Bone 116 (2018) 103–110

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

In vivo identification of Bmp2-correlation networks during fracture healing by means of a limb-specific conditional inactivation of Bmp2

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ARTICLE INFO

Keywords:
Bmp2
Fracture healing
Co-expression
Differential gene expression
Conditional knockout
Networks

ABSTRACT

Bmp2 is known to play an essential role in the initiation of fracture healing via periosteal activation.

Specifically, activation and subsequent differentiation of periosteal progenitor cells requires Bmp2 signaling for activation of the osteo-chondrogenic pathway. Here, we explored the interactive transcriptional gene-gene interplays between Bmp2 and 150 known candidate genes during fracture repair. We constructed the interactive Bmp2 signaling pathways in vivo, by comparing gene expression levels prior and 24 h post femur fracture, in presence (wild type) and in absence of Bmp2 (Bmp2c/c;Prx1::cre limb-specific conditional knockout). Twenty-six differentially expressed genes (pre- vs. post-fracture), which demonstrated high correlations within each experimental condition, were used to construct the co-expression networks. Topological dynamic shifts across different co-expression networks characterized the 26 differentially expressed genes as non-redundant focal linking hubs, redundant connecting hubs, periphery genes, or non-existent. Top-ranked up- or down-regulated genes were identified and discussed. Protein-protein interactions in public databases support our findings. Thus, the co-expression networks from this study can be used for future experimental hypotheses.

1. Introduction

Since its identification as a key regulator of bone formation [1], bone morphogenetic protein 2 (Bmp2) has been shown to induce osteoblastic differentiation in vitro [2] and in vivo [3], and to be clinically effective in bone regenerative therapy [4]. Specifically, Bmp2 expression is essential to initiate fracture healing [5] and regulate embryonic patterning [6], and is an indispensable multifunctional regulator of vertebrate development [7,8]. Polymorphisms of the human *Bmp2* gene have been linked to osteoporosis [9–12] and osteoarthritis [13,14]. Thus, Bmp2 plays a crucial role in biological processes associated with bone formation, homeostasis, and regeneration.

Evidence has shown that selective genes in the Wnt and TGF-beta signaling pathways are active in bone fracture healing [15]. Our goal is to explore the gene-gene interplays between Bmp2 and a list of 150 candidate genes during the bone fracture healing process. To do so, we compared gene expression in fractured femurs of animals expressing Bmp2 (wild type; denoted as WT) vs. those fractured femurs of animals not expressing Bmp2 (*Bmp2*$^{c/c}$;*Prx1::cre* limb-specific conditional knockout; denoted as cKO) [16]. The 150 candidate genes, selected for their roles in bone development, are enriched in such pathways as Wnt, Bmp, PTH and TGF-beta signaling. Details are provided in Suppl. Table 1.

Previous studies using a mouse femur fracture [5] model found maximum Bmp2 expression at 24 h post-fracture. Therefore, in this report, gene expression profiles were obtained and compared at two time points – before fracture and 24 h post-fracture. We demonstrated that markers for chondrogenesis and osteogenesis were absent at 24 h post-fracture in Bmp2 cKO mice. This is consistent with human studies that examined co-expression of BMPs in non-unions [17]. We further investigated differential gene expression signatures (DGEs) and their correlation strength in the Bmp2 WT vs. cKO groups. Interplays among the DGEs were characterized with four co-expression networks. The identified changes in topological characteristics and the associated correlation strength between Bmp2 and DGEs provided an in-depth dynamic understanding of Bmp2 networks during fracture repair.

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https://doi.org/10.1016/j.bone.2018.07.016
Received 9 November 2017; Received in revised form 9 May 2018; Accepted 21 July 2018
Available online 23 July 2018
8756-3282/

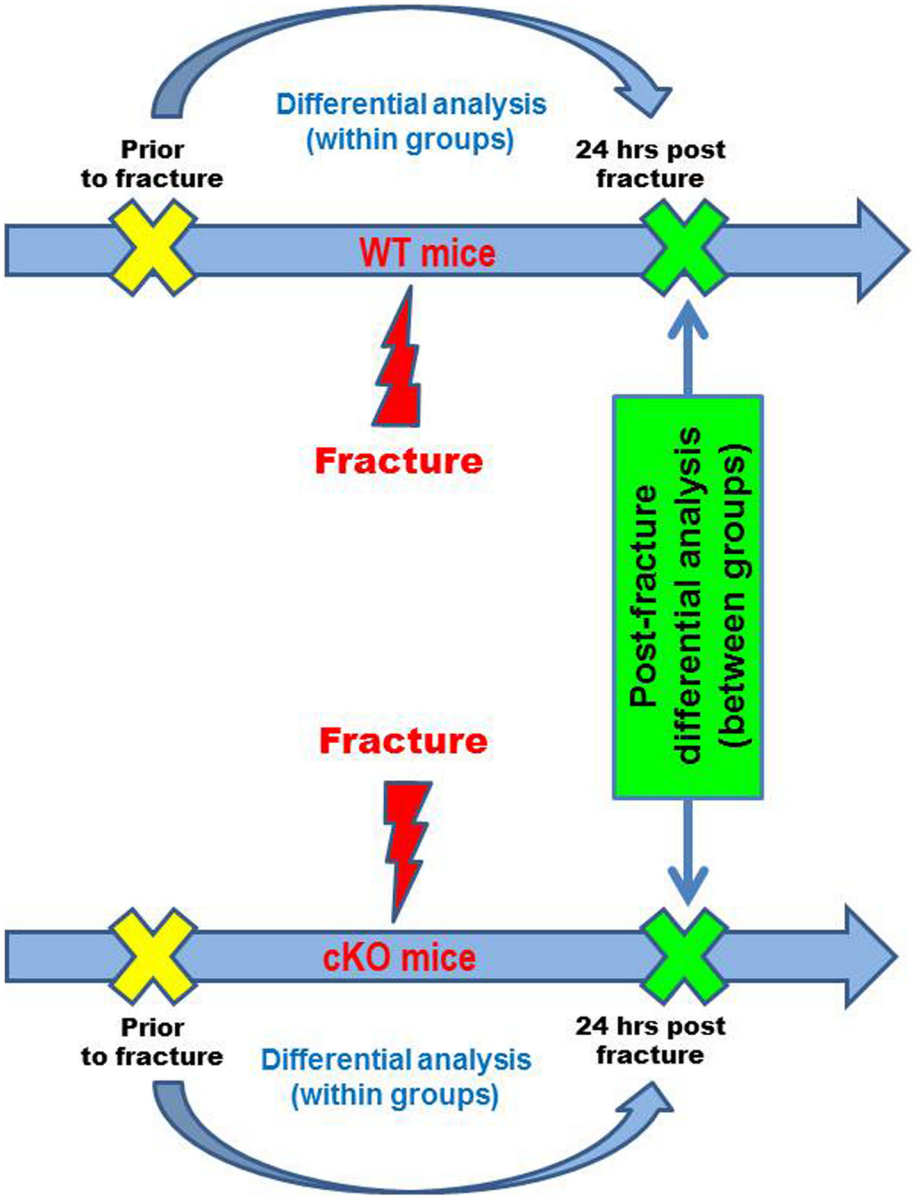

Fig. 1. Flow chart of experimental design and differential expression analysis.
Genes with induced or suppressed expression activities were identified within the WT and the cKO mice. Post-fracture transcriptional variations were compared between the two groups and corrected for multiple testing.

2. Materials and methods

2.1. Experimental design

Our research design follows the flowchart shown in Fig. 1. In the WT group, 5 animals were sacrificed post-fracture vs. 6 pre-fracture, leading to 30 combinatory differences in gene expression activity between post- vs. pre-fracture (denoted WT $\Delta_{post\text{-}pre}$). Similarly, in the cKO group, we calculated 36 combinatory differences of gene activity by comparing 6 animals post-fracture vs. 6 pre-fracture (denoted cKO $\Delta_{post\text{-}pre}$). Student *t*-test was used to compare gene activities between two groups (WT vs. cKO) at the baseline before fracture, 24 h post-fracture, as well as combinatory differences in gene expression activity (WT $\Delta_{post\text{-}pre}$ vs. cKO $\Delta_{post\text{-}pre;}$ with Bonferroni correction) [18]. Analysis of Variances (ANOVA) was performed for statistical analyses among four groups.

2.2. Animal experiment and humane endpoints

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and were approved by the Harvard Medical Area Institutional Animal Care and Use Committee. Mice carrying floxed Bmp2 alleles ($Bmp2^{c/c}$) with C57BL/6 background [16] were crossed with heterozygous Prx1::cre mice with C57BL/6 background [19] to obtain the following littermates: 1) $Bmp2^{c/c}$ mice (WT); 2) $Bmp2^{c/c}$;*Prx1::cre* (cKO) mice. Mice were born at the expected Mendelian ratios. Tail biopsies were collected for genotyping by PCR as described by Tsuji et al. [16] Four groups of 8–10 weeks old mice (un-fractured WT, fractured WT, un-fractured cKO, fractured cKO) were created by randomly distributing 11 males and 12 female mice in each group (n = 5–6). Using a method previously described [20], unilateral fractures were produced in the right femurs of the fractured-group mice. X-rays were taken using Micro50 (Microfocus Imaging, Faxitron Bioptics LLC, Tucson, AZ, USA) at 50 kV for 100 s, to ensure that the fracture sites were consistently located in the same central area of the