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A distinct regulatory region of the *Bmp5* locus activates gene expression following adult bone fracture or soft tissue injury

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Abstract

Bone morphogenetic proteins (BMPs) are key signaling molecules required for normal development of bones and other tissues. Previous studies have shown that null mutations in the mouse *Bmp5* gene alter the size, shape and number of multiple bone and cartilage structures during development. *Bmp5* mutations also delay healing of rib fractures in adult mutants, suggesting that the same signals used to pattern embryonic bone and cartilage are also reused during skeletal regeneration and repair. Despite intense interest in BMPs as agents for stimulating bone formation in clinical applications, little is known about the regulatory elements that control developmental or injury-induced BMP expression. To compare the DNA sequences that activate gene expression during embryonic bone formation and following acute injuries in adult animals, we assayed regions surrounding the *Bmp5* gene for their ability to stimulate *lacZ* reporter gene expression in transgenic mice. Multiple genomic fragments, distributed across the *Bmp5* locus, collectively coordinate expression in discrete anatomic domains during normal development, including in embryonic ribs. In contrast, a distinct regulatory region activated expression following rib fracture in adult animals. The same injury control region triggered gene expression in mesenchymal cells following tibia fracture, in migrating keratinocytes following dorsal skin wounding, and in regenerating epithelial cells following lung injury. The *Bmp5* gene thus contains an “injury response” control region that is distinct from embryonic enhancers, and that is activated by multiple types of injury in adult animals.

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Graphical abstract

Introduction

Enhancing bone formation is an important objective in medicine. In the Western world, approximately 300–400 individuals per 100,000 suffer long bone fractures each year leading to billions of dollars in health care costs [1–3]. While the majority of injuries heal within several months, approximately 5–10% of bone fractures exhibit delayed healing or a failure of the repair process [4, 5]. Although, recombinant growth factors such as Bone Morphogenetic Proteins (BMPs) are widely used clinically in orthopedic applications to stimulate bone fusion in cases ranging from trauma to spinal fusion, recent studies suggest that supra-physiological doses are sometimes associated with adverse side effects [6, 7]. Therefore, a better understanding of regulatory mechanisms underlying the normal bone fracture response may suggest new therapeutic routes to treat skeletal injuries through increased expression of endogenous growth factors [8].

The process of skeletal repair mimics many aspects of skeletal formation [9, 10]. Mesenchymal cells initially proliferate and condense, followed by differentiation into cartilage, vascularization, and endochondral ossification of tissue at either fracture sites or in skeletal precursors. Shared patterns of gene expression further support the similarities between embryonic bone formation and adult regeneration. Transcriptional analysis of bone repair has shown that many genes are reactivated upon injury in the same temporal and spatial patterns that can be observed during earlier skeletal formation [10–12]. For example, transforming growth factor- β (TGF- β) proteins, early signals of chondrogenic condensations, appear during the inflammatory phase, followed by other markers of cartilage formation including *Runx2*, *Wnt2*, and *Sox9*. Although, these similar molecular and histological signatures suggest that fracture healing occurs by reactivation of a program of embryonic bone formation [11, 13, 14], the regulatory relationship between these two processes, including the mechanism that reactivates developmental signaling molecules in injured adults, is still unclear.

BMPs play important roles during the embryonic development of many tissues, including the skeleton [15]. Members of the TGF- β superfamily of secreted signaling molecules [16], BMPs are expressed within the mesenchymal stem cell anlagen that prefigure future skeletal structures [17], continue to be expressed in the perichondrium and periosteum layers surrounding growing bones [18, 19], and are upregulated following bone injury [20–23]. Several BMP mutants display characteristic skeletal and soft tissue defects [24–26],

providing strong genetic evidence that BMPs are endogenous signals used to control cartilage and bone formation during embryogenesis [18].

The *Bmp5* locus provides particularly useful genetic tools for studying both the embryonic and adult functions of BMPs. Encoded by the classical mouse *short ear (se)* locus [24], *Bmp5* is expressed in early mesenchymal condensations, as well as in the perichondrium and periosteum layers surrounding growing cartilage and bones [19, 27]. *Bmp5* null mutants are fully viable and fertile and exhibit defects in several skeletal structures, including the pronounced shortening of the external ear pinna due to defects in the formation and growth of ear cartilages [18, 28, 29]. Importantly, *Bmp5* null mutations also cause bone regeneration defects in adult animals. Following rib cage injury, the formation and maturation of cartilaginous fracture calluses are delayed in *Bmp5* mutants [30]. In addition, the fracture calluses are approximately half the volume of calluses in comparably staged controls [30]. Gene expression profiles of fracture repair have shown that *Bmp5* expression begins during the chondrogenic phase of bone healing and remains elevated throughout the regeneration process [12, 22]. Thus, *Bmp5* is both expressed during, and required for, normal injury responses. Notably, exogenous BMP5 has been shown to have potent osteogenic capacity *in vitro* and in an *in vivo* model of fracture repair [31, 32]. Finally, recent genetic studies show that epidermal stem cell numbers and ear regeneration differences in adult animals are also linked to the *Bmp5* gene [33–36], suggesting that *Bmp5* may play an important role in the maintenance and repair of multiple tissues.

Molecular studies of radiation and chemical mutagen-induced DNA lesions revealed several interesting alleles that disrupt *Bmp5* regulation without altering the *Bmp5* coding sequence [37]. Notably, two of these regulatory alleles involve structural rearrangements that occur approximately 6 kb and 105 kb downstream of the last *Bmp5* exon (Fig. 1A) [38]. *Bmp5* expression is lost at particular anatomical locations in the corresponding mutants, confirming that a large 3'-region is required for normal *Bmp5* regulation [38]. Hundreds of kilobases (kb) of the surrounding DNA have subsequently been screened for functional enhancers using *lacZ* reporter genes in transgenic mice. These studies have identified multiple modular enhancers that control expression in particular developing skeletal structures, or in soft tissues [38–40]. Here we use *lacZ* transgenic assays to further characterize the regulatory sequences that control where and when *Bmp5* is expressed during embryonic development, and during recovery from acute tissue injury in adults.

Materials and methods

Plasmid and BAC clones

Mouse sequences were assembled from BAC RP23-426K2 (Genbank accession #AC079245) and BAC RP23-343K17 (Genbank Accession #AC079244) [40]. Evolutionarily conserved regions (ECRs) shared between mice and humans were identified using global sequence alignment programs as previously described [41]. Genomic clones corresponding to single or small clusters of ECRs were amplified using primers listed in Supplementary Table 2. Single copy PCR products were cloned directly into the NotI site of the *Not5'hsp68lacZ* reporter [38]. Multiple-copy inserts of concatenated PCR products were cloned into the SfiI site of the p*Sfi*-hsp*lacZ* reporter [41]. The number of tandem copies

inserted was determined by digestion with NotI. For clones 8, 17, 18, 26, and 28, two genomic PCR products were attached using an XmaI linker, and the combined products were cloned into the NotI site of the *Not5'hsp68lacZ* reporter. All plasmids were verified by Sanger sequencing.

BAC A (RP23-377M6), BAC B (RP23-317C9) and BAC C (RP23-458E23) were isolated from the RPCI-23 Female (C57Bl/6J) Mouse BAC library (Invitrogen) as described [40]. The 17.8 kb Ph7 and 6.2 kb Ex4r clones have been previously described [38, 40].

Mouse strains and transgenics

BAC DNAs, co-injected with a minimal *hsp68-lacZ* reporter, and all plasmids were prepared for microinjection as described [39]. Taconic (NY) or the Stanford Transgenic Core performed pronuclear injections. E14.5 day post-coitum (dpc) embryos collected by Taconic were fixed for 1 hour in 4% paraformaldehyde (PFA) in 1X PBS at 4 °C, placed in cold 1X PBS and shipped overnight on ice.

Ph7, Ex4r, BAC A, BAC B, and BAC C founder animals were screened using primers Kg827: 5'TCACCAGACGCTGACAGCTACTCAG3' and Kg828: 5'TAACCGTGCATCTGCCAGTTTGAGG3'. Transgene positive animals were bred to FVB mice to check for germline transmission and embryonic expression. Stable lines were maintained on the FVB background.

All procedures were done in accordance with protocols approved by the Stanford University Institutional Animal Care and Use Committee.

Surgical procedures

Surgical procedures were performed in 6–7 week old male mice that were anesthetized with inhaled isoflurane. Mice were euthanized at the endpoint of each experiment, and whole rib cage, limb, or skin tabs were collected for further processing.

Rib fracture model—The back of the mouse was shaved, sterilized with Betadine, and a 2 cm long transverse incision was made approximately 0.5 cm below the right scapula. The incision was carried down to expose the ribs and an adjustable wire retractor was placed. The intercostal muscles were separated from the fourth and sixth ribs using an 18-gauge needle roughly 0.5 cm away from the vertebral column. Individual ribs were held using pierce tip fixation forceps at the site where the muscle and rib had been separated, and ribs were cut with fine scissors (14 mm cutting edge) to create rib fractures without entering the pleural space. The fracture ends were realigned and the incisions were closed with 6-0 absorbable sutures.

Tibia fracture model—The hind limb was shaved and sterilized with Betadine. A 0.5 cm incision through the skin and underlying muscles was made along the limb exposing the tibia. A malleus bone nipper was used to create a fracture approximately 0.6 cm below the knee. After fracture, the bones were realigned, and the incision was closed using 6-0 absorbable sutures.

Excisional skin wound model—The back of the mouse was shaved and sterilized using Betadine. A single 1 cm circumferential full thickness wound was created with scissors on the back just below the scapula. The underlying fascia and muscle were left intact, and the wound was allowed to heal in an open manner.

Naphthalene lung injury model—Mice were injected with naphthalene (Arcos Organics) intra-peritoneally at a dose of 275 mg/kg [42]. Corn oil was used as the vehicle. All injections were performed between 9 am and 12 pm, and mice were euthanized at different times following injection for analysis of lung histology and gene expression.

Gene expression analysis

Whole-mount staining for β -galactosidase activity in embryos was carried out as described [40]. Whole rib cage, limb, and skin tabs were fixed in 4% PFA in 1X PBS at 4 °C for 1 hour and then stained for lacZ activity as described [38] with the following modification: Tissues were incubated in X-gal stain overnight at room temperature. Following staining, specimens were washed in 1X PBS and fixed for 48 hours in 4% PFA in 1X PBS at 4 °C. Prior to embedding in paraffin, whole rib cages and limbs were decalcified by incubation in 19% EDTA, pH 7.4, at 4 °C for 3–4 weeks. The EDTA solution was changed every 48–72 hours. 8–10 μ m paraffin sections were collected, dewaxed, rehydrated and counter-stained with nuclear fast red (Vector Laboratories).

To detect reporter activity in naphthalene and vehicle-treated lungs, whole lungs were isolated and inflated with 4% PFA through the trachea using a 5 ml syringe attached to a 25-gauge needle. Tissues were fixed for 10 minutes at room temperature with rocking. Samples were washed once in 1X PBS before incubating in X-gal stain overnight at room temperature. Tissues were post-fixed in 4% PFA overnight at 4 °C, processed for paraffin embedding, and 8 μ m sections were collected.

For additional time course studies, lungs were inflated with 4% PFA and fixed for 1 hour on ice in order to retain β -galactosidase enzyme activity. After a brief rinse in 1X PBS, lungs were incubated in 30% sucrose overnight at 4 °C before embedding in OCT for cyrosectioning. 8 μ m sections were collected and stained in X-gal stain at 37 °C for 24 hours. Slides were post-fixed in 4% PFA overnight at 4 °C, counterstained with nuclear fast red, dehydrated through an ethanol series and orange terpene (Histo-clear, National Diagnostics) and mounted in Cytoseal 60 (Richard-Allan Scientific).

For whole-mount *in situ* hybridization, embryos from timed pregnancies were collected at E13.5 or E14.5 dpc, fixed overnight in 4% PFA in 1X PBS at 4 °C, and stored at –20 °C in methanol. The *Bmp5* antisense probe has been previously reported [38]. The *in situ* hybridizations were performed essentially as described [43] with the following modification: BM purple (Roche #1442074) was used as the color reagent. The color reaction was stopped by incubation in 100 mM glycine in PBT (1X PBS with 0.1% Tween-20) for 5 minutes at room temperature, followed by dehydration with two 5-minute washes in ethanol. Samples were rinsed in PBT and post-fixed in 4% PFA overnight at 4 °C.

For *in situ* hybridization on skeletal tissue sections, rib and tibia fractures were collected at post-operative day (POD) 4, bone on either side of the callus was cut away, and the samples were embedded in OCT. 12 µm sections were collected and processed according to the RNAscope 2.0 BROWN kit (Advanced Cell Diagnostics) instructions for fresh frozen sections with the following modifications: Slides were incubated in Pretreatment 2 for 15 minutes at 40 °C and incubated in Pretreatment 4 for 15 minutes at room temperature. Advanced Cell Diagnostics *Mm-Bmp5* (#401241) and *E.coli-lacZ* (#313451) probes were used.

For *in situ* hybridization on skin and lung sections, tissues were collected at POD4, fixed in 10% neutral buffered formalin (BDH) overnight at room temperature, dehydrated, and embedded in paraffin. 5 µm sections were collected and processed according to the RNAscope 2.0 RED kit (Advanced Cell Diagnostics) instructions with the following modifications: Slides were boiled in Pretreatment 2 for 4 minutes (lung) or 12 minutes (skin), incubated in Pretreatment 3 for 30 minutes, hybridized with RNA probes for 150 minutes, and incubated in RED solution for 15 minutes.

Immunohistochemistry was performed on 10 µm paraffin-embedded tibia sections. Samples were incubated at 50 °C for 1 hour, rehydrated and incubated at 37 °C with 240 ng/ml Proteinase K in 1X PBS for 10 minutes. Sections were rinsed and blocked for 1 hour using Dako's Protein Serum-free Block solution (Agilent Technology). Samples were incubated with primary antibodies directed against Sox9 (1:50 dilution; sc-20095, Santa Cruz Biotechnology), or ColIII (1:500 dilution; ab34712, Abcam) overnight at 4 °C. Detection was performed using the Vectastain Elite ABC Kit (Vector Laboratories) according to the manufacturer's instructions. Samples were counter-stained with Hematoxylin (Bethyl Laboratories), dehydrated, mounted in Permount (Fisher Scientific), and photographed. For all immunohistochemistry experiments, adjacent sections were used to compare different protein expression profiles.

CC10 antibody staining was performed on 8 µm paraffin-embedded lung sections by standard methods using PBS/0.1% Tween-20 with 10% normal donkey serum for blocking and staining steps. The CC10 rabbit antibody (sc-25555, Santa Cruz Biotechnology) was used at a 1:200 dilution. Secondary staining was performed using an anti-rabbit Cy3 antibody (Jackson ImmunoResearch), and sections were mounted in Prolong Gold (Invitrogen).

Tartrate Resistant Acid Phosphatase (TRAP) staining was performed with a commercially available kit (Sigma Aldrich) according to the manufacturer's instructions with the following modification: Slides were incubated in pre-warmed TRAP stain solution at 37 °C for 40 minutes.

Results

Tissue specific enhancers are distributed throughout the *Bmp5* genomic region

To further characterize the regulatory landscape surrounding *Bmp5*, global sequence alignment programs were used to compare sequences representing 418,315 bp of the mouse

Bmp5 locus (GRCm38/mm10 chr9:75,797,064–76,215,378, Fig. 1A) and the corresponding human *BMP5* gene locus (Materials and methods). Evolutionarily conserved regions (ECRs) greater than 100 bp showing at least 60% identity between mouse and human sequences were selected for further analysis using a *lacZ* transient transgenic enhancer assay (Fig. 1A, Materials and methods). As previous studies have demonstrated expression of *Bmp5* in a wide range of developing skeletal structures and soft tissues at mid-gestation [27], *lacZ* expression from 34 clones was evaluated at E14.5 days post-coitum (dpc) (Materials and methods).

Seventeen of the constructs drove consistent *lacZ* activity (blue staining) at particular anatomical sites in multiple independent founder embryos (Fig. 1B–G, Supplementary Fig. 1, Supplementary Table 1). As predicted by previous genetic and regulatory studies of *Bmp5* mutants, positive enhancer sequences were distributed across a broad genomic region, including both introns and downstream noncoding regions. Many of the clones produced *lacZ* expression at sites that correspond to known locations of *Bmp5* expression during development, including: facial mesenchyme (Fig. 1B, $n = 10/11$ clone 2 embryos), tongue muscle (Fig. 1C, arrow, $n = 6/9$ clone 6 embryos), elbow and knee joints (Fig. 1D, asterisks, $n = 8/8$ clone 11 embryos), ribs (Fig. 1D, $n = 7/8$ clone 12 embryos), digit joints (Fig. 1E, $n = 5/7$ clone 13 embryos), fingertips (Fig. 1E, $n = 6/8$ clone 18 embryos), thyroid cartilage (Fig. 1F, arrowhead, $n = 6/7$ clone 23 embryos), and the head mesenchyme adjacent to the developing ear (Fig. 1G, $n = 3/3$ clone 34 embryos). A few clones produced *lacZ* activity at sites that did not correspond to previously known locations of *Bmp5* expression at E14.5 dpc (clones 20, 21, and 24; Supplementary Fig. 1D–F). These results provide a more detailed map of the complex regulatory sequences necessary to coordinate the spatial expression of *Bmp5* during development.

Identification of a novel rib injury response module

To identify regulatory sequences that drive gene expression in adult animals following skeletal injury, we surveyed a 391 kb region of the locus using three overlapping BAC clones that cover the entire *Bmp5* gene and flanking regions (Fig. 1A). Each BAC clone was coinjected with a *lacZ* reporter construct (Materials and methods), and founder animals were bred to establish transgenic mouse lines that could be used to study expression at multiple temporal stages. BAC clones A and B both contain a sequence that is known to drive expression in the lateral perichondrium of embryonic ribs (Fig. 1A, Ex4r), while BAC C contains separate sequences controlling expression in the medial perichondrium of embryonic ribs [40]. As expected, all three stable BAC transgenic lines showed robust embryonic rib expression at E14.5 dpc (data not shown).

To screen for injury control regions within the *Bmp5* locus, we fractured ribs in adult BAC-*lacZ* transgenic animals by cutting the ribs near the dorsal midline, just distal to the neck of the rib protruding from the vertebral column (Fig. 2A, Materials and methods). We then assayed *lacZ* activity at post-operative day 4 (POD4), a time when *Bmp5* expression is elevated [12, 22] and *Bmp5* null mutants show a delay in rib fracture repair [30]. Although the BAC A clone contains a known embryonic rib enhancer, adult BAC-A-*lacZ*⁺ mice did not show significant *lacZ* activity in POD4 rib fractures, similar to wild type controls (Fig.

2B, C, dashed circles, $n = 2$; $n = 7$). The absence of rib expression from BAC A sequences was confirmed using animals generated from a second, independent stable BAC A transgenic line (data not shown, $n = 5$).

In contrast, robust lacZ activity within rib fracture calluses was observed from two independent transgenic lines for both *BAC-B-lacZ*/ $+$ (Fig. 2D, $n = 2$; $n = 4$) and *BAC-C-lacZ*/ $+$ transgenic mice at POD4 (Fig. 2E, $n = 4$; $n = 3$). Importantly, the lacZ expression observed in the fracture callus, triggered by the BAC regulatory sequences, overlaps endogenous *Bmp5* expression following rib injury (Fig. 2F–H). More detailed time course studies showed very little induction of lacZ reporter activity at POD1 (Supplementary Fig. 2A, $n = 1$), weak lacZ staining by POD2 (Supplementary Fig. 2B, $n = 2$; $n = 2$) and strong lacZ expression at POD4 (Fig. 2D and Supplementary Fig. 2C), and POD7 (Supplementary Fig. 2D, $n = 2$; $n = 3$). Examination of lacZ expression at later time points was confounded by the increase in endogenous β -galactosidase activity in rib fracture calluses, even in wild type controls (Supplementary Fig. 2H, and data not shown). This is likely due to osteoclasts, which express β -galactosidase, and which rise in number at later stages of repair as the callus is remodeled into lamellar bone [44, 45].

BAC B and BAC C share an 83 kb region of the *Bmp5* locus (Fig. 1A). Strikingly, transgenic mice carrying a 17.8 kb subregion of this overlapping interval (Ph7 clone in Fig. 1A, chr9:75,985,673–76,003,508), also showed activation of reporter gene activity following rib injury. lacZ activity from two independent stable lines was visible in rib fracture calluses at POD4 (Fig. 3A, arrows, $n = 2$; $n = 2$), and cross-sections (10 μ m) through *Ph7-lacZ*/ $+$ ribs show small clusters of blue cells scattered throughout the fracture callus (Fig. 3B, arrowheads).

Note that the adult injury response region identified in these experiments maps 110 kb downstream of previously identified sequences that drive embryonic expression in the lateral rib perichondrium (Fig. 1A, 6.2 kb *Ex4r* region clone [40]). Rib fractures made in a stable mouse line carrying the *Ex4r-lacZ* transgene did not show a lacZ injury response in adult animals (Fig. 3C dashed circles, Fig. 3D; $n = 2$), but did produce robust laterally restricted lacZ activity in the developing ribs of E14.5 dpc embryos (Fig. 3G, H). In contrast, *Ph7-lacZ*/ $+$ mice, which show strong lacZ expression following rib fracture in adults, did not show lacZ expression in the developing ribs at embryonic stages (Fig. 3E, F). Therefore, regulatory sequences functioning during rib development and following rib fracture map to distinct regions of the *Bmp5* locus.

The injury response module is activated upon fracture of tibia as well as ribs

Many of the enhancers that drive reproducible *Bmp5* expression during embryonic development are specific for highly localized anatomical locations in the skeleton (Fig. 1) [38–40]. To further test the spatial specificity of the adult injury response, we generated fractures in the diaphysis of the tibia (Fig. 4A) and assayed lacZ expression at POD4 in the same BAC transgenic lines used for the rib fracture analysis. As with rib injury, wild type controls and *BAC-A-lacZ*/ $+$ transgenics did not show any lacZ activity following tibia fracture (Fig. 4B, C, dashed line, $n = 1$; $n = 2$). In contrast, strong lacZ expression was present in tibia fracture calluses from both *BAC-B-lacZ*/ $+$ (Fig. 4D, $n = 3$) and *BAC-C-*

lacZ/+ (Fig. 4E, $n = 2$) transgenic mice. A tibia injury response was also seen in *Ph7-lacZ*/+ transgenic mice carrying the smaller 17.8 kb region (Fig. 4F, $n = 2$). Further examination of *BAC-B-lacZ*/+ tibia fracture calluses showed overlapping domains of *lacZ* and endogenous *Bmp5* expression (Fig. 4G–I). Thus, injury responses in both adult ribs and tibia map to a similar region of the *Bmp5* locus. Further, this injury response module is distinct from the multiple embryonic enhancers that separately drive expression in the either rib or limb skeletal structures during development (Fig. 3 and Supplementary Fig. 3).

To examine in more detail the activity of the skeletal injury enhancer during bone healing, we compared *lacZ* expression in tibia calluses to two markers of chondrogenesis, *Sox9* and type II collagen (*Col2a1*). *Sox9* is one of the earliest genes expressed by chondrocyte progenitors and is activated in response to BMP signaling [46, 47]. Immunohistochemical analysis of cross-sections (10 μ m) through *BAC-B-lacZ*/+ tibia fractures with *Sox9* antibodies shows *lacZ*-positive cells surrounding and scattered among *Sox9*-expressing immature chondrocytes (Fig. 5A, B). *Sox9* regulates *Col2a1* expression, which is a marker of chondrocyte differentiation and results from direct binding of *Sox9* to a *Col2a1* enhancer [48–51]. As expected, *Col2a1* expression mirrors *Sox9* activity; it is localized to differentiating chondrocytes surrounding the fracture site, adjacent to the major domains of *lacZ* activity (Fig. 5C). The expression of *Sox9* and *Col2a1* in close proximity to *lacZ*-positive cells is consistent with transcriptional activation of *Bmp5* through the skeletal injury enhancer triggering the formation of new cartilage. Osteoclasts play an important role in fracture repair and can also express endogenous β -galactosidase [45]. Staining for the osteoclast marker tartrate-resistant acid phosphatase revealed some TRAP-positive cells in tibia sections (Fig. 5D, asterisks). However, these cells were largely absent from the region of the fracture callus that was labeled with *lacZ* activity at POD4 in transgenic mice (Fig. 5D).

The injury response module is also activated after skin wounding

To test whether the *Bmp5* regulatory region also activates gene expression in response to other types of injuries, we examined *lacZ* activation following dorsal skin wounding. Skin is a continually renewing tissue with discrete populations of stem cells necessary for normal epithelial maintenance and repair. Previous studies have shown that *Bmp5* is expressed in both the interfollicular epidermis and hair follicles, including the follicular bulge, the site of one population of epidermal stem cells [36, 52]. Genetic studies have also shown that *Bmp5* is required for normal epidermal stem cell numbers [35, 36].

A full-thickness skin excisional biopsy on the upper back of *Ph7-lacZ*/+ mice was created and allowed to heal normally for 4 days before assaying *lacZ* activity (Materials and methods). Notably, the *Ph7-lacZ*/+ animals showed robust *lacZ* expression appearing in a ring around the periphery of the wound and in some nearby hair follicles (Fig. 6A, $n = 2$). A time course showed detectable *lacZ* activity as soon as 4–6 hours after injury (Supplementary Fig. 4A, B). Activity peaked between POD1 and POD4 (Supplementary Fig. 4D–F) and continued throughout the remainder of wound closure (data not shown). Cross sections (8 μ m) through the skin identified a stream of *lacZ*-positive keratinocyte cells in the superbasal layer migrating into the junction between the wound and uninjured skin

(Fig. 6B). *In situ* hybridization of skin punch cross sections (5 μ m) showed that the pattern of *lacZ* expression triggered by the *Bmp5* regulatory region overlaps domains of endogenous *Bmp5* expression following skin injury (Fig. 6C, D).

The injury response module is also activated after lung injury

Activation of BMP signaling following airway inflammation has been demonstrated in rodents and humans [53–55]. In addition, on some genetic backgrounds, *Bmp5* null mutants display a high incidence of fluid-filled cysts throughout the lungs [56, 57], suggesting *Bmp5* may participate in normal lung maintenance. To further investigate the activity of the *Bmp5* injury control region in soft tissues, *Ph7-lacZ/+* transgenic mice were injected intra-peritoneally with the xenobiotic naphthalene (Materials and methods). Naphthalene is a lipophilic compound metabolized by cytochrome P450-mediated oxidation into toxic epoxides that selectively ablate non-ciliated secretory cells (Club (Clara) cells), the predominant epithelial cell type lining the bronchiolar airways of the lungs [58, 59].

Examination of *Ph7-lacZ/+* mice at POD4 showed robust *lacZ* activity in the bronchioles of naphthalene-treated but not control lungs (Fig. 7A, B). The *Bmp5* injury response was activated soon after naphthalene-induced lung injury (6 hours, Supplementary Fig. 5A,B), and continued for several days (Supplementary Fig. 5C–F). Closer examination of the bronchioles in naphthalene-treated mice revealed *lacZ*-positive cells dotted along the bronchiolar epithelium (Fig 7C, D). Immunohistochemical staining with antibodies against CC10, a specific marker for Club cells, revealed a marked reduction in Club cells lining the bronchioles in injured lungs (Fig. 7E, F) [59]. *lacZ*-positive cells in naphthalene-treated lungs may include either non-Club epithelial cells, or a variant population of Club cells that survive naphthalene treatment and contribute to repopulation of the epithelial lining of the bronchioles [60]. The bronchiolar epithelium also shows induction of endogenous *Bmp5* expression following injury (Fig. 7G, H) in regions similar to the domains of *lacZ* activity controlled by the injury response module.

Discussion

Despite the importance of BMPs in development and disease, relatively little is known about the regulatory mechanisms controlling BMP activation at particular times and places during development and following injury. Here, we have characterized a set of tissue-specific enhancers that coordinate *Bmp5* developmental expression. Importantly, we find that activation following adult bone or soft tissue injury occurs through a distinct regulatory region that we hereafter refer to as the “injury response element” (IRE).

The presence of multiple *Bmp5* regulatory modules in both intronic and distant flanking regions has previously been inferred from studies of classical *Bmp5* regulatory mutations and tests using *BAC-lacZ* reporter mice [38, 39]. The locations of specific rib, fingertip, lung, and thyroid cartilage enhancer sequences reported here are consistent with the broad regulatory predictions made in earlier studies. Indeed, the cumulative *lacZ* expression patterns seen with cloned enhancers in this survey recapitulate many aspects of endogenous *Bmp5* expression during development. However, although more than 30 well-conserved ECRs from the *Bmp5* locus were tested, no enhancers were found for some known sites of

Bmp5 expression, such as the xiphisternum and ear cartilage [19, 27]. Regulatory elements driving *Bmp5* expression in these anatomical locations may reside in ECRs that did not meet the conservation criteria used to select clones for testing in the transgenic assay. Alternatively, sequences necessary for expression in one of these tissues may map even further from the *Bmp5* gene than the regions covered by our current BAC, sequencing, and ECR studies.

Half of the clones tested using the *lacZ* reporter assay demonstrated consistent enhancer activity at E14.5 dpc in some embryonic tissues (Supplementary Table 1). This is comparable to the 53% rate of regulatory element identification observed in the large-scale survey for enhancers in many genes, scored at E11.5 dpc, and summarized at the VISTA Enhancer Browser site (<http://enhancer.lbl.gov>) [61]. However, several highly conserved *Bmp5* genomic fragments, with homology extending to zebrafish, failed to show any response in this reporter assay (Supplementary Table 1). In *Drosophila*, compact clusters of well-conserved transcription factor binding sites are predominantly located in regulatory modules required early in development [62, 63]. *Bmp5* expression begins at E6.5–7.5 dpc in the presumptive anterior mesoderm, shortly after gastrulation begins [64]. Therefore, well-conserved ECRs that failed to show expression at E14.5 dpc may be driving some of the earliest *Bmp5* activity. *Bmp5* is also expressed and required in late-developing structures, such as the ear pinna cartilage [29, 65, 66]. Thus some ECRs that are inactive in our tests may function at late embryonic or postnatal stages. Unfortunately, the costs associated with the transgenic reporter assays make it difficult to examine a full range of time points for each tested construct. Additionally, insulator or repressor elements would also fail to exhibit activity in our transgenic enhancer assay, so ECRs that appear inactive may have other, non-enhancer functions.

During embryogenesis, at least two enhancers coordinate *Bmp5* expression in developing ribs, each of which is active in subcompartments of the developing rib perichondrium [40]. These dual rib enhancers are located at least 84 kb apart, and are also physically distinct from other enhancers for developing bone and cartilage. Interestingly, despite the analogous gene transcription profiles observed for bone formation and fracture repair, we have found that a distinct IRE activates gene expression in adult ribs following injury. In contrast to the previously identified embryonic rib enhancers, the IRE drives expression in cells scattered throughout the fracture callus, rather than in highly compartmentalized medial and lateral domains. Also unlike the embryonic rib enhancers, the IRE sequence drives gene expression in multiple bones after injury (both ribs and tibia).

The same IRE is also rapidly activated following very different types of injury, including dorsal skin wounding and naphthalene-induced lung damage. Similar to our findings in bones, we have identified separate developmental enhancers in the *Bmp5* locus that drive expression in skin and lung tissue during embryogenesis. An embryonic skin enhancer maps more than a hundred kb upstream of the IRE sequences; and BAC clone A (which contains this enhancer) fails to activate reporter gene expression in response to acute dorsal skin injury. A separate *Bmp5* enhancer also controls embryonic lung expression. This region maps 4.2 kb outside of the IRE region, and *Ph7-lacZ/+* transgenic mice do not show embryonic lung expression (CG, unpublished data). Thus, although *Bmp5* is expressed both

during embryonic development and during injury repair, the regulatory sequences controlling *Bmp5* activation in embryonic development and adult injury response are clearly distinct.

Developmental expression of *Bmp5* is highly programmed and reproducible, with stereotyped patterns of local expression that help generate the characteristic size, shape, and number of particular skeletal structures during embryogenesis. In contrast, adult wounds occur at unpredictable times and locations in the body, and trigger an inducible response through a distinct regulatory region (Fig. 8). Although the details of wound response vary depending on the type of tissue damaged, some steps appear to be shared between multiple types of injury, including: 1) immediate release of damage-induced signals and rapid hemostasis; 2) an inflammation phase lasting approximately 24–48 hours; 3) a proliferation phase in which new tissue is formed, typically occurring between 2 and 10 days post-injury; and 4) a remodeling phase beginning 2–3 weeks after injury that can last up to a year [67, 68]. Immediately after wounding, local calcium and ATP stores are released from damaged cells, and lead to hydrogen peroxide production by dual oxidase Duox [68–70]. Platelets also rapidly aggregate at sites of damaged blood vessels, and create a plug to prevent further loss of blood. Local damage signals and cytokines from degranulating platelets trigger an inflammatory reaction that includes activation of complement, recruitment of neutrophils, and eventually the influx of monocytes that differentiate into macrophages [67, 68]. The macrophages in turn release numerous cytokines into the injury site [71–73]. While an inflammatory response is common to multiple injuries, the consequences of this signaling cascade can vary depending on tissue type. For example, in fractured bones, cytokine signaling triggers osteoblast and chondrocyte differentiation from nearby stem cell progenitors, commencing the proliferative phase of healing [11, 74]. In the skin, signaling cascades lead to the activation of epidermal stem cell progenitors, followed by migration and proliferation of keratinocytes at the wound edge [67]. In the lung, cytokines and growth factors promote Club cell proliferation leading to re-epithelialization of the airways [75].

It is possible that the *Bmp5* IRE contains multiple response modules, each of which is coupled to distinct injury-induced signals present in fractured bones, injured skin, or damaged lung tissue. Alternatively, the IRE may respond to a common signal that occurs at diverse types of tissue injury, such as hydrogen peroxide, platelet or complement factors, anoxia due to tissue damage, or cytokines released during the early inflammatory period.

Several transcription factors are known to be important for the regulation of wound responses and regeneration [12, 76]. NF- κ B factors are sensitive to redox state, and likely act downstream of early hydrogen peroxide signaling to stimulate inflammatory responses [77, 78]. The basic-Helix-loop-Helix transcription factor *Hypoxia-inducible factor 1 alpha* (*HIF-1 α*) is expressed at many injury sites following blood vessel changes [76]. Targeted disruption of *HIF-1 α* in osteoblasts, keratinocytes, or Club cells results in impaired healing of bone or skin [79, 80], or asthma [81]. In contrast, increased *HIF-1 α* expression or HIF-1 α stabilization has been shown to improve regeneration in both bone and skin [79, 82]. Transcription factor activator protein-1 (AP-1) mediates gene transcription in response to cytokines and growth factors [83], and AP-1 family members like Fos and Jun are upregulated in response to many types of tissue injury [84–89]. Interestingly, overexpression

of *Fra1*, a Fos family member that heterodimerizes with Jun proteins to form AP-1 [90], leads to impaired fracture healing that is thought to result from suppression of inflammation responses [91]. Kruppel-like and Grainy-head family transcription factors are expressed in injured epithelia and are required for establishment and repair of epithelial barrier functions [76, 92–95]. Following skin injury, KLF4-positive epidermal derivatives migrate into the junction between wounded and uninjured tissue [96], in a pattern very similar to *Bmp5* IRE-*lacZ* expressing cells.

The IRE sequence contains multiple predicted Fos-Jun and KLF binding sites [97], and also shows an experimental peak of c-Jun binding in chromatin immunoprecipitation surveys with cultured mouse CH12 B cell lymphoma cells, suggesting it may be activated through the AP-1 pathway in some contexts [98]. In the future, additional narrowing of the minimal sequences necessary for skeletal and soft tissue injury responses should make it possible to test whether AP-1, KLF, or other factors are the key factors that lead to IRE activation following diverse types of injury *in vivo*. Adult fracture repair ability, skin stem cell numbers, and ear regeneration capacity have all previously been linked to the *Bmp5* locus in mice [30, 33–36]. Thus, further understanding of the molecular basis of *Bmp5* activation following injury may suggest new strategies for enhancing repair and regeneration of multiple mammalian tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

We survey the mouse *Bmp5* locus for both embryonic and postnatal enhancers.
Embryonic expression is controlled by many stereotyped locally-acting enhancers.
Expression after adult injury is activated by a separate “injury response region.”
The same “injury response region” turns on after bone fracture, skin, or lung injury.
Development and repair activate the same gene, but via distinct control sequences.

Fig. 1.

Multiple evolutionarily conserved sequences control *Bmp5* expression at specific anatomical sites in developing embryos. (A) Schematic of the *Bmp5* locus on chromosome 9 (mm10: chr9:75,725,358–76,225,357) displaying the approximate breakpoints of two *short ear* (*se*) regulatory mutations that define a large 3' prime regulatory region. The positions of three BAC clones and two plasmids, Ex4r and Ph7, from which stable transgenic mouse lines were generated, are shown. The multi-species alignment track from the UCSC Genome Browser indicates sequence conservation (blue peaks), which was used to select evolutionarily conserved regions (ECRs) for the *lacZ* enhancer assays (constructs 1–34). The locations of cloned ECRs exhibiting consistent expression patterns in transgenic embryos (black) and those with no specific activity (gray) are noted. (B–G) Representative *lacZ* transgenic embryos showing mid-gestation (E13.5–E14.5 dpc) reporter gene expression (blue), and similarly staged embryos showing corresponding expression of the endogenous *Bmp5* gene by *in situ* hybridization (purple) in: facial mesenchyme (B, clone 2), tongue muscle (arrow in C, clone 6), limb joints (asterisks in D, clone 11), ribs (D, clone 12), digit joints (E, clone 13), apical epidermal ridge (E, clone 18), thyroid cartilage (arrow in F, clone 23), and lateral head mesenchyme (G, clone 34). Scale bars: B, C, D, F and G = 1 mm, E = 400 μ m.

Fig. 2.

Identification of a rib fracture regulatory region in the *Bmp5* locus. (A) Mouse rib cage showing the locations of breaks, denoted by white jagged lines in the fourth and sixth ribs, assayed in the rib fracture model. (B–E) Medial views of post-operative day 4 (POD4) whole rib cages processed for *lacZ* activity (blue staining). No *lacZ* expression is evident within the rib fracture calluses of wild type or *BAC-A-lacZ/+* mice (dashed circles in B, C). Darkly stained rib fracture calluses are observed in both the *BAC-B-lacZ/+* (D) and *BAC-C-lacZ/+* (E) transgenic mice. (F, G) *In situ* hybridization analysis of gene expression in *BAC-B-lacZ/+* POD4 rib fractures. (F) Low magnification image of a transverse section (12 μ m) through a *BAC-B-lacZ/+* rib fracture at POD4 showing the adjacent ends of the fractured rib (dashed lines). Area of detail in (G, H) is boxed. Endogenous *Bmp5* expression (brown dots indicated by arrows in G) is observed throughout the fracture callus, corresponding to a region with robust *lacZ* activity (blue dots indicated by arrows in H) controlled by the *BAC-B-lacZ* clone. Scale bars: E = 2 mm, F = 200 μ m, H = 20 μ m.

Fig. 3.

Rib fracture in adults activates a regulatory region that is distinct from known embryonic rib enhancers. A 17.8 kb genomic fragment (Ph7) lies within the area of overlap between BAC B and BAC C (Fig. 1A) and also activates *lacZ* expression in POD4 rib fractures (A, arrows). Cross-sections (10 μ m) through injured ribs reveal that *lacZ* expression is distributed randomly throughout the fracture callus (B, arrowheads). In contrast, transgenic animals carrying a 6.2 kb region containing a known embryonic rib enhancer (Ex4r clone; Fig. 1A), exhibit little *lacZ* activity in rib calluses (dashed circles in C, sections in D). (E–H) Mid-gestation (E14.5 dpc) *lacZ* expression from Ph7 and Ex4r genomic clones underscores differences between repair and developmental processes. *Ph7-lacZ*/ $+$ transgenic embryos show no evidence of *lacZ* activity in embryonic ribs (asterisks in E and F). Conversely, *Ex4r-lacZ*/ $+$ transgenic embryos show strong *lacZ* expression in embryonic ribs (arrowhead in G), which is spatially restricted to the lateral rib perichondrium (H, arrowheads) [40]. Scale bars: C = 1 mm, D = 200 μ m, G = 2 mm, and H = 50 μ m.

Fig. 4.

Tibia fracture in adults activates the same regulatory region activated by rib fracture. (A) Mouse limb showing the location of the break, denoted by jagged lines, assayed in the tibia fracture model. (B–F) Lateral views of POD4 tibia fractures assayed for *lacZ* expression (blue staining). No induced *lacZ* activity was observed in wild type or *BAC-A-lacZ/+* transgenic mice following tibia fracture (dashed lines in B, C). In contrast, clear *lacZ* activity was detected in the tibia fracture calluses of *BAC-B-lacZ/+* (D), *BAC-C-lacZ/+* (E), and *Ph7-lacZ/+* (F) transgenic mice. (G–I) *In situ* hybridization analysis of gene expression in *BAC-B-lacZ/+* POD4 tibia fractures. (G) Low magnification image of a transverse section (12 μ m) through a *BAC-B-lacZ/+* tibia fracture at POD4. Area of detail in (H, I) is boxed. Endogenous *Bmp5* expression (brown dots indicated by arrows in H) is observed in the fracture callus in a region overlapping the domain of *lacZ* expression activated by the *BAC-B-lacZ* clone (brown dots indicated by arrows in I). Scale bars: E = 2 mm, G = 0.5 mm, I = 50 μ m.

Fig. 5.

Histological analysis of a *BAC-B-lacZ/+* tibia fracture callus shows that *lacZ* expression overlaps sites of forming cartilage. (A) Low magnification image of a transverse section (10 μ m) through a *BAC-B-lacZ/+* tibia fracture at POD4. Area of detail in (B–D) is boxed. (B) Immunohistochemical staining for Sox9 protein, which labels chondrocytes, partially overlaps the *lacZ* expression domain (arrows). (C) In contrast, Col2a1 protein, a marker for cartilage, is seen predominantly in regions adjacent to the major domain of *lacZ* expression. (D) TRAP staining, which labels osteoclasts, is largely absent from the fracture callus (asterisks). Scale bars: A = 1 mm, and D = 100 μ m.

Fig. 6.

The *Bmp5* bone injury regulatory region also activates gene expression following skin wounding. (A) Whole-mount dorsal skin tab from a *Ph7-lacZ/+* transgenic mouse showing *lacZ* activity (blue staining) at POD4. Expression is restricted to the periphery of the healing wound. (B) Cross-section (8 μ m) through the skin shows *lacZ* expression predominantly along a thin, upper layer of the epithelial tongue (To), an area of hyperproliferative epithelium. The dotted line represents the location of the original wound edge. Es, eschar (scab); Un, uninjured skin. (C, D) *In situ* hybridization analysis of gene expression in a *Ph7-lacZ/+* skin wound at POD4. *Bmp5* expression (red dots indicated by arrows in C) is observed in both epithelial and dermal cells, corresponding to a region with robust *lacZ*

expression activated by the Ph7 injury clone (D). Dashed lines: boundary between epidermis and dermis. Scale bars: A = 2 mm, B = 200 μ m, C and D = 30 μ m.

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Fig. 7.

The *Bmp5* injury regulatory module responds to chemically induced lung damage. (A–D) *lacZ* activity in POD4 lung cross-sections (8 μm) following intra-peritoneal injection of *Ph7-lacZ/+* transgenic mice with corn oil (vehicle; A, C), or naphthalene (B, D). Low magnification images (A, B) show *lacZ* activity distributed throughout the lung in naphthalene-treated (B) but not vehicle-treated (A) animals. High magnification images (C, D) reveal *lacZ* expression is restricted to a subset of epithelial cells lining the bronchiolar airway in naphthalene-treated (D) but not vehicle-treated (C) lungs. (E, F) High magnification images of lung cross-sections (8 μm) stained with antibodies against CC10, a marker for Club cells. CC10-positive Club cells are abundant in uninjured airway epithelium (E). In contrast, Club cells are greatly reduced 4 days after naphthalene treatment, confirming injury to the lung epithelium (F). (G, H) Endogenous *Bmp5* expression analysis in vehicle-treated (G) or naphthalene-treated (H) POD4 lung cross-sections (5 μm) from *Ph7-lacZ/+* transgenic mice. Low levels of *Bmp5* transcript (red dots indicated by arrows in G) are detected in uninjured lungs. In contrast, four days after naphthalene treatment,

elevated *Bmp5* expression is visible in many of the epithelial cells lining the bronchiolar airways (H, arrows). Scale bars: A, B = 0.5 mm, C H = 50 μ m.

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Fig. 8.

Distinct regulatory pathways control *Bmp5* expression during embryonic development and adult regeneration. The stereotyped patterns of *Bmp5* expression during normal embryonic development are controlled by a series of enhancers that act in particular bones, tissues, and body regions. In addition to this pre-programmed activity, *Bmp5* expression can also be induced by tissue injury in adult animals. Bone fracture, skin wounding, and lung injury all induce gene expression through a newly mapped region of the *Bmp5* locus, which is distinct from other embryonic enhancers, and allows local reactivation of *Bmp5* during repair and regeneration of adult tissues.