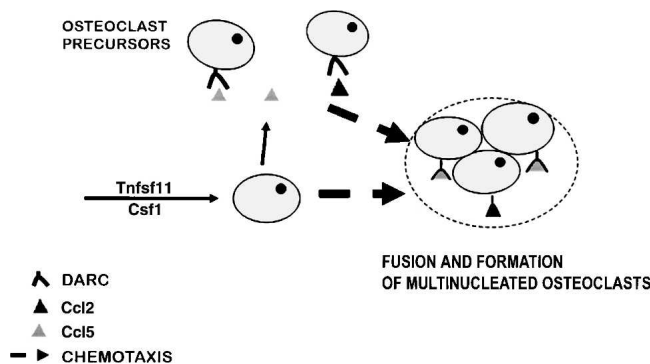


Figure 6. Model of *Darc* involvement in osteoclast generation. Activation of osteoclast precursors leads to increased expression of *Ccl2* and *Ccl5*, which bind to *Darc* protein. *Darc*-chemokine signaling is involved in osteoclast precursor trafficking for subsequent fusion into differentiated osteoclasts.

mapping, and the LOD score significant thresholds were calculated using MapQTL permutation tests at a confidence level of 99%.

Generation of congenic sublines and genotyping

The B6.CAST-1^{D1mit370-D1mit152} congenic (C168–185) subline, previously described by Edderkaoui et al. (2006), was used to generate more congenic sublines and break down the cast region first transferred to the B6 background. Additional crosses between the C168–185 congenic subline and the B6 progenitor produced new recombinations carrying smaller overlapping segments that were fixed by crossing the heterozygous mice of same recombination between one another. Each subline was genotyped using the polymorphic markers that encompass the region 168–185 Mb from the centromere (Edderkaoui et al. 2006).

Darc-KO mice were generated as previously described (Papaioannou and Johnson 1993; Luo et al. 2000) and backcrossed with C57BL mice. DNA extraction and genotyping were performed as described by Edderkaoui et al. (2006).

Phenotypic analyses

Based on data from previous studies, which showed that rapid acquisition of vBMD peaked at ~16 wk, resulting in significant higher density values in the CAST females compared with the B6 females (Beamer et al. 1999), only females were used in this study and all measurements were performed at 16 wk of age.

All the measurements for the B6.CAST F₂ mice were performed at TJL as described by Beamer et al. (1999). For the B6 control mice and B6.CAST-1 congenic sublines of mice, bones were stored and processed as described by Edderkaoui et al. (2006).

The measured parameters for the congenic sublines of mice as well as knockout mice were expressed as the percentage of B6 control mice and were mean $\pm$ SEM. Characteristics of the congenic sublines as well as knockout mice were compared with the B6 control progeny using a Student's *t*-test, and differences were judged statistically significant when $P < 0.05$. Data from the congenic sublines mice were compared with B6 progenitors provided by TJL and bred in LL, while the *Darc*-KO mice data were compared with the B6 control mice provided by Dr. Chaudhuri (New York Blood Center, New York) and bred in LL.

RNA isolation and real-time PCR analyses

We isolated total RNA from femurs without bone marrow following the protocol supplied by Life Technologies Company using Trizol reagent. Since the BMD phenotype was analyzed in 16-wk-old female mice, only female mice 14 wk of age were used for gene expression studies to screen for candidate genes responsible for this phenotype. We synthesized cDNA by priming 500 ng of total RNA with oligodT in the presence of M-MLV-reverse transcriptase following the manufacturer's protocol (<http://www.promega.com/tbs/9pim170/9pim170.pdf>).

The quantification of mRNAs was performed in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using SYBR Green as a detector dye. The reaction mixture contained 12.5 μ L of Brilliant SYBR Green (Stratagene), 2 pmol of each primer, 1 μ L of template cDNA, and RNase-free water to a final volume of 25 μ L. The oligonucleotide primers were designated in order to amplify the most specific sequence in the coding region of each gene or EST. The relative differences in expression between the groups were determined using cycle time (Ct) values as follows: The Ct values of the genes of interest were first normalized with 18S of the same sample; then the relative differences between the B6 mice and congenic sublines of mice were

calculated and expressed as relative increases or decreases, setting the control as 100%. Assuming the Ct value reflects the initial starting copy and there is 100% efficiency, a difference of one cycle is equivalent to a twofold difference in starting copy.

If a significant difference in the expression between the congenic sublines and B6 mice was found for a certain gene, another pair of primers was used to confirm the results, so only the genes that showed a difference with at least two pairs of primers are reported herein. For the *Darc* gene, we have used four pairs of primers. The first two pairs of primers showed a 12-fold increase of the RNA expression in C168–176 compared with B6 mice. However, after sequence analysis, we found that the two primers used for real-time PCR (RT-PCR) covered the polymorphism sequences between B6 and CAST, so we decided to design new primers to determine whether the difference showed by RT-PCR was due to the mismatch or due to any difference in mRNA levels. The new primers that do not cover any SNP showed a sixfold increase of *Darc* expression in the C168–176 compared with B6 control mice.

The primers used to determine *Darc* gene expression level are:

Darc-F1. TCGGGTGGAAACCCTTTCA
Darc-R1. AGCTGGTGTGTCAGGCTGTAGTCA
Darc-F2. CCTGAATCCCAGACTGAATATTGG
Darc-R2. GAAAGGTGCAGAAGCCATTGTAA
Darc-F3. CCCATTCTGGCAGAGTTAGCA
Darc-R3. TCAACAGAGCTTGGGGAAAAA
Darc-F4. TTCTTCCACTGGCAGATTTCG
Darc-R4. ACCATACCCAGTAGCCCAGGTT

SNPs analyses

We identified SNPs by direct DNA sequence analysis of PCR products. To sequence the promoter region, we have designated primers using the DNA sequence reported by Luo et al. (2000). More primers were designated to amplify overlapping regions from the start codon to the stop codon of the *Darc* gene using a B6 sequence from NCBI, accession no. AC084073.

Chemokine binding to nonadherent bone marrow cells

Bone marrow cells were obtained from femurs isolated from 6-wk-old female B6, C168–176 subline, and *Darc*-KO mice. Femurs were dissected, the ends of the bones were cut, and the marrow was flushed out using a 2-mL syringe and transferred into 15-mL Falcon tubes. Bone marrow cells were dispersed by repeated aspiration of the cell preparation containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone). Cells were seeded in 10-cm petri dishes and incubated at 37°C in a humidifier incubator with 5% CO₂. After 24 h, all NABMC were transferred to new 50-mL Falcon tubes and were then counted with a hemocytometer. Cells were then spun down, the supernatant was removed, and the pellet was resuspended in binding medium (DMEM supplemented with 10 mM HEPES, 0.1% BSA at pH 7.4). Each reaction contained 2–4 $\times 10^6$ cells in a total volume of 250 μ L of binding buffer. Cells were incubated for 1 h at 37°C with 200,000 CPM ¹²⁵I-MCP1 (Ccl2)/II8 and with or without unlabeled murine Ccl2/Human recombinant II8 (PeproTech). We have found that Ccl5 binds better at room temperature (RT), so the cells were incubated with ¹²⁵I-RANTES (Ccl5) for 3 h at RT with and without unlabeled murine RANTES (Ccl5). The reactions were terminated by centrifugation for 10 min at 3000 rpm. The supernatant was removed and the precipitates were washed three times with the binding buffer. Then the radioactivity was

quantified using a gamma counter. Each reaction was carried out using 8–10 replicates.

Cell culture

NABMC were isolated as described above. The pellet was resuspended in DMEM supplemented with 10% FBS at a density of 8×10^5 cells/mL. Cells were then seeded into 24-well plates at a density of 2×10^5 cells/well and treated with murine soluble Tnfsf11 (100 ng/mL, Peprotech) and murine colony-stimulating factor (Csf1, 100 ng/mL, Peprotech). The cultures were maintained for up to 10 d and re-fed twice a week. After 10 d of treatment, the adherent osteoclasts were rinsed with phosphate-buffered saline (PBS), fixed with citrate (4%)–acetone (60%), and then stained using a Leukocyte acid phosphatase kit (Sigma-Aldrich). A Darc-specific polyclonal antibody (Darc-Ab) generated in a goat model against the N-terminal domain of the Darc mouse protein (Santa Cruz Biotechnology) was used to neutralize the Darc protein of B6 wild-type mice. NABMC were treated for 6 d with Tnfsf11 and Csf1 (as described above) in presence of Darc-Ab or control IgG (Sigma-Aldrich). Then the TRAP-positive (pink/purple) multinucleated (MNC) osteoclasts were visualized and counted under light microscopy, and only cells with more than two nuclei were considered.

Histomorphometry study

Femurs from 8-wk-old *Darc*-KO mice and wild-type B6 mice were defleshed and embedded in methylmethacrylate. Thick cross sections (0.5-mm thickness) were cut from the midpoint of the shaft with a diamond wire Histo-saw (Delaware Diamond Knives). The cross sections were ground lightly, mounted in aqueous Fluoromount-G (Fisher Scientific), and examined under an Olympus BH-2 fluorescent/brightfield microscope to measure bone areas and calcein labels. All bone histomorphometric parameters were measured as previously described (Kasukawa et al. 2003).

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