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Impact of broad regulatory regions on *Gdf5* expression and function in knee development and susceptibility to osteoarthritis

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Abstract

Objectives—Given the role of growth and differentiation factor 5 (*GDF5*) in knee development and osteoarthritis risk, we sought to characterise knee defects resulting from *Gdf5* loss of function and how its regulatory regions control knee formation and morphology.

Methods—The *brachypodism* (*bp*) mouse line, which harbours an inactivating mutation in *Gdf5*, was used to survey how *Gdf5* loss of function impacts knee morphology, while two transgenic *Gdf5* reporter bacterial artificial chromosome mouse lines were used to assess the spatiotemporal

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Contributors

TDC conceived and oversaw the project. HC and MS designed BAC transgenic mice. MS, HC and TDC performed mouse rescue experiments. SKP, JC, MY, and ZL performed mouse breeding and collected bp and BAC-LacZ tissue samples. SKP performed histology and lacZ staining. AMK performed all morphometric and statistical analyses on bp and BAC-rescue mice. SKP, AMK and TDC wrote the manuscript, with input from VR and all other authors.

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activity and function of *Gdf5* regulatory sequences in the context of clinically relevant knee anatomical features.

Results—Knees from homozygous *bp* mice (*bp/bp*) exhibit underdeveloped femoral condyles and tibial plateaus, no cruciate ligaments, and poorly developed menisci. Secondary ossification is also delayed in the distal femur and proximal tibia. *bp/bp* mice have significantly narrower femoral condyles, femoral notches and tibial plateaus, and curvier medial femoral condyles, shallower trochlea, steeper lateral tibial slopes and smaller tibial spines. Regulatory sequences upstream from *Gdf5* were weakly active in the prenatal knee, while downstream regulatory sequences were active throughout life. Importantly, downstream but not upstream *Gdf5* regulatory sequences fully restored all the key morphological features disrupted in the *bp/bp* mice.

conclusions—Knee morphology is profoundly affected by *Gdf5* absence, and downstream regulatory sequences mediate its effects by controlling *Gdf5* expression in knee tissues. This downstream region contains numerous enhancers harbouring human variants that span the osteoarthritis association interval. We posit that subtle alterations to morphology driven by changes in downstream regulatory sequence underlie this locus' role in osteoarthritis risk.

INTRODUCTION

The mammalian knee involves complex articulations between the distal femur, proximal tibia and patella that are passively controlled by bony anatomy and surrounding connective tissues. Knee morphology is initially determined during embryogenesis, when an interzone delineates the boundary between the presumptive femur and tibia. This zone provides progenitor cells for articular cartilage, ligaments, meniscus and synovium, while adjacent cells give rise to the bony elements. Over the course of late prenatal and postnatal development, knee joint matures and is remodelled, during which alterations to morphology and integrity of knee structures can compromise joint stability, alter joint loading, and ultimately lead to joint injury or degeneration (i.e., osteoarthritis (OA)).

The steps underlying knee development, growth, remodelling and homeostasis require coordinated signalling pathways from many gene families. Growth and differentiation factor 5 (*Gdf5*)/cartilage-derived morphogenetic protein (*CDMPI*)/bone morphogenetic protein 14 (*BMP14*), a BMP only found in vertebrates,^{1–5} is one of the earliest markers of joint development, including the knee.⁵ During knee development, cells from the interzone and cells that later enter the knee joint express *Gdf5*, and give rise to articular cartilage, menisci, and ligaments.⁶ Mutations disrupting the *GDF5* coding sequence, resulting in complete loss of GDF5 protein, underlie Hunter-Thompson-type acromesomelic chondrodysplasia in humans,⁷ as well as the *brachypodism* (*bp*) phenotype in mice,² both of which are severe appendicular skeletal dysplasias with profound effects on the knee.^{8–11}

Genome-wide association studies (GWAS) have revealed a robust and highly reproducible correlation between *GDF5* and knee OA susceptibility.^{12–14} Specifically, single nucleotide polymorphisms (SNPs) spanning a 130 kb interval containing *GDF5* and downstream *UQCC1* (*ubiquinol-cytochrome C reductase complex assembly factor 1*) are associated with a 1.2-fold to 1.8-fold increase in knee OA risk.^{12–14} This association interval reflects a haplo-type found in millions, if not billions, of people worldwide, the frequency of which

has been attributable to an SNP (rs4911178) residing within a growth plate enhancer (*GROW1*) that affects long bone size.¹⁵ Interestingly, no SNPs affecting the *GDF5* or *UQCC1* protein coding sequences account for the OA associations, leading researchers to focus on linked *GDF5* 5'UTR variants (rs143383 and rs143384). These variants reduce transcriptional activity of the core *GDF5* promoter in vitro,¹⁶ and along with other variants in strong linkage disequilibrium correlate with reduced *GDF5* transcript levels in vivo.¹⁷

A recent investigation into the cis-regulatory architecture of the human and mouse *GDF5* locus using a bacterial artificial chromosome (BAC) scan and fine-mapping approach revealed a number of distinct *GDF5* joint regulatory enhancers (R1-R5), many of which reside downstream (R3-R5), control knee expression and harbour putative OA risk variants.¹⁸ Given these findings, as well as the association of *GDF5* with knee OA, and the relevance of knee morphology in cartilage loading and OA risk, here we perform a series of refined experiments highlighting the functional importance of this downstream regulatory region with respect to knee morphology.

METHODS

Mouse strains and rescue experiments

To study *Gdf5* loss of function, the BALB/cJ *bp*^{3J} strain was used, in which a CG dinucleotide is replaced by a T at position 876 within the *Gdf5* coding sequence.² This mutation shifts the *Gdf5* open reading frame, producing a premature translational stop and truncation of the entire mature sequence, thus rendering the allele non-functional. Mice with two copies of this allele (*bp/bp*) completely lack *Gdf5* and exhibit the classic *bp* phenotype, while mice with one copy (*bp/+*) have no overt developmental abnormalities.¹⁰¹¹¹⁹ Because the latter are indistinguishable from wild-type mice,¹⁰ and because the typical breeding strategy for this line generates only *bp/bp* and *bp/+* animals, we used *bp/+* mice as controls in this study. Two transgenic FVB-NJ (Friend Virus B - NIH Jackson) strains were also used (*upstream BAC* and *downstream BAC*),¹⁵¹⁸ each expressing *Gdf5* and *LacZ* from a BAC transgene containing mouse *Gdf5* and ~110 kb of upstream or downstream flanking sequence. Accordingly, each BAC contains an IRES-P-Geo cassette within the *Gdf5* 3'UTR, allowing for bicistronic transcription of *Gdf5* and *LacZ*. For rescue experiments, stable upstream BAC and downstream BAC (n=3 lines each) lines were separately crossed to *bp/bp* animals. *Upstream BAC;bp/+* and *downstream BAC;bp/+* were back-crossed to *bp/bp* mice, and progeny genotyped for the *LacZ* transgene and the *bp* allele in separate PCR reactions.¹⁸ All analyses were performed on male mice.

Histology and whole mount skeletal preparations

bp and BAC transgenic mouse tissues were fixed in 10% neutral buffered formalin (NBF) at 4°C for 48 hours and decalcified in 10% EDTA for 1 week. *bp* mouse (n=2 per genotype per time point) tissues were paraffin-embedded, sectioned at 10 μ m and H&E-stained. BAC transgenic mouse (n=3 per line, per genotype, per time point) tissues were O.C.T.-embedded (Sakura, Tokyo, Japan), sectioned at 10 μ m and nuclear fast red-counterstained (Sigma, St Louis, Missouri, USA). Bacterial β -galactosidase (*LacZ*) activity was detected via X-gal staining (n = 3 per genotype) as described.¹⁵¹⁸ All stained samples were either prepared for

histology or transferred to glycerol for imaging and permanent storage. Skeletons from 8-week-old *bp/+*, *bp/bp*, and *bp;BAC* rescue mice (n=5 per genotype) were stained with Alcian blue (cartilage) and Alizarin red (bone) as described,¹⁵¹⁸ and imaged.

Micro-CT imaging, anatomical index measurements, and statistical methods

To quantify phenotypic differences related to *Gdf5* and the functions of its upstream and downstream regulatory sequences, right femur and tibia of twenty 8-week-old mice (n=5 per genotype) were scanned using high-resolution micro-CT (μ CT 40, SCANCO Medical AG, Bruttisellen, Switzerland). Digital Imaging and Communications in Medicine (DICOM) images were exported for measurements of several femoral and tibial anatomical indices in OsiriX MD V7.5 (Pixmeo Sarl, Bernex, Switzerland), using clinically established protocols (online supplementary figure 1).^{20–24} The measurer (AMK) was blinded to specimens' genotype. In the *bp/+* group, one femur had incomplete coverage of its medial condyle and one tibia had incomplete coverage of its plateau during imaging. Thus, the medial femoral condyle and tibial plateau measurements for those samples were discarded. Data normality was assessed by Shapiro-Wilk's test in SPSS (Version 25, IBM SPSS). Normally distributed data were compared using analysis of variance with a post hoc Tukey correction for multiple comparisons. Non-normally distributed data were compared between the groups using Kruskal-Wallis test with a Benjamini-Hochberg post hoc correction for multiple comparisons (Prism, GraphPad Software, La Jolla, California, USA). P values are two-sided and statistical significance was assessed at $\alpha=0.05$.

RESULTS

Assessment of *Gdf5*'s role in prenatal and postnatal gross knee morphology

We began our study with a histological assessment of knee joints in prenatal *bp/bp* and *bp/+* mice. At E14, when knee joints become discernible by histology, cavitation between the presumptive femur and tibia was apparent in *bp/+* and *bp/bp* embryos (figure 1); however, no clear morphological differences between genotypes were found, likely due to the normal, poorly defined joint shape at this early stage. However, by E16, *bp/+* knee morphology was more clearly defined and was accompanied by soft-tissue structures, the meniscus and cruciate ligaments (figure 1), whereas the *bp/bp* knee had minor morphological alterations to the femoral condyles and patella, each of which were smaller than *bp/+* elements. Furthermore, compared with *bp/+* knees, the *bp/bp* tibia had slight anterior subluxation relative to the femur (figure 1).

At birth (P0), *bp/+* knees were similar in morphology and registration to those at E16 (figure 1), whereas *bp/bp* knees had dysmorphic femoral and tibial epiphyses and anteriorly dislocated tibiae relative to their femora (figure 1). Additionally, *bp/bp* cruciate ligaments consisted of a rudimentary fibrous structure emanating from the anterior tibial plateau, unlike the well-formed ligaments in *bp/+* knees (online supplementary figure 2). By 2 weeks of age, secondary ossification centres were apparent throughout the distal femur and proximal tibia of *bp/+* mice (figure 1 and online supplementary figure 2), whereas in *bp/bp* mice, only the proximal tibia possessed a rudimentary secondary ossification centre, identifiable only in knee mid-sections (figure 1 and online supplementary figure 2).

However, this difference is attributable to a delay, as both *bp/bp* knee epiphyses appeared fully ossified by 8 weeks (figure 1 and online supplementary figure 2). Indeed, by 8 weeks, mature articular cartilage was readily identifiable in *bp/bp* knees and was histologically indistinguishable from that of *bp/+* mice. Finally, by 6 months, whereas the meniscus of *bp/+* knees was completely ossified (figure 1), no ossification was observed in the residual secondary structures of *bp/bp* knees (figure 1).

Early prenatal activity of *Gdf5* regulatory domains

Given the conservation of the mouse and human *GDF5* gene as well as regulatory sequence function,¹⁵¹⁸ we next analysed chromatin conformation capture data acquired from human and mouse cell types²⁵ to gain an understanding of the broader, stable regulatory neighbourhood containing *GDF5*.^{26–28} Across cell types and species, we found conservation in the topologically associated domain (TAD) structure of the locus (figure 2), indicating that the large majority of regulatory interactions occur within the higher level TAD module. Within this conserved TAD are two broad upstream and downstream regulatory sequence domains contained within previously reported BAC transgenes (see Methods), containing distinct regulatory enhancer (R1-R5) combinations.¹⁵¹⁸

Given these findings, and our work on the conservation of upstream and downstream regulatory domain function,¹⁵¹⁸ we next assessed the prenatal expression patterns of the *Gdf5* upstream and downstream BAC transgenes in developing E12- E16 knees (figure 3). At E12, the upstream BAC was weakly expressed in the nascent knee interzone (white arrowheads), but expressed in more distal hindlimb structures near the base of digit 1. In contrast, the downstream BAC drove wide interzone expression (red arrowheads) extending between the tibial and femoral condensations (empty red arrowhead). From E13 to E14, the upstream BAC was expressed in knee anlagen (white arrowheads), although it appeared fainter and restricted to ventral structures, whereas the downstream BAC expression encompassed the entire knee including the outer capsule (red arrowheads), although the signal between the tibia and fibula receded (empty red arrowhead (E13) to no arrowhead (E14)). Furthermore, the downstream BAC expression was observed in chondrogenic growth zones (black arrowheads).¹⁵ We next characterised BAC expression histologically at E14 and found the upstream BAC expression was faintly visible within the joint cavity (figure 3). In contrast, downstream BAC expression was strongly visible throughout the joint mesenchyme and the outer edges of the presumptive femur and tibia (figure 3). By E16, the upstream BAC was expressed at very low levels (figure 3), while downstream BAC expression occurred throughout the rudimentary meniscus, in the articular surfaces of the femur and tibia, in the deeper layers of the femoral epiphysis and in the developing cruciate ligaments, although weakly (figure 3).

Late prenatal and postnatal activity of *Gdf5* regulatory domains

We next assessed each BAC's expression in late fetal and postnatal knees. From E17 to P2, the upstream BAC was expressed, although weakly, on the trochlear groove (figure 4; black arrow heads), along the articular cartilage surface of the femoral condyles (figure 4; red arrowheads) and tibial plateau (figure 4; green arrowheads), and in cruciate ligaments (figure 4; white arrowheads). The downstream BAC was broadly expressed in the outer articular

capsule (figure 4; empty black arrowhead), along the articular cartilages of the femoral condyles (figure 4; red arrowheads) and tibial plateau (figure 4; green arrowheads), within the collateral ligaments (figure 4; yellow arrowheads), and in the meniscus (figure 4; empty red arrowheads) and cruciate ligaments (figure 4; white arrowheads). By 6 months, the upstream BAC was no longer expressed in the knee (the staining observed in each element's metaphyses (figure 4; blue arrowheads) was observed in non-transgenic control mice and likely reflects endogenous beta-galactosidase activity (online supplementary figure 3)). The downstream BAC was expressed along the patellar articular cartilage surface (not shown), femoral trochlear groove (figure 4; black arrowheads), and in the articular cartilage of the femoral condyles (figure 4; red arrowheads) and tibial plateau (figure 4; left green arrowhead). Finally, it was also expressed in the collateral ligaments (figure 4; yellow arrowheads) and meniscus (figure 4; right green arrowhead), but no longer in cruciate ligaments. Each BAC's expression patterns are extensively catalogued in online supplementary table 1.

We also characterised BAC postnatal expression via histology (figure 5). At P0, the upstream BAC was faintly expressed by tibial and femoral articular chondrocytes (figure 5; red arrowheads), and in the meniscus (figure 5, black arrowhead) and cruciate ligaments (online supplementary figure 4). Two weeks later, its expression diminished to only a handful of cells within articular cartilages and menisci, and vanished from the cruciate ligaments. By 8 weeks and 6 months, it was no longer expressed in the knee (figure 5 and online supplementary figure 4). Conversely, at birth the downstream BAC was robustly expressed by tibial and femoral articular chondrocytes, with expression extending into the femoral epiphysis (figure 5). It was also expressed in the meniscus, although restricted to outer cells (figure 5), and in the cruciate ligaments (online supplementary figure 4). By 2 weeks, the downstream BAC was robustly expressed by tibial and femoral articular chondrocytes (figure 5, red arrowheads); however, it was no longer expressed within the femoral epiphysis. Expression also persisted in the meniscus (figure 5, black arrowhead) and cruciate ligaments (online supplementary figure 4), although to a lesser extent. Finally, downstream BAC expression persisted in adult articular cartilage (figure 5, red arrowheads) and meniscus (figure 5, black arrowheads), changing little between 8 weeks and 6 months (figure 5, black and red arrowheads), although it disappeared from the cruciate ligaments (online supplementary figure 4).

BAC rescue experiments of the *bp* knee

Given the dynamic upstream and downstream BAC expression patterns, and preliminary findings indicating their influence on long bone morphology,¹⁵¹⁸ we more deeply explored the regulatory control that each broad region has on clinically relevant knee dimensions related to knee biomechanics as well as OA risk/progression. Our bicistronic construct design facilitates coexpression of both *LacZ* and *Gdf5*; thus, we introduced a copy of each BAC transgene onto the *bp* background and gauged phenotypic rescue at 8 weeks. The mean ($\pm$ SD) of all quantified indices along with the P values for all pairwise comparisons are presented in online supplementary table 2. Compared with *bp/+* mice, *bp/bp* mice had shorter femora with smaller knees, indicated by narrower femoral condyles and tibial plateaus (figures 6 and 7A-C; $P<0.02$). They also exhibited anterior subluxation relative to

the femur (figure 6, top row), which is likely indicative of absent or underdeveloped anterior cruciate ligaments (ACL). The presence of an upstream BAC allele could not rescue *bp/bp* morphology, whereas the downstream BAC allele restored femoral length, and the width of the femoral condyles and tibial plateaus to levels observed in *bp/+* mice (figure 7A-C). Compared with *bp/+* mice, *bp/bp* mice also had smaller intercondylar notches, lateral femoral condyles and more curved medial femoral condyles (figure 7D,E and online supplementary table 2; $P < 0.02$). Whereas the upstream BAC restored only the curvature of the medial femoral condyle, the downstream BAC fully rescued all of these abnormal phenotypes (figure 7D,E and online supplementary table 2). Compared with *bp/+* mice, *bp/bp* mice possessed a shallower trochlear groove depth and larger sulcus angle (figure 7F,G; $P < 0.001$). The upstream BAC significantly improved trochlear groove morphology, but did not restore it to the depth observed in *bp/+* mice (figure 7F,G; $P < 0.02$). Conversely, the downstream BAC restored the trochlear groove measurements to levels observed in *bp/+* mice (figure 7F,G). *bp/bp* mice also had a less posteriorly sloped lateral tibial plateau with smaller tibial spines, compared with *bp/+* mice (figure 7H,I; $P < 0.01$). The upstream BAC failed to rescue these morphologic features, whereas the downstream BAC restored the tibial slope and spine height to levels observed in *bp/+* mice (figure 7H,I).

DISCUSSION

We found that *bp/bp* mice are characterised by grossly abnormal knees, a feature that becomes apparent during prenatal development and that fails to resolve postnatally. Further, we found that *Gdf5* expression in prenatal knee structures is governed by sequences contained within the upstream and downstream BACs, while its expression in postnatal knees is mediated primarily by sequences present in the downstream BAC. We also found that the specific defects in knee morphology exhibited by *bp/bp* mice can be completely rescued by transgenic expression of *Gdf5* from the downstream BAC only. Taken together, these findings highlight a key role for *Gdf5* in determining knee structure and demonstrate the importance of non-coding sequences from the downstream region in mediating this role.

To our knowledge, this is the first study showing that *Gdf5* loss-of-function mutations affect several key morphological features of the knee in a quantitative manner. We showed that *bp/bp* mice have curvier medial femoral condyles, flatter lateral tibial slopes, smaller tibial spines, and shallower trochlear grooves. In humans, increased medial condyle curvature results in lower contact area and increased local pressure in the medial compartment,²⁹ while decreased lateral plateau slope may contribute to a more varus alignment, also resulting in increased cartilage loading in the medial compartment.³⁰ A smaller tibial spine compromises knee stability resulting in increased knee axial rotation and medial-lateral translation,²⁰ both of which increase shear loads across the medial compartment.³¹ Increased cartilage pressure and shear loads in the medial compartment, the most common site of knee OA, result in excessive cartilage wear and tear and are strongly linked to OA development and progression.³² A shallow trochlear groove results in less congruent patella-femur articulation, with smaller contact area and increased local pressure on the articular cartilage.³³ A shallow trochlea also fails to stabilise the patella during range of motion, leading to unwanted medial-lateral translation of the patella.^{24,33} This patellar maltracking imposes non-physiological shear loads on the cartilage and may shift the centre of pressure to areas

of the cartilage that have not evolved to bear heavy loads. The combination of increased cartilage pressure and shear as well as non-physiological centre of pressure results in accelerated wear of the patellofemoral cartilage, another common site of knee OA, and may ultimately lead to joint degeneration.³² The observed morphological changes suggest that the associations between *GDF5* mutations and OA risk are primarily mediated through joint anatomy. Indeed, a recent analysis of genetic associations between *GDF5* and OA suggests a strong and significant association between self-reported knee OA and joint morphology, indicating that anatomical configuration, as well as chondrocyte biology (prior to joint OA) are important risk factors in OA aetiology.¹⁴ Several studies have also shown substantial morphological variations, including those reported here, between subjects with progressive radiographic knee OA and age-matched and sex-matched controls.^{34,35} Despite these observations, the relationships between the genetic variants in the *GDF5* locus, knee morphology, and OA susceptibility are not clearly understood for individual OA phenotypes, including post-traumatic and inflammatory OA.

Remarkably, most of the specific *bp/bp* knee defects were completely rescued by transgenic expression of *Gdf5* from the downstream BAC. This BAC also mediated robust reporter expression throughout prenatal and postnatal life in all knee joint structures that are directly affected by *Gdf5* loss of function. In contrast, upstream BAC *Gdf5* expression was insufficient to rescue morphology, despite its activity in a subset of prenatal and early postnatal knee tissues, such as the ACL/ posterior cruciate ligament (PCL), directly affected by *Gdf5* loss of function. Our analysis therefore suggests that regulatory elements in the upstream BAC likely need to interact with downstream elements to execute their effects. Importantly, both BACs span a conserved mouse and human TAD (figure 2) possessing joint enhancers (R1-R5) active in human prenatal and postnatal tissues.^{15,18} The singular ability of the downstream BAC to rescue knee morphology indicates that sequences unique to this transgene are critical to mediating the role of *Gdf5* in determining knee morphology. The downstream BAC contains several joint-specific enhancers (R3, R4 and R5) that are not found in the upstream BAC, and removal of these sequences may have the same or similar effect as disruption of the *Gdf5* coding sequence, although this remains to be verified experimentally. Alternatively, small changes to these sequences may lead to subtle alterations in hard and soft tissue morphology and/or joint homeostasis. Importantly, the downstream enhancers contain a number of variant sequences that correlate with increased OA risk.^{15,16,18}

In this study, we took advantage of the *bp* mouse line as a classic model of *Gdf5* deficiency that phenocopies loss of *GDF5* in humans.^{7,10} This model's inherent limitation is that the mutation occurs in the germline, resulting in a global knockout of *Gdf5*. Consequently, *bp/bp* mice have severe limb abnormalities that dramatically affect the manner in which they load joints, confounding the interpretation of any OA phenotype or lack thereof. Conversely, *bp/+* mice have no overt limb abnormalities,^{10,15,18,19} yet they displayed no frank signs of OA up to 6 months of age. A previous study found that these mice have an altered gait that aggravates experimentally induced knee OA,¹⁹ consistent with the notion that reduced *Gdf5* levels increase OA susceptibility. Nevertheless, the global nature of the *bp* mutation makes it difficult to separate the effects of *Gdf5* on the developing knee from a potential homeostatic role in the adult knee.

In light of our anatomical and genetic findings, and given that variants in the *GDF5* association interval as well as knee shape and soft tissue morphology are risk factors for OA, we propose a model in which risk-associated variant sequences from the downstream region reduce local *GDF5* levels in knee structures, leading to subtle alterations in knee morphology or tissue composition that, in turn, predispose the joint to failure. This model primarily describes events that occur prior to or during knee maturation; however, it is possible that decreased *GDF5* levels in fully formed adult knees may also influence OA risk by impairing homeostasis in healthy joints or by accelerating degeneration due to injury. *GDF5* transcripts are present in articular cartilage from adult knees,¹⁷ and the downstream BAC is expressed in adult knees, but it is unclear whether *Gdf5* has any role in the knee beyond determining its shape or soft tissue composition. The development of a conditional allele for disrupting *Gdf5* expression at specific prenatal and postnatal time points and spatial domains, along with the targeted deletion of its knee joint enhancers, will be essential for teasing apart potential roles of *Gdf5* in knee development and/or homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of *bp/bp* and control knees from E14 to 6 months. H&E-stained sagittal sections from *bp/+* and *bp/bp* knees at E14, E16, P0, 2 weeks, 8 weeks, and 6 months are shown. Insets show lower magnification views of each section. Scale bars=250 μm. *bp*, brachypodism; f, femur; m, meniscus; t, tibia.

Figure 2.

Topologically associated domains across the *GDF5-UQCC* locus in mouse and human embryonic stem cells. Embryonic stem cells from mice (mESCs) and humans (hESCs) were probed via Hi-C to detect TADs containing the *GDF5-UQCC* locus as described.²⁵ Heatmaps depicting frequency of genomic interactions around the *GDF5-UQCC* locus in both cell types are shown, along with inferred topologically associating domains (light grey bars adjacent to genomic coordinates). The relative locations of *GDF5* joint enhancers (R1-R5)¹⁸ and long bone enhancers (GROW1, G1) are shown,¹⁵ along with the relative locations

of the upstream BAC (*UP-BAC*) and downstream BAC (*DOWN-BAC*) sequences. Protein coding genes within the locus are also shown (blue arrows). While there is generally low resolution in discerning specific intrachromosomal interactions between individual genes and their regulatory elements within TADs, both BAC sequences and the regulatory elements they contain reside within a TAD conserved between mice and humans. This pattern is highly conserved across the large majority of cell types (n=41) examined in mice and humans (<http://promoter.bx.psu.edu/hi-c/view.php>).²⁵ BAC, bacterial artificial chromosome; *GDF5*, growth and differentiation factor 5; TADS, topologically associated domain; *UQCC*, ubiquinol-cytochrome C reductase complex assembly.

Figure 3.

Comparison of upstream and downstream BAC expression during early knee joint formation. X-gal-stained hindlimbs from upstream BAC (*UP-BAC*) and downstream BAC (*DOWN-BAC*) lacZ transgenic mice at E12, E13, E14 and E16 are shown. Intact hindlimbs are shown for E12 through E14 (left three panels, top and bottom rows), and sections are shown for E14 and E16 (right three panels, top and bottom rows). Expression in the knee field is indicated for the upstream BAC (white arrowheads) and the downstream BAC (red arrowheads). Expression of the downstream BAC in between the presumptive tibia and fibula (empty red arrowheads) and in the developing long bones (black arrowheads) is also indicated. A single section is shown for each BAC at E14, containing the interzone region appearing between the presumptive femur (f) and tibia (t). Two sections are shown for each BAC at E16 showing the femur, tibia, and either the menisci (m) or cruciate ligament (cl). Insets show lower magnification views of each section. Scale bars=500 μ m. BAC, bacterial artificial chromosome.

Figure 4.

Comparison of upstream and downstream BAC expression in later prenatal knee development and in the postnatal knee. X-gal-stained knees from upstream BAC (*UP-BAC*) and downstream BAC (*DOWN-BAC*) mice at E17, P2 and 6 months are shown. Images taken prior to removal of the outer joint capsule are shown at E17 (outer capsule), and images taken after its removal are shown at E17 and P2 (inner capsule, frontal view). Various angles of the distal femur (anterior femur, posterior femur, lateral condyle, medial condyle, femoral notch) and the proximal tibia (tibial plateau) are shown at 6 months. X-gal staining is highlighted in the joint capsule (empty black arrowheads), as well as the trochlear groove (black arrowheads), the femoral condyles (red arrowheads), the tibial plateau (green arrowheads), and the lateral condyle (yellow arrowheads).

arrowheads), cruciate ligaments (white arrowheads), collateral ligaments (yellow arrowheads), menisci (empty red arrowheads, and right green arrowhead at 6 months in downstream BAC experiment), and metaphysis (blue arrowheads). Scale bars=1 mm. BAC, bacterial artificial chromosome

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Figure 5.

Histological comparison of upstream and downstream BAC expression in postnatal knees. Sections of X-gal-stained knees from upstream BAC (*UP-BAC*) and downstream BAC (*DOWN-BAC*) mice at P0, 2 weeks, 8 weeks, and 6 months are shown. Staining is highlighted in the articular cartilage (red arrowheads) and meniscus (black arrowheads). Insets show lower magnification views of each section. Scale bars=1 mm. BAC, bacterial artificial chromosome; f, femur; m, meniscus; t, tibia.

Figure 6. Morphological appearance of femur, tibia, and knee joint structures in *bp* and *bp:BAC* rescue mice. (Top row) Medial views of representative knees from each genotype stained with Alizarin red/Alcian blue are shown. (Bottom row) Reconstructed three-dimensional models of a representative specimen from each genotype are shown. Scale bars=1 mm. BAC, bacterial artificial chromosome; *bp*, brachypodism; *DOWN-BAC*, downstream BAC; *UP-BAC*, upstream BAC.

Figure 7.

Quantitative morphological analysis of the femur, tibia, and knee joint structures in the BAC rescue experiment. Group differences in (A) femoral length (* $P < 0.02$), (B) bicondylar width of the femur (* $P < 0.04$), (C) width of the tibial plateau (* $P < 0.05$), (D) width of the intercondylar notch (* $P < 0.02$), (E) curvature radius of the medial femoral condyle (* $P < 0.03$), (F) depth of the trochlear groove (* $P < 0.02$), (G) trochlear groove sulcus angle (* $P < 0.01$), (H) posterior slope of the lateral tibial plateau (* $P < 0.01$), and (I) height of the lateral tibial spine (* $P < 0.03$). Mean $\pm$ SD ($n = 5$ /group). BAC, bacterial artificial chromosome; *bp*, brachypodism; *DOWN-BAC*, downstream BAC; *UP-BAC*, upstream BAC.