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Co-expression of BMPs and BMP-inhibitors in human fractures and non-unions

Peter Kloen [a,*], Dominique Lauzier [b,c], Reggie C. Hamdy [b,c]

[a] Department of Orthopaedic Surgery, G4-N, Academic Medical Centre, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands
[b] Division of Orthopaedic Surgery McGill University, 1529 Cedar Avenue, Montreal, Quebec, Canada H3A 1A6
[c] Shriners Hospital for Children, Montreal, Quebec, Canada

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ABSTRACT

Bone morphogenetic proteins (BMPs) are increasingly being used clinically to enhance fracture repair and healing of non-unions. However, the potential efficacy of supraphysiological dosing for clinical results warrants further clarification of the BMP signaling pathway in human fracture healing. As BMP signaling can be fine-tuned at numerous levels, the role of BMP-inhibitors has become a major focus. The aim of the present study was to document co-expression of BMPs, pSmad 1/5/8, and BMP-inhibitors in human fracture callus and human non-unions. Using human tissue of fracture callus (n = 14) and non-unions (n = 4) we documented expression of BMPs (BMP2, BMP3 and BMP7), pSmad 1/5/8 and the BMP-inhibitors noggin, gremlin, chordin, Smad-6, Smad-7 and BAMBI. Co-expression of pSmad 1/5/8, BMPs and BMP-inhibitors was noted in the osteoblasts of fracture callus as well as of non-unions. Expression of BMP-inhibitors was generally stronger in non-unions than in fracture callus. The most pertinent differences were noted in the cartilaginous tissue components. Expression of BMP2 in chondrocytes was markedly decreased in non-unions compared to fracture callus and that of BMP7 was almost completely absent. Expression of BMP-inhibitors was almost the same in osteoblasts, chondrocytes and fibroblasts of fracture callus and well as in non-unions. Interestingly, although BMP ligands were present in the chondrocytes and fibroblasts of non-unions, they did not co-express pSmad 1/5/8 suggesting that BMP signaling may have been inhibited at some point before Smad 1/5/8 phosphorylation.

These results suggest co-expression of BMP, pSmad 1/5/8 and BMP-inhibitors occurs in human fracture callus as well as non-unions but the relative expression of BMPs vs. BMP-inhibitors was different between these two tissue types. In contrast to our expectations, the expression of BMP inhibitors was comparable between fracture callus and non-unions, whereas the expression of BMPs was notably lower in the cartilaginous component of the non-unions in comparison to fracture callus. Based on these results, we believe that aberrations in the BMP-signaling pathway in the cartilaginous component of fracture healing could influence clinical fracture healing. An imbalance between the local presence of BMP and BMP-inhibitors may switch the direction towards healing or non-healing of a fracture.

Introduction

Bone healing is a complex regenerative process initiated in response to a fracture; with the final aim of restoring skeletal function. Over the last 2 decades, this well orchestrated cascade of events has become increasingly understood [1]. Interestingly, bone healing seems to recapitulate many events seen in bone development and embryogenesis [1–3]. The key drivers of this process are cytokines, platelets and growth factors, of which bone morphogenetic proteins (BMPs) have emerged as critical players.

BMPs are members of the pleiotropic Transforming Growth Factor-Beta (TGF-β) family [4]. More than 20 BMPs are currently known, and their characteristic feature is the capacity to induce endochondral bone formation [4–12]. Starting after birth, BMPs play a critical role in maintenance of bone mass through inducing commitment of mesenchymal cells towards cells of the osteoblastic lineage, and they also enhance the differentiated function of the osteoblast. Analysis of genetically modified mouse models with various null mutations, dominant-negative or conditional knockouts of BMP ligands, BMP receptors (BMPRs) or Smad proteins, has clearly shown the functional relevance of the BMP signaling cascade in skeletal formation and repair [13]. In addition, naturally occurring mutations of BMPs and BMPR in humans are associated with skeletal abnormalities [14].

The BMP signals are mediated by type I and type II serine/threonine kinase transmembrane receptors (BMPRIA, BMPRIB and BMPRII) [4,11]. These receptors are expressed at different levels in different tissues. BMP binding to BMPRs activates Smad signaling that is translocated to the nucleus. The Smads are intracellular proteins than can be broadly divided in three classes: 1) receptor regulated Smads (R-Smads) such as Smad 1/5/8; 2) co-Smads, such as Smad-4; 3) inhibitory Smads (Smad-6 and Smad-7). It has also been shown that the actions of BMPs are tempered by inhibitors or antagonists, indicating the existence of local

* Corresponding author. Fax: +31 205669117.
E-mail addresses: p.kloen@amc.uva.nl (P. Kloen), dominique.lauzier@hotmail.com (D. Lauzier), rhamdy@shriners.mcgill.ca (R.C. Hamdy).

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feedback mechanisms to modulate BMP cellular activities [14–16]. The antagonists function at different levels of the BMP-signaling cascade: extracellular at the BMP-BMPR interaction (e.g. prevention of BMP binding to its receptors by noggin, chordin, and gremlin), by expression of membrane pseudo-receptors (e.g. BAMBI), and at the intracellular level (Smad-6 and Smad-7). Others have also been described (e.g. Ski).

After numerous animal studies showed the presence of BMPs, BMPRs and some of their antagonists [6,17–19] in fracture healing and distraction osteogenesis [20–26], we were the first to show expression of BMPs, BMPRs and intracellular signaling proteins (Smads) in human fracture and non-union tissue [7,8]. Surprisingly, our work showed that expression patterns did not differ between healing and non-healing fractures, suggesting that differences in healing capacity are not directly due to level of expression of BMPs, their receptors, and/or intracellular Smads. The first description of BMP-inhibitors in human fracture tissue was done by Kwong et al. in 2009 [27].

Although many questions remain for a complete understanding, scientists and clinicians are keen to leverage what is already known for clinical application. Preclinical studies have led to the clinical use of BMP2 and BMP7 [11,28,29]. So far, however, efficacy seems to be no better than autologous bone graft, with a key disadvantage being exogenous application is more costly [30]. Also, the clinical dosage needed is 100–1000 times higher than endogenous BMPs [28], and complications mostly related to the off-label use of BMPs have been reported [11,29].

To improve the effectiveness of BMPs as treatment, there are many aspects that still need clarification. What is well known is that BMP signaling can be fine-tuned at numerous levels at almost any step along the pathway [13–16,31]. Recently, the role of BMP-inhibitors (e.g. noggin, gremlin, chordin) and the extent to which they can be used as a control mechanism have received much attention [13–16,31]. Therefore, it seems possible that abnormal BMP signaling caused by increased expression of BMP-inhibitors could be related to unsuccessful bone healing. We and others have speculated on focusing on the BMP-inhibitors in bone healing, which is now emerging as a therapeutic target [32].

The objective of the current study was to document naturally occurring levels of BMPs and their inhibitors in human fractures and non-unions. Our hypothesis was that the balance between BMP and BMP-inhibitors differs between healing and non-healing human fractures, which would imply an interventional opportunity. In addition, we also set out to study their co-expression using double and triple immunohistochemistry staining. Fundamental to our hypothesis is a better understanding at the molecular level of why certain fractures heal and others do not.

Materials and methods

Specimens

Fracture callus and non-union tissue was obtained during surgery of 16 different patients at the time of operative repair or revision surgery of the fracture (n = 12) or hypertrophic non-union (n = 4). Three fractures involved the acetabulum (n = 2) or pelvis (n = 1). All other fractures and non-unions pertained to the appendicular skeleton. Although more patients were treated during this period, representative tissue availability was limited. The definition of a non-union was a fracture that had not healed within 6 months. All patients were treated by the senior author (PK) between 2001 and 2010. Patient characteristics are listed in Table 1.

Fracture patients were between 10 and 70 years of age and otherwise in good health. There were 10 males and 2 females. Time to callus harvest ranged from 2 to 10 weeks. Non-union patients were between 37 and 69 years of age and otherwise in good health. There were 3 males and 1 female. Approval of the Institutional Review Board (IRB) was obtained where appropriate. Oral consent for removal of the tissue and its storage in the tissue bank for research purposes was obtained from each patient. Individual consent for this specific project was waivered by the ethics committee of the remaining two hospitals since the research was performed on "waste" material, stored in a coded fashion. Indications for surgery were nascent (impending) malunion, non-union, and failure of fixation or fractures that were operated on in a delayed fashion. All fractures and non-unions have subsequently successfully healed.

Table 1
Summary of patient data.

Specimen	Age/sex	Location	Time since fracture (weeks)
Fracture callus	21 M	Acetabulum	2
	15 M	Acetabulum	5
	34 M	Distal radius	4
	39 M	Proximal femur	8
	43 F	Distal femur	6
	44 M	Humerus shaft	10
	28 M	Distal humerus	3
	35 M	Femur shaft	3
	10 F	Foot	2
	43 M	Pelvis	3
	33 M	Distal fibula	5
	70 M	Humerus shaft	6
Non-union	69 M	Tibia shaft	56
	38 M	Ulna shaft	24
	37 M	Tibia shaft	24
	51 F	Femur shaft	272

After removal from patients, specimens were placed in 10% neutral buffered formalin for 24 h and subsequently decalcified – if needed – in 10% ethylenediamine tetra acetic acid (EDTA), pH 7.2. The tissue was then routinely processed and embedded in paraffin wax. Sequential sections of 5–7 μm thick were prepared for haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC).

Immunohistochemistry

For immunohistochemistry, samples were fixed in 4% paraformaldehyde overnight, decalcified in 20% ethylene diamine tetra-acetic acid for 3 weeks, embedded in MMA (methylmethacrylate), and sectioned using a Leica RM 2255 microtome (Leica Microsystems, Richmond Hill, ON, Canada). Following deparaffinization and hydration, endogenous peroxidase activity was blocked using 10% hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubating samples in phosphate-buffered saline containing 10% normal horse serum for 20 min. Commercially available polyclonal goat antibodies were used for the detection of the following proteins: BMP2, BMP3, BMP7, noggin, gremlin, chordin, pSmad 1/5/8, Smad-6, Smad-7, and BAMBI (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA, 1:100 dilution in 1% goat serum).

Tissue sections were probed with the polyclonal goat antibody overnight at 4 °C in a humidified chamber. For negative controls, we omitted the primary antibody. We then incubated the sections with a biotinylated mouse anti-goat secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA, 1:400 dilutions in 1% normal goat serum) for 30 min at room temperature in a humidified chamber. Sections were stained using the avidin–biotin complex method for 30 min, followed by DAB-peroxidase staining. Finally, we counterstained sections with hematoxylin and mounted with Permount (Fisher Scientific, Montreal, Canada). Photomicrographs of the tissues were taken under 10× and 40× magnifications using a Leica microscope (Leica Microsystems, Richmond Hill, ON, Canada) attached to a Q-Imaging camera (Olympus DP70, Japan). For each frame tested, we also performed a negative control where the same procedure was performed but omitting the primary antibodies.

Table 2
Immunohistochemistry results of BMP2, BMP3, BMP7, BMP-antagonists and pSmad 1/5/8.

Protein	Tissue	OB	OC	Non-HC	C	FB
BMP2	Fracture callus	+	−	++	++	+
	Nonunion	++	+	+	+	+
BMP3	Fracture callus	+	+	+	++	+
	Nonunion	+	+	−	−	−
BMP7	Fracture callus	+	+	+	++	+
	Nonunion	+	+	−	−	−
Noggin	Fracture callus	+	+	+	++	+
	Nonunion	+	+	+	+++	+
Chordin	Fracture callus	++	++	++	+++	++
	Nonunion	++	++	−	−	−
Gremlin	Fracture callus	++	++	+++	++	++
	Nonunion	++	++	++	+++	+++
BAMBI	Fracture callus	+++	−	−	−	−
	Nonunion	++	−	−	−	−
Smad-6	Fracture callus	+	+	+	−	+
	Nonunion	++	+	++	+	−
Smad-7	Fracture callus	+++	+	+++	++	++
	Nonunion	+++	++	++	+	+
pSmad 1/5/8	Fracture callus	+	+	−	++	++
	Nonunion	++	+	−	−	−

−, No positive staining; +, less than 25% of cells stained positive, ++, 25 to 50% cells stained positive, +++, 50 to 75% cells stained positive, ++++, over 75% cells stained positive. OB: Osteoblasts; OC: Osteoclasts; Non-HC: Non-hypertrophic chondrocytes; C: Chondrocytes; FB: Fibroblasts.

Grading of the immunostained sections

Although immunohistochemistry is known as a qualitative technique used for description of cellular and extracellular components that stain positively, our laboratory has successfully developed a technique to semi-quantitatively evaluate our results, based on the percentage of positively staining cells. This technique has been previously reported for mandibular [21] and long bone distraction osteogenesis [20,22–24]. We have utilized the same technique in the current study. The grading scheme included: + represents <25% of the cells stained positively for the protein of interest, ++ represents 25% to 50% of cells stained positive, +++ represents 25% to 75% of cells stained positive, ++++ represented more than 75% of cells stained positive, and lastly − denoted no cells stained positively. The number of cells expressing the various proteins is assessed by cell counting in a superimposed grid. Sections were analyzed blindly in triplicates by an immunohistochemistry specialist (DL). The averages of all results were recorded (Table 2). Chondrocytes (hypertrophic and non-hypertrophic), osteoblastic, and fibroblastic cells were identified morphologically and scored independently. There were 16 samples: twelve fracture callus and four non-unions and there were 10 sections from each sample for a total of 160 sections that were analyzed for cell count.

Immunofluorescence

The fixed tissues were embedded in paraffin. Sections of 6 μm were deparaffinized and hydrated. Nonspecific binding was blocked by incubation in phosphate-buffered saline containing 2% normal donkey serum for 30 min. For immunofluorescence staining, sections were incubated with both polyclonal primary goat and rabbit (or goat and monoclonal mouse) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) in a humidified chamber at 4 °C overnight (1:100 dilution in phosphate-buffered saline with 1% normal donkey serum). For the second antibody, combination of Alexa Fluor donkey anti-goat 488 and donkey anti-rabbit 555 (Invitrogen, Grand Island, NY, USA) were incubated for 60 min at room temperature at a dilution of 1:600 for the anti goat and 1:800 for the anti-rabbit.

For the combination of polyclonal goat and monoclonal mouse, both Alexa Fluor donkey anti-goat 488 and donkey anti-mouse 555 were incubated for 60 min at room temperature at a dilution of 1:600. Finally for nuclear staining, sections were incubated 30 min with DAPI (Sigma-Aldrich, Oakville, Ontario, Canada) then mounted with permanent aqueous mounting media. Before taking the pictures for immunofluorescence, the tissues were first examined under "phase contrast" in order to visualize the various types of cells, then the pictures were acquired with different fluorochromes; DAPI (UV), Alexa Fluor 488 (green) and the Alexa Fluor 555 (red).

Images were captured with a fluorescent microscope (Leica model with software ACDSee, magnification 630×). Superposition of images was performed with Adobe Photoshop software. The following were analyzed BMP2, BMP7, BMP3, BAMBI, noggin, gremlin, pSmad 1/5/8, chordin, Smad-6 and Smad-7.

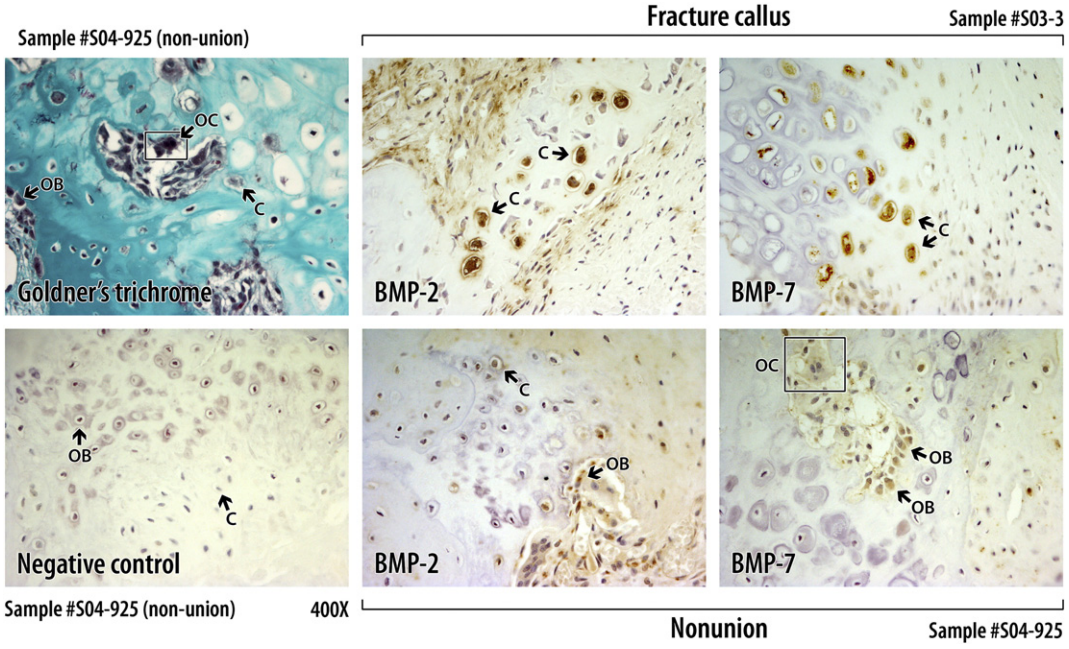

Fig. 1. Histological section (Goldner's trichrome) of fracture callus (upper row) and non-union (lower row). The most significant finding is decreased staining of BMP2 and BMP7 in hypertrophic chondrocytes (C) of a non-union. Also shown, positive staining of BMP2 and BMP7 in osteoblasts (OB) of non-unions, BMP2 in osteoblasts and fibroblasts of fracture callus and of BMP7 in osteoclasts (OC) of a non-union (magnification 400×).

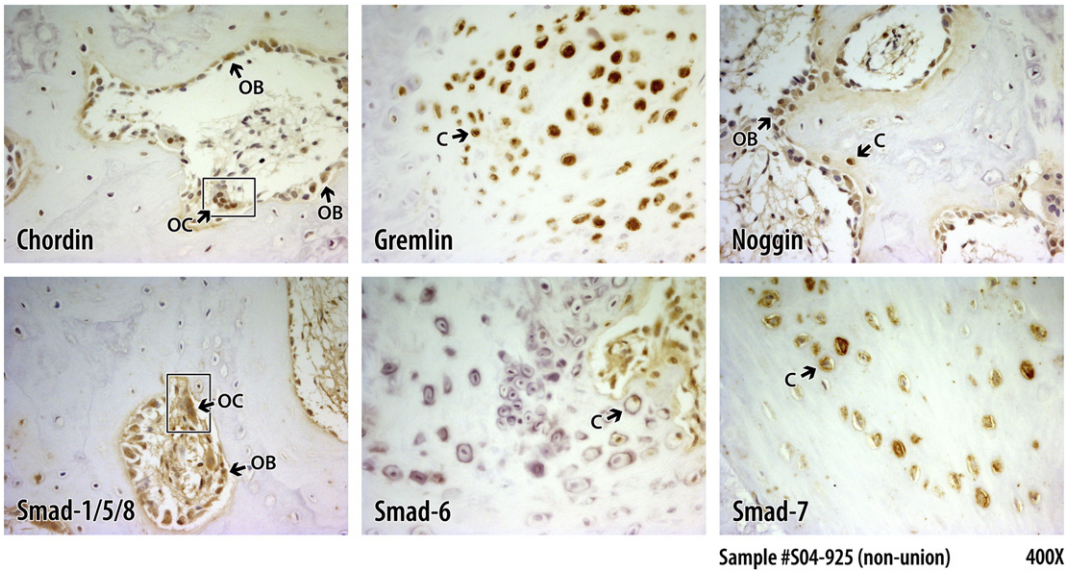

Fig. 2. Histological section showing expression of BMP antagonists and pSmad 1/5/8 in a non-union (magnification 400×). No staining for chordin in hypertrophic and non-hypertrophic chondrocytes (C) and positive staining for chordin in osteoblasts (OB) and osteoclasts (OC). Very strong staining of gremlin in cartilaginous cells (hypertrophic and non-hypertrophic chondrocytes). Staining of noggin in cartilaginous cells (hypertrophic and non-hypertrophic chondrocytes) and osteoblasts. Minimal staining of pSmad 1/5/8 in hypertrophic chondrocytes. Smad-6 and Smad-7 showing positive and negative staining in hypertrophic and non-hypertrophic chondrocytes.

Results

Histology

Similar to our previous work, standard light microscopy of H&E-stained histological sections revealed callus formation at various stages of development in all fracture cases [7]. Most specimens contained a mixture of endochondral and intramembranous ossification. There were also interspersed areas of stroma formed by fibroblast-like cells and areas of new blood vessel formation. We did not attempt to correlate the maturity of the callus with the time since fracture. For ethical reasons we could only remove callus tissue that was

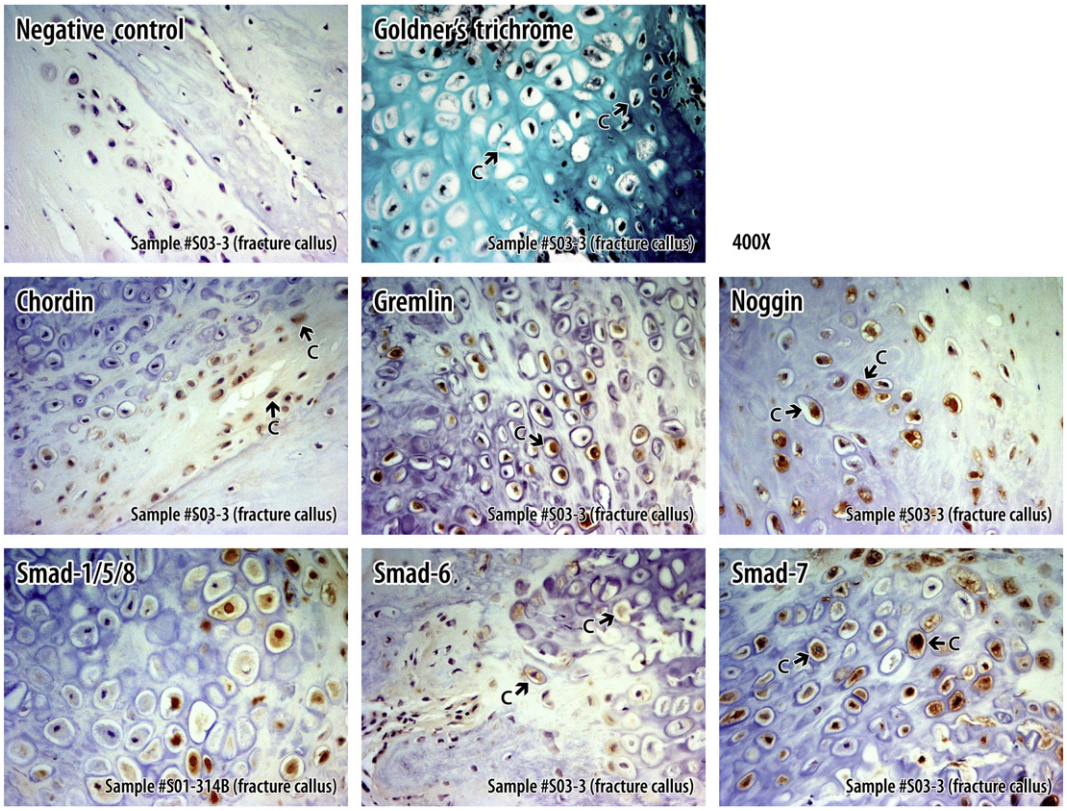

Fig. 3. Histological section showing expression of BMP antagonists and pSmad 1/5/8 in fracture callus (magnification 400×). Chordin showing mixed positive and negative staining in cartilaginous cells (C). Gremlin showing intense staining in hypertrophic chondrocytes. Noggin showing staining in hypertrophic and non-hypertrophic chondrocytes. pSmad 1/5/8 showing mixed negative and positive staining in hypertrophic chondrocytes. Smad-6 showing almost no staining in hypertrophic chondrocytes while Smad-7 showing intense staining in hypertrophic chondrocytes.

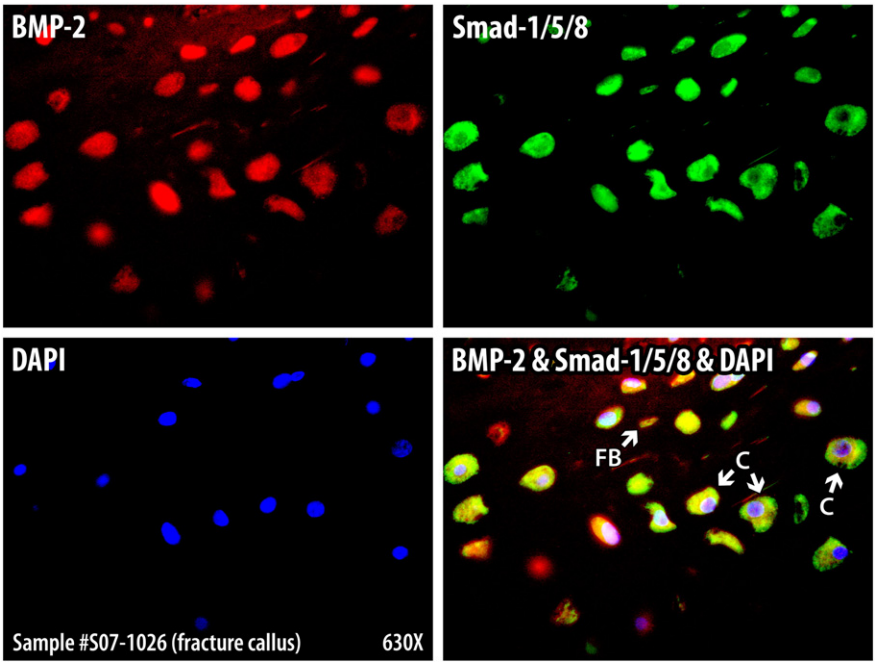

Fig. 4. Immunofluorescent triple staining showing co-localization of BMP2, pSmad 1/5/8 and DAPI in fracture callus in hypertrophic chondrocytes (C) and fibroblasts (FB) (magnification 630×).

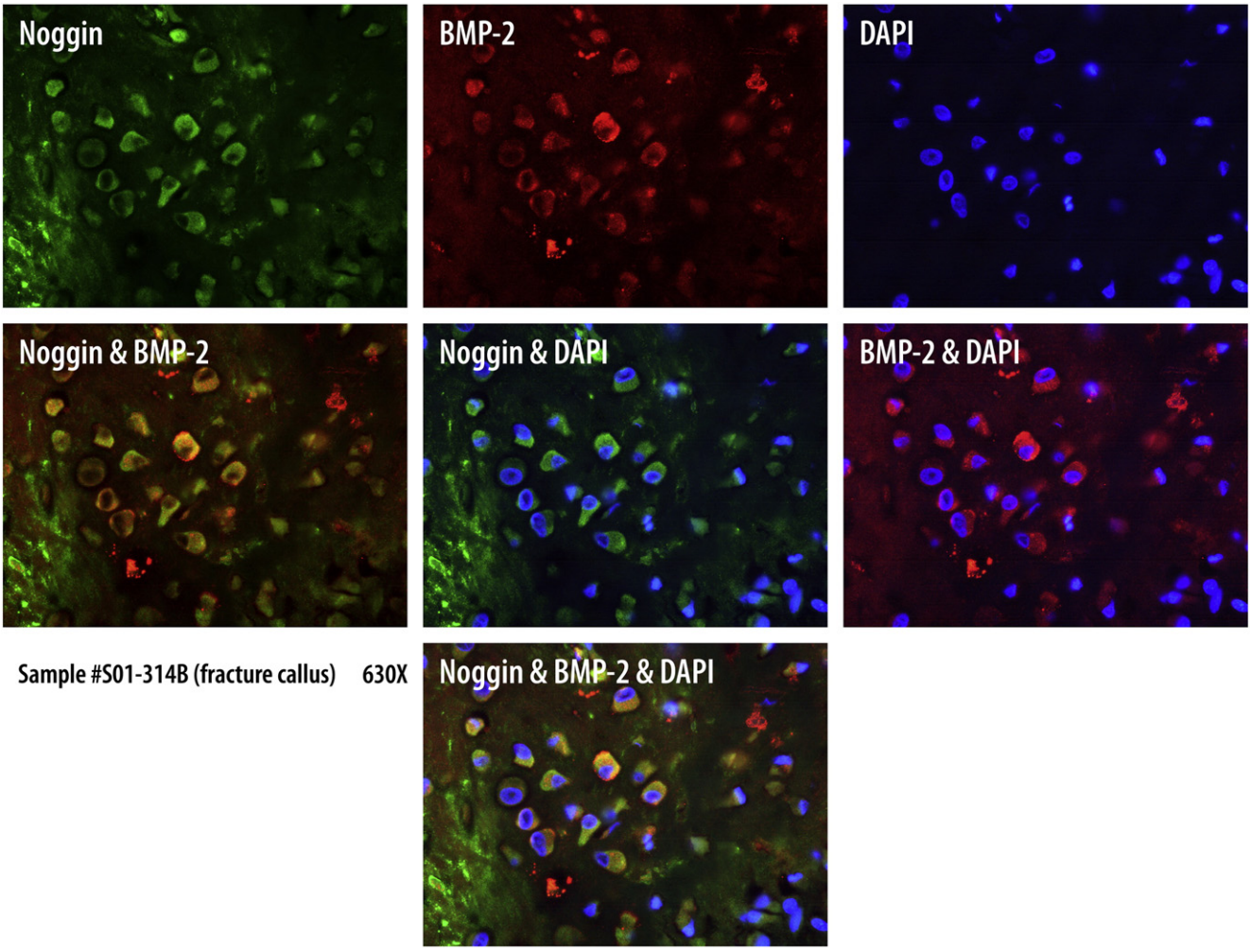

Fig. 5. Immunofluorescent triple staining showing co-localization of BMP2, noggin and DAPI in hypertrophic chondrocytes of fracture callus (magnification 630×).

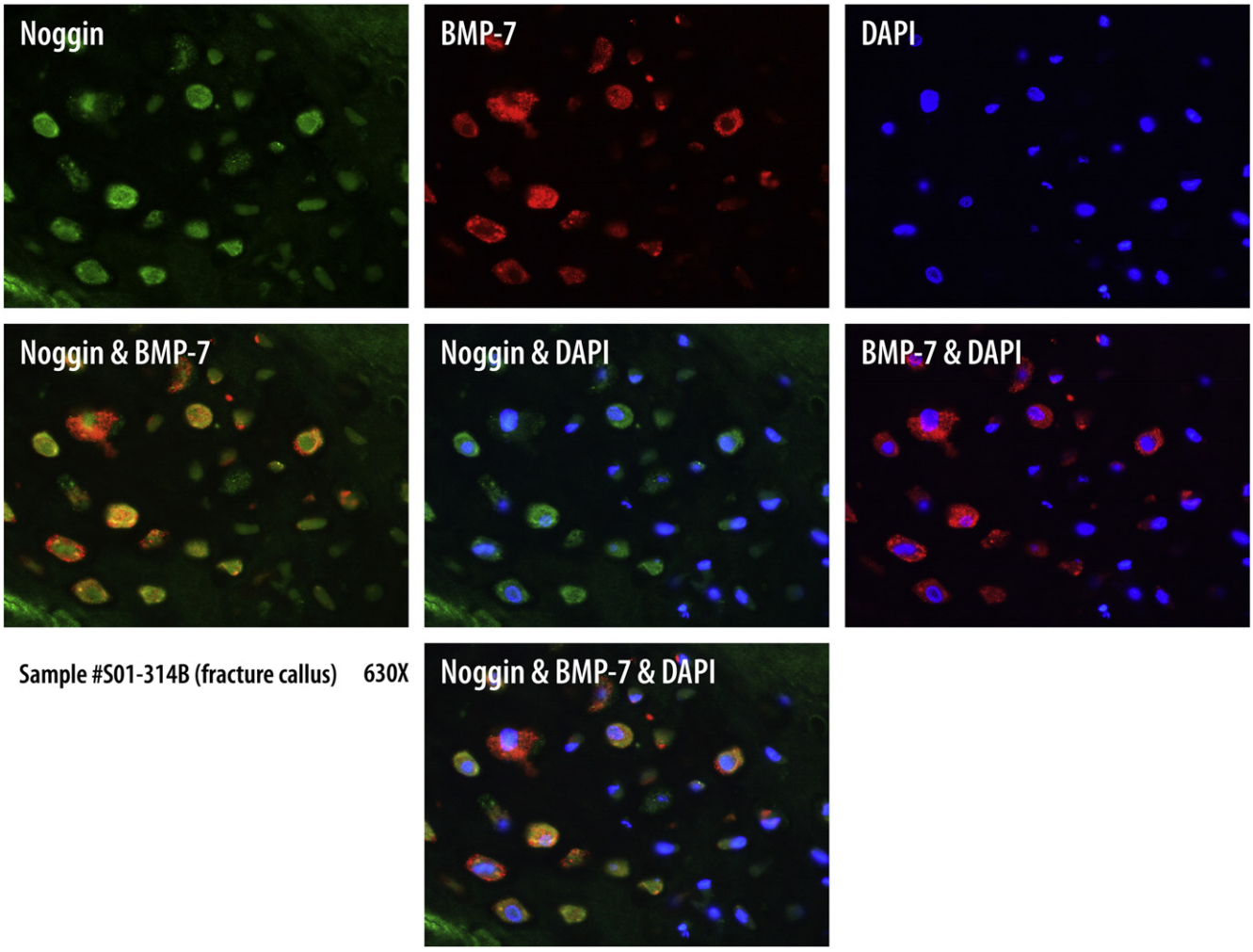

Fig. 6. Immunofluorescent triple staining showing co-localization of BMP7, noggin and DAPI in hypertrophic chondrocytes of fracture callus (magnification 630×).

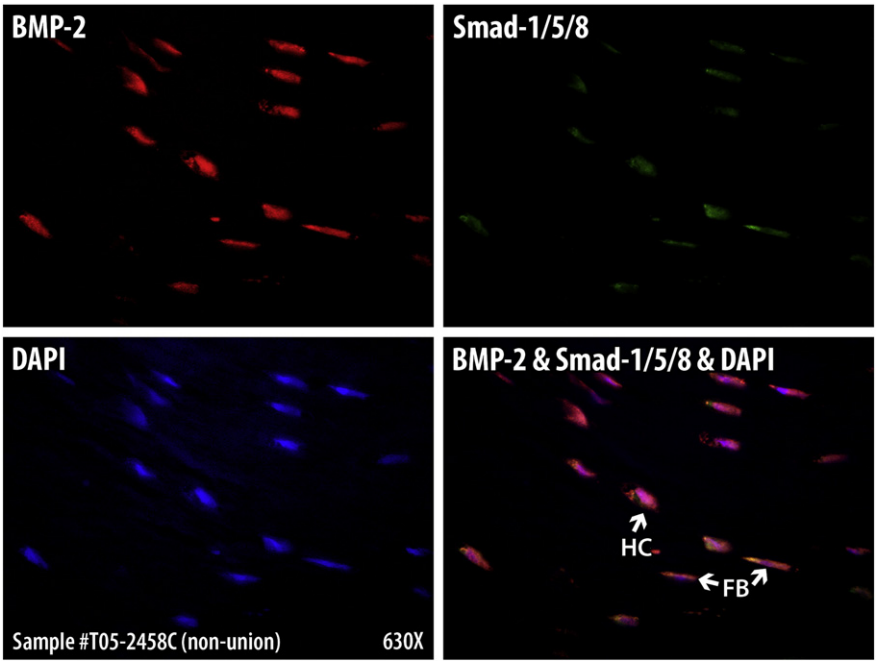

Fig. 7. Immunofluorescent triple staining showing co-localization of BMP2, pSmad 1/5/8 and DAPI in hypertrophic chondrocytes (HC) of a non-union (magnification 630×, FB: fibroblast).

interfering with operative repair of the bone and we could not obtain control tissue from the same patient. Non-unions revealed a mixture of different tissue types. There were foci of woven bone interspersed by areas of fibrous tissue with presence of blood vessels.

Immunohistochemistry

In general, our results showed that expression of BMP-inhibitors was stronger than BMP ligands. In addition, active BMP signaling as exemplified by presence of pSmad 1/5/8 was present in osteoblasts of all specimens, fracture callus and non-union. The main differences were found to be in the chondrocytes and fibroblasts.

Expression of BMP2, BMP7 and pSmad 1/5/8

Overall, our results showed decreased or no expression of BMPs in cartilaginous cells (hypertrophic and non-hypertrophic) of non-unions compared to fracture callus. The expression of BMP2 was decreased in cartilaginous cells (hypertrophic and non-hypertrophic chondrocytes) of non-unions while it was increased in osteoblasts and osteoclasts of non-unions. On the other hand, there was little expression of BMP7 in hypertrophic and non-hypertrophic chondrocytes and fibroblasts of non-unions, but equal expression in osteoblasts and osteoclasts of both groups (Table 2 and Fig. 1). Although the expression of pSmad 1/5/8 was decreased in cases of non-unions compared to fracture callus, it was still present in osteoblasts and hypertrophic chondrocytes of non-unions (Table 2 and Figs. 1–3), confirming our previous report showing active BMP signaling in non-unions [8].

Expression of BMP-inhibitors

The expression of noggin and gremlin was present in all cell types of all specimens. On the other hand, BMP3 (generally referred to as a BMP-inhibitor) and chordin were not expressed in chondrocytes (hypertrophic and non-hypertrophic) of non-unions. Results of the expression of Smad-6 and Smad-7 were mixed. Although both Smad-6 and Smad-7 are inhibitors, their expression did not follow the same pattern. When comparing sections of fracture callus with those of non-unions, our results showed increased expression of Smad-6 in osteoblasts, hypertrophic and non-hypertrophic chondrocytes of non-unions, Smad-7 showed equal expression in osteoblasts of both fracture callus and non-unions, while decreased expression in hypertrophic and non-hypertrophic chondrocytes of non-unions. Representative staining images are shown in Figs. 2 and 3.

Co-staining and co-localization by double and triple immunofluorescence analysis

In general, results of double and triple immunofluorescence staining showed co-localization of BMP ligands with inhibitors, in all sections of both fracture callus and non-unions. There was also decreased staining of BMP2 in the non-unions (representative images are shown in Figs. 4–8). A summary of the expression data is shown in Table 2.

Discussion

The results of this study support our hypothesis that the balance between expression of endogenous BMP ligands and BMP-inhibitors in non-unions is different than in normal fracture healing. Specifically, our results show that in chondrocytes, expression of BMP2 was markedly decreased in non-unions and that of BMP7 was almost completely absent. On the other hand, expression of BMP-inhibitors (noggin, gremlin, Smad-6 and Smad-7) was almost the same in osteoblasts, chondrocytes and fibroblasts of both fracture callus and non-unions. Although these data are consistent with our hypothesis, we had expected that this "imbalance" was due to an increased expression of BMP-inhibitors in non-unions. The current data suggest, however, that it is due to decreased expression of BMPs. In our previous study on delayed and non-unions, we demonstrated that BMP2, BMP4 and BMP7, BMPRs and pSmad 1/5/8 were present in most non-unions in osteoblasts and fibroblasts [8]. However, in that study, we did not specifically analyze the expression of these BMP-related proteins in cartilage cells and we did not compare our findings with those of normal fracture healing.

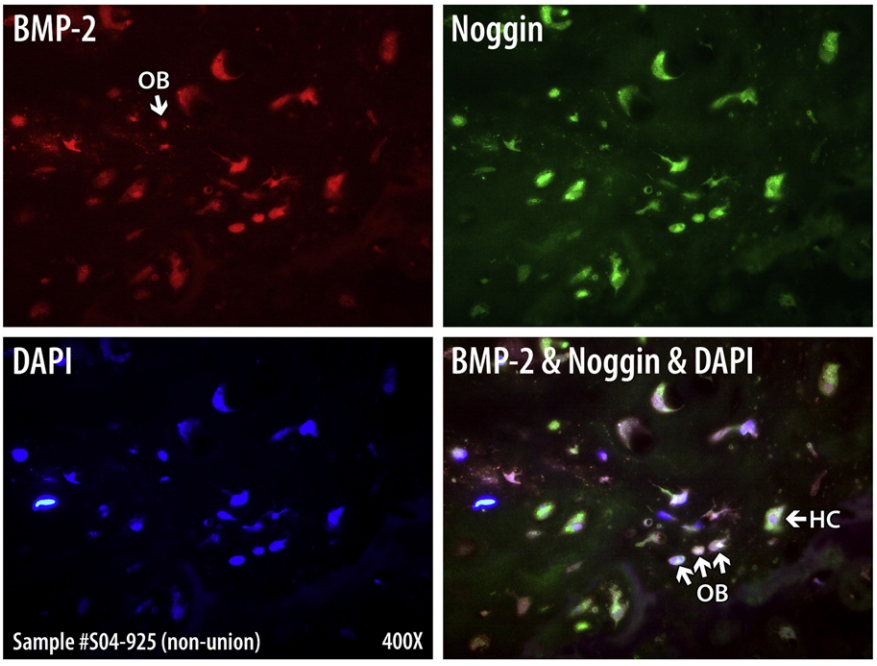

Fig. 8. Immunofluorescent triple staining showing co-localization of BMP2, noggin and DAPI in hypertrophic chondrocytes (HC) of a non-union (magnification 400×, OB: osteoblast).

The concept of imbalance between BMP ligands and their antagonists, being a potential cause of the development of non-unions, was first suggested by Niikura et al. who compared global gene expression for osteogenic BMPs and their inhibitors in a rat fracture and atrophic non-union model [33]. They concluded that non-unions were not accounted for by up-regulation of BMP-inhibitors. Others studies have investigated the same question with various results [27,34–37] (see Tables 3 and 4 for a summary of the current literature on the balance between BMPs and BMP-inhibitors in human and animal fractures and non-unions). Thus, although we and others agree on the presence of a different balance between BMP and BMP-inhibitors in fractures vs. non-unions, there is disagreement on the nature of this "imbalance". Namely, the question remains as to whether the disconnect is caused by a suboptimal expression of BMPs, or by increased presence of BMP-inhibitors, or possibly by both of these factors.

A potential explanation of these differences in expression of BMPs and their inhibitors could be the difference in timing of the non-union analysis, species, location of the non-union and type of non-union (atrophic vs. hypertrophic) and, most importantly, by the complexity and tight control of the BMP signaling pathway. Results of our immunofluorescence studies emphasize the magnitude of this control, where almost all staining for BMP2 and BMP7 was co-localized with BMP-inhibitors, suggesting an intimate interaction between them.

There is enough evidence in the literature that BMP-inhibitors do play a major role in bone healing and formation [38–42]. However, to date, there are no studies evaluating the effects of inhibiting one or more of these inhibitors on fracture healing in humans. We and others have hypothesized that local application of BMPs in humans will lead to a dose-dependent increase in expression of antagonists, limiting their functional therapeutic application [32]. Ideally, using inhibition, we would be able to maximize BMP intrinsic activity and eliminate the need for high – and expensive – exogenous BMP dosing. Furthermore, another advantage of addressing the inhibitors rather than the ligands is that noggin, gremlin and chordin bind to several BMPs [43–45]. This has tremendous therapeutic potential, as pharmacological targeting of any of these inhibitors should up-regulate the expression of not a single but several BMPs. Interestingly, recently BMP variants have been engineered to overcome inhibition by noggin. This has the additional potential to allow development of more effective, second generation BMPs with more potent clinical applicability [43,46].

Inherent weaknesses of the current study are the obvious heterogeneity of the patients, relatively small sample size, the different time to sampling and the variety in location of the fractures and non-unions. Although it is not possible to rule out intrinsic variability in the current data, it is not feasible to obtain a large number of comparable fracture and/or non-unions in similar bones and patients.

The current study, although limited in sample size, has several important and relevant strengths. For example, due to timing of most fracture surgery, which is mostly done within 2–3 weeks after injury, the availability of human fracture callus is notoriously limited. At this early stage, there rarely is any substantial callus that can be removed without ethical concerns. In addition, we used a double and triple staining technique that allows us to document co-expression of the

Table 3
Summary of reports on BMP signaling components in human fracture healing and non-unions.

Author–year	Tissue analyzed	BMPs analyzed	Cells analyzed	Technique	Results
Kloen–2002 [8]	Delayed union (n = 4) Non-union (n = 17)	BMP2, BMP3, BMP4, BMP7, BMPRIA, BMPRIB, BMPRII, pSmad 1/5/8	Fibroblasts Osteoblasts	IHC	All proteins detected in most specimens (17/21) No difference in BMP expression in delayed healing vs. non-unions
Kloen–2003 [7]	Fracture callus (n = 5)	BMP2, BMP3, BMP4, BMP7, BMPRIA, BMPRIB, BMPRII, pSmad 1/5/8	Chondrocytes Fibroblasts Osteoblasts	IHC	Expression of all BMPs in osteoblasts Co-localization of BMPs with receptors in osteoblasts Variable staining in chondrocytes and fibroblasts First to report BMP signaling in human fractures
Kwong–2009 [27]	Fracture callus (n = 15)	BMP2, BMP14, noggin, chordin	Chondrocytes (hypertrophic and non-hypertrophic) Osteoblasts	IHC	Expression of all 4 proteins in endochondral ossification and to lesser extent in osteoblasts First report of BMP-inhibitors in human fractures
Fajardo–2009 [35]	Hypertrophic non-union (n = 15) extracting healing tissue as well as non-union tissue from the same patient	BMP2, BMP4, BMP5, BMP6, BMP7, BMP8, BMPRIA, BMPRIB, BMPRII, noggin, chordin, drm, gremlin, follistatin		qPCR, Western blot, IHC	Up-regulation of BMP4, drm, gremlin, follistatin and noggin in non-unions Up-regulation of BMP7 in healing tissue Suggested that up-regulation of BMP4 and inhibition of BMP7 may play role in non-unions
Kwong–2009 [34]	Fracture callus (n = 8) Non-union (n = 7)	BMP2, BMP14, noggin, chordin	Only cartilaginous tissue examined	IHC	Decreased expression of BMP2 and BMP14 in non-unions No difference in expression of noggin and chordin
Present study–2012	Fracture callus (n = 12) Non-union (n = 4)	BMP2, BMP3, BMP7, pSmad 1/5/8, noggin, chordin, gremlin, BAMBI, Smad-6, Smad-7	Chondrocytes Fibroblasts Osteoblasts Osteoclasts	IHC	In general, stronger expression of inhibitors than BMPs Active BMP signaling in osteoblasts of all sections Decreased expression of BMP2 and absent expression of BMP7 in cartilage cells of non-unions, leading to an different balance between ligand and inhibitor in non-unions compared to fractures

BMPR: BMP receptor; IHC: immunohistochemistry; qPCR: quantitative PCR.

Table 4
Summary of reports on BMP signaling components in animal fracture healing and non-unions.

Author–year	Tissue analyzed	BMPs analyzed	Cells	Technique	Results
Ishidou–1995 [26]	Rat femoral fracture	BMPRIA, BMPRIB	Chondrocytes Fibroblasts Osteoblasts	IHC	Up-regulation of both receptors in all cell types
Onishi–1998 [6]	Rat femoral fracture	BMP2, BMP4, BMP7 BMPRII	Chondrocytes Osteoblasts Osteoclasts	IHC	BMP2 and BMP4 expressed in all stages of both intramembranous and endochondral ossification BMP7 up-regulated in early phases BMPRII co-localized with BMP2, BMP4 and BMP7
Yoshimura–2001 [25]	Mouse rib fracture	noggin, BMP4	Chondrocytes Osteoblasts Periosteal and Endosteal cells	*In situ* hybridization, Northern blotting	Co-localization of BMP4 and noggin was the same in osteoblasts, chondrocytes and hypertrophic chondrocytes First to report noggin in animal model First to suggest that balance between noggin and BMP4 important for fracture healing
Niikura–2006 [33]	Rat femoral fracture Normal healing vs. atrophic non-union	Microarray analysis of >31,000 genes		Microarray, qRT-PCR	Gene expression of BMP2, BMP3, BMP3B, BMP4, BMP6, BMP7, GDF5, GDF7 and noggin, drm, sclerostin and BAMBI was significantly lower in non-unions compared to healing fractures at several time points Concluded that down-regulation of BMP components may account for the non-unions of fractures Balance between BMPs and their antagonists is critical for fracture healing
Yu–2010 [37]	Mouse tibia fracture Stabilized (intramembranous ossification) vs. nonstabilized (endochondral ossification)	BMP2, BMP8, BMPRIA, BMPRIB, pSmad 1/5/8, noggin	Chondrocytes Endothelial cells Osteoblasts Osteocytes, Periosteal cells	IHC	In stabilized fractures BMPs and noggin were detected in isolated inflammatory cells and not detectable in osteoblasts or osteocytes within new bone. In non- stabilized fractures, all BMP components are expressed Concluded that BMP pathway mostly activated during endochondral ossification, and at the chondro-osseous junction
Dean–2010 [36]	Mouse femoral fracture	BMP2, BMP4, BMP7, BMPRIA, BMPRII, noggin, Dan, chordin, Smad-6, BAMBI, PRDC, SOST, Smad-7, GREM1, cerberus		qRT-PCR	Noggin up-regulated early, down-regulated at weeks 2 and 3. Other BMP-inhibitors divided into 2 groups: (1) PRDC, SOST, Smad-7, GREM1, cerberus down-regulated during early fracture healing (2) Dan, chordin, Smad-6, BAMBI up-regulated 7 days after fracture

BMPR: BMP receptor; IHC: immunohistochemistry; qRT-PCR: quantitative real-time PCR; GDF: Growth Differentation Factor; PRDC: protein related to Dan/cerberus; GREM1: gremlin; SOST: sclerostin.

ligands and its inhibitors in the same cell. Rather than using sequential slides, we show the co-expression of BMPs, pSmad 1/5/8 and BMP-inhibitors on the same slide. To the best of our knowledge this has not been described in human or animal fractures and non-unions.

Conclusion and future directions

Our results add to a growing body of evidence suggesting that BMP-inhibitors may play a crucial role in bone healing. The potential of inhibiting the inhibitors is great because of the fact that a single BMP-inhibitor controls several BMPs, which theoretically would allow natural synergy to regenerate bone in a more physiological state. Given this, molecular therapeutics (including gene therapy, small interfering RNAs, neutralizing antibodies and small molecule antagonists) might eliminate the need for high doses of BMPs to stimulate fracture healing [47,48]. The data from this study will help our understanding of the roles of BMPs and their inhibitors in fracture healing, and further develop new strategies for the treatment of delayed and non-unions. Future studies should aim at evaluating the effects of inhibiting BMP-inhibitors on the healing of delayed and non-unions in various (animal) models.

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