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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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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

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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′*hsp*68lacZ* 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′*hsp*68lacZ* 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 h in 4% paraformaldehyde (PFA) in 1× PBS at 4 °C, placed in cold 1× 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.

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