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Characterization of a Murine Type IIB Procollagen-specific Antibody

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Abstract

Type II collagen is the major collagenous component of the cartilage extracellular matrix; formation of a covalently cross-linked type II collagen network provides cartilage with important tensile properties. The *Col2a1* gene is encoded by 54 exons, of which exon 2 is subject to alternative splicing, resulting in different isoforms named IIA, IIB, IIC and IID. The two major procollagen protein isoforms are type IIA and type IIB procollagen. Type IIA procollagen mRNA contains exon 2 and is generated predominantly by chondroprogenitor cells and other non-cartilaginous tissues. Differentiated chondrocytes generate type IIB procollagen, devoid of exon 2. Although type IIA procollagen is produced in certain non-collagenous tissues during development, this developmentally-regulated alternative splicing switch to type IIB procollagen is restricted to cartilage cells. Though a much studied and characterized molecule, the importance of the various type II collagen protein isoforms in cartilage development and homeostasis is still not completely understood. Effective antibodies against specific epitopes of these isoforms can be useful tools to decipher function. However, most type II collagen antibodies to date recognize either all isoforms or the IIA procollagen isoform. To specifically identify the murine type IIB procollagen, we have generated a rabbit antibody (termed IIBN) directed to a peptide sequence that spans the murine exon 1-3 protein junction. Characterization of the affinity-purified antibody by western blotting of collagens extracted from wild type murine cartilage or cartilage from *Col2a1*^{+ex2} knock-in mice (which generates predominantly the type IIA procollagen isoform) demonstrated that the IIBN antibody is specific to the type IIB procollagen isoform. IIBN antibody was also able to detect the native type IIB procollagen in the hypertrophic chondrocytes of the wild type growth plate, but not in those of the *Col2a1*^{+ex2} homozygous knock-in mice, by both immunofluorescence and

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immunohistochemical studies. Thus the IIBN antibody will permit an in-depth characterization of the distribution of IIB procollagen isoform in mouse skeletal tissues. In addition, this antibody will be an important reagent for characterizing mutant type II collagen phenotypes and for monitoring type II procollagen processing and trafficking.

Keywords

Type II collagen; antibody; type IIA procollagen; type IIB procollagen; cartilage; chondrocyte

1. Introduction

Type II collagen is one of the most studied and well-characterized matrix components of the cartilage extracellular matrix (ECM). The primary function of type II collagen lies in the development of a specialized ECM that provides cartilage tissue with important tensile properties while the proteoglycans that make up the majority of non-collagenous ECM components (e.g. aggrecan) provide the tissue with compressive properties (Sandell, 2007). Type II collagen is an indispensable fibrillar collagen in cartilage as exemplified by mouse models that lack type II collagen in the ECM (Li et al., 1995; Patra et al., 2011; Yang et al., 1997). It is composed of three identical alpha 1 chains, derived from the *Col2a1* gene in mice (Sandell et al., 1991b) (or the *COL2A1* gene in humans (Ryan et al., 1990)) which assemble to form a triple-helical procollagen molecule that is secreted into the surrounding matrix. Mandatory processing of the procollagen to remove the amino (N-) and carboxy (C-) terminal propeptides results in formation of the mature protein consisting of a triple helical, collagenous domain (containing tripeptide Gly-X-Y repeats where X and Y are frequently proline or hydroxyproline) and short, non-collagenous N- and C-terminal telopeptide domains (von der Mark, 2006). These processed type II collagen triple helical molecules are then covalently cross-linked to each other as well as to other minor cartilage collagens (type IX and XI collagens) to form stable heterotypic fibrils in the ECM (Eyre et al., 2002).

The gene structure of type II collagen is interesting. The procollagen is encoded by 54 exons, but exon 2 is known to be alternatively spliced in a developmentally-regulated manner. Specifically, chondroprogenitor cells synthesize type IIA procollagen mRNA containing exon 2, while differentiated chondrocytes generate mainly the type IIB procollagen isoform, devoid of exon 2 (Ryan and Sandell, 1990; Sandell et al., 1991a). Recently, other type II procollagen transcripts have been identified that differ from IIA mRNA by the inclusion of an additional three nucleotides at the 3' end of exon 2 (the IID isoform) or from utilization of an alternative 5' splice site within exon 2 to generate a truncated mRNA (the IIC isoform) (McAlinden et al., 2008). The IID isoform has been found to be expressed in human mesenchymal stem cells and ATDC5 cells undergoing chondrogenesis (McAlinden et al., 2008), but has not been detected *in vivo* in mouse cartilage tissue (McAlinden et al., 2012). No IIC protein isoform has been detected since the truncated IIC mRNA contains premature stop codons and is likely degraded by nonsense-mediated decay mechanisms (McAlinden et al., 2008). While the transition from type IIA to type IIB procollagen during chondrocyte differentiation was thought to be essential for overt

cartilage development, recent analysis of a knock-in mouse model expressing predominantly the embryonic IIA isoform (*Col2a1*^{+ex2} mice) have suggested otherwise (Lewis et al., 2012).

The detection of persistent type IIA procollagen protein expression in *Col2a1*^{+ex2} heterozygous and homozygous mice was possible by the use of the well-characterized IIA antibody (Oganesian et al., 1997), which specifically recognizes the exon 2-encoded cysteine-rich domain in the N-propeptide of the type IIA procollagen. At the mRNA level, the design of specific oligonucleotide probes has permitted the localization and distinction of IIA and IIB mRNA transcripts by *in situ* hybridization (Sandell et al., 1994). However, as type IIB protein differs from IIA only by the exclusion of the exon 2 coded sequences (exon 1 is spliced directly to exon 3 that also introduces a new amino acid at the junction formed by sequences derived from both exons 1 and 3) there has been no antibody that can specifically detect the type IIB procollagen protein isoform in mouse. An antibody that can specifically detect the human type IIB procollagen was recently reported (Aubert-Foucher et al., 2013). In this report we present the characterization of the IIBN antibody that can specifically detect the murine type IIB procollagen. This antibody was directed to a peptide sequence that spans the unique exon 1-3 protein junction in mice. Using both wild type (+/+) and the *Col2a1*^{+ex2} mouse skeletal tissues we have demonstrated the ability of this antibody to distinguish between types IIA and IIB procollagen isoforms. This antibody was also able to detect the native type IIB procollagen in the chondrocytes of the hypertrophic zone in the wild type growth plate by both immunofluorescence and immunohistochemical applications. This IIBN antibody will be useful in the characterization of mutant mouse phenotypes with disruptions in type II collagen synthesis or processing, and is likely to augment our knowledge on the dynamics of type IIB procollagen-specific trafficking from the endoplasmic reticulum to the cartilage ECM.

2. Results

To create a murine type IIB procollagen-specific antibody, we took advantage of the unique peptide junction which is created when exon 1 is spliced to exon 3 during the formation of type IIB messenger RNA. This is the only portion of the protein that is unique to the type IIB isoform and does not exist in type IIA, IIC, or IID collagen spliced variants. The exon 1-3 junction peptide sequence QGQDARKLGP, where QGQDA is specific to exon 1 and KLGP to exon 3 with R forming at the splice junction from DNA sequences contributed by both exons, was used to immunize rabbits after coupling to KLH using a cysteine added to the amino terminus of the peptide. Figure 1 shows the primary structures of type IIA and IIB procollagen isoforms and the antibodies used in the laboratory for their detection.

To analyze the properties of the exon 1-3 junction antibody, termed IIBN, and its specificity for the type IIB procollagen isoform, the antibody was affinity purified from rabbit sera and first analyzed in western blot applications. Figure 2 shows IIBN antibody tested in a western blot to study its specificity to the type IIB procollagen isoform. Epiphyseal cartilage was harvested from the limbs of embryonic (E) 17.5 wild type (+/+), *Col2a1*^{+ex2} homozygous (ki/ki) and the *Col2a1*^{+ex2} heterozygous (ki/+) mice. In embryonic skeletal tissues, the type IIA procollagen isoform is synthesized primarily by chondroprogenitor cells while differentiated chondrocytes splice exon 1 to exon 3 forming predominantly type IIB

procollagen isoform. In the transgenic knock-in *Col2a1^{+ex2}* ki/ki mice constitutive splicing of exon 2, rather than alternative splicing, results in predominant expression of type IIA procollagen even in differentiated chondrocytes (Lewis et al., 2012). Thus, the *Col2a1^{+ex2}* ki/ki mouse model is a good control to investigate the type IIB procollagen specificity of the IIBN antibody.

Protein extracts from E17.5 epiphyseal cartilage were processed in a reducing 8% SDS-PAGE and analyzed by western blotting using IIF, IIA, and IIBN antibodies (Fig. 2). *Col2a1^{+ex2}* heterozygous (ki/+) E17.5 pups were also included in the analysis; these mice generate both type IIA and IIB procollagen isoforms (Lewis et al., 2012). The IIF antibody (Fig. 2A) strongly detected the mature, fully processed, type II collagen [$\alpha 1(\text{II})$] from all three genotypes as expected as this antibody detects the type II collagen fibrillar or triple-helical domain (THD). Unequivocal identification of the fully processed $\alpha 1(\text{II})$ form was done by simultaneously analyzing pepsin cleaved type II collagen as a control in the SDS-PAGE (not shown). The IIA antibody, specific to the exon 2 coded cysteine-rich domain of type IIA procollagen, strongly detected the pN procollagen isoform [pN $\alpha 1(\text{IIA})$] and the type IIA procollagen [pro $\alpha 1(\text{IIA})$] in the *Col2a1^{+ex2}* ki/ki mice as expected, and to a slight extent in the ki/+ mice (Fig. 2B). The IIBN antibody strongly detected the type IIB procollagen [pro $\alpha 1(\text{IIB})$] and the pN $\alpha 1(\text{IIB})$ in wild type cartilage extracts (Fig. 2C). In heterozygous ki/+ cartilage extracts, where the expression of both type IIB specific procollagen and pN $\alpha 1(\text{IIB})$ would be expected to be reduced by half, the detection of these two isoforms is reduced when compared to that seen in the wild type (+/+) mice, thus validating the identity of these bands as being type IIB-specific. Importantly, in extracts derived from the *Col2a1^{+ex2}* ki/ki mouse, no type II collagen bands were detected by IIBN. Comparison of the protein bands in the western blot analysis from the wild type, ki/ki, and ki/+ mice demonstrated that the IIBN antibody is indeed type IIB procollagen-specific.

In order to confirm the validity of the IIBN antibody, we repeated the analysis on extracted collagens from the cartilage tissue of postnatal 14 day old (P14) mice and similar results were observed (Fig. 3). The IIF antibody detected the fully processed mature type II collagen from the wild type and ki/ki mice (Fig. 3A) and the IIA antibody detected the pro $\alpha 1(\text{IIA})$ and pN $\alpha 1(\text{IIA})$ forms strongly in the ki/ki mice as expected (Fig. 3B), but also in the wild type. However, the IIBN antibody again detected the pro $\alpha 1(\text{IIB})$ and pN $\alpha 1(\text{IIB})$ forms only in the wild type but not in cartilage extracts from ki/ki mice (Fig. 3C). Thus these data indicated that we were able to raise a type IIB procollagen specific antibody that does not react with the type IIA procollagen isoform or to the mature, fully processed type II collagen.

Next, we analyzed the specificity of IIBN antibody in immunofluorescence applications to study the distribution of type IIB procollagen *in vivo*. We analyzed IIBN antibody in the femur of E17.5 wild type (+/+) and ki/ki mice and compared signals from IIBN to that seen with IIF and IIA antibodies in double-labeled immunofluorescence applications (Fig. 4). In the composite image showing both IIA and IIF signals (Fig. 4A), the mature matrix showed signals primarily from the IIF antibody (in red, detecting the type II collagen fibrillar domain) in the proliferative region, with those overlapping with IIA seen as yellowish due to colocalization of the signals. The IIA antibody strongly detected the presence of the exon 2-

containing type IIA procollagen shown as a lattice network in green in the matrix (Fig. 4B). However, when we analyzed type II collagen distribution using the IIBN antibody in combination with IIF in the wild type growth plate (Fig. 4C), we were not able to see any intracellular signals or any signals in the matrix, whether in the resting or the proliferating zone. The wild type (Fig. 4C) and *ki/ki* (Fig. 4D) mice looked identical in this respect. Figures 4E, F show a higher magnification image of the resting zone and Figures 4G, H show a higher magnification of the proliferative zone in the wild type demonstrating that all type II collagen signals in these two zones are from the fibrillar/THD domain detected by the IIF antibody in the cartilage ECM. No signals from IIBN were seen in the resting or the proliferating zones in the wild type or *ki/ki* growth plate.

Interestingly, signals from IIBN were detected in the hypertrophic zone of the wild type growth plate (Fig. 4C, D). These signals were seen as intracellular clumps of type IIB procollagen in the hypertrophic chondrocytes. Figures 4I, J show higher magnification images of the hypertrophic zone in the wild type which demonstrated that signals from IIBN often colocalized with signals from IIF in the hypertrophic chondrocytes. In the insets in Figures 4I, J, signals from IIF (in red) are seen surrounding the signals from IIBN (yellowish in Fig. 4I inset due to the colocalization of IIF and IIBN signals, and green in Fig. 4J inset when only IIBN signals are shown). These data indicated that IIBN is detecting the native type IIB procollagen in the hypertrophic chondrocytes of the wild type growth plate. These observations were further validated by the fact that these signals were absent in the hypertrophic cells of the *ki/ki* mice (Fig. 4D, K, L), indicating that this phenomenon is specific to the wild type chondrocytes. These data indicated that IIBN antibody does not cross-react with other type II collagen isoforms, i.e. with the type IIA procollagen or with the type II fibrillar domain validating the results from the western blot analyses (Fig. 2, 3); these data also indicated that IIBN can be used in immunofluorescence applications to detect the native type IIB procollagen in chondrocytes. However, these results also suggest that IIBN cannot detect type IIB procollagen in the proliferative zone, presumably due to the rapid processing of the procollagen molecule to create the cartilage matrix as demanded by the rapid growth rate of the skeletal structure in mice.

In order to confirm the specificity of IIBN to cartilage, we tested IIBN in immunohistochemical applications on E15.5 femur in wild type mice. As seen in the immunofluorescence studies, no signals from IIBN were detected in the resting to the proliferating zone (Fig. 5A), but positive signals were detected from the chondrocytes in the hypertrophic zone, shown under higher magnification in Figure 5B. A panoramic view of the entire femur and surrounding tissues (Fig. 5A) demonstrated that IIBN does not cross-react with the cortical bone, endochondral bone, or other surrounding tissues. The lack of signals from bone, tendon and other tissues indicated that IIBN antibody does not cross-react with type I or type V collagens either. In summary, these data indicate that the IIBN antibody is specific for type IIB procollagen isoform and can be used in immunofluorescence and immunohistochemical studies to detect the native type IIB procollagen.

3. Discussion

In this report, we describe the generation and characterization of the murine type IIB procollagen-specific antibody, IIBN. IIBN was made against a peptide sequence that spanned the murine exon 1-3 protein junction unique to the type IIB procollagen isoform, and was raised in rabbits. As dictated by antibody design and the nature of type IIB procollagen processing, IIBN is specific to the unprocessed procollagen forms pro α 1(IIB) and pN α 1(IIB) which contain the amino-propeptide as part of their procollagen structure. As the mature type II collagen is incorporated into the cartilage ECM, it is processed to remove both the N- and C-propeptides. Consequently, IIBN antibody does not recognize the fibrillar (the triple helical domain) type II collagen and therefore no positive antibody signals are found in the ECM. This observation is validated by both the lack of signals in western blots where IIBN did not recognize the fully processed α 1(II) and its lack of colocalization with signals from both IIA and IIF antibodies in immunofluorescence applications. The ability to detect procollagen bands only in wild type (+/+) and *Col2a1*^{+ex2} heterozygotes in western blots and its inability to recognize any collagen bands in cartilage extracts from *Col2a1*^{+ex2} homozygous ki/ki mice (which synthesizes predominantly the type IIA procollagen isoform) confirmed the type IIB procollagen-specificity of the IIBN antibody. Both immunofluorescence and immunohistochemical analysis also demonstrated that IIBN does not cross-react with fibrillar collagens in other tissues of the limb such as the cortical bone, endochondral bone, tendon or the surrounding musculature.

While western blots clearly demonstrated the presence of type IIB procollagen in both embryonic and postnatal cartilage, IIBN was unable to detect any intracellular type IIB procollagen by immunohistochemical or immunofluorescence applications in the resting and proliferative zones of the wild type (+/+) growth plate. These findings indicate that chondrocytes are able to rapidly traffic type IIB procollagen from the endoplasmic reticulum to the cartilage ECM which likely reduces procollagen levels to below the detection level of the antibody under these circumstances. No signals from IIBN were seen in the ECM either where the mature fibrillar type II collagen domain was detected, which may suggest a rapid removal of the N-propeptide from the procollagen molecule. The simultaneous use of the IIF and IIA antibodies in immunofluorescence studies had the advantage that we could analyze the relative distribution of the mature, processed type II collagen and exon 2 containing collagen sequences. The type IIA procollagen is more easily detected in the cartilage matrix as it is likely retained longer in the cartilage ECM as demonstrated in previous studies (Zhu et al., 1999).

Surprisingly, in the normal wild type growth plate we detected the native type IIB procollagen in chondrocytes in the hypertrophic zone. Hypertrophic chondrocytes are unique in that they predominantly synthesize and secrete massive amounts of type X collagen, in addition to other ECM components, into the matrix of the hypertrophic zone (Poole, 1991). It is therefore possible that the rates of type IIB procollagen transcription and translation are altered, possibly lowered, in the hypertrophic zone which decreases its secretion into the matrix allowing its detection. It is also possible that excessive secretion of type X collagen disrupts the type II collagen secretion machinery thereby making it feasible to detect the type IIB procollagen isoform only in the hypertrophic chondrocytes of the wild type growth

plate. While it is not surprising that no signals from IIBN were detected in the resting to the hypertrophic zones in the ki/ki growth plate, no type II collagen intracellular clumps were detected by the IIF antibody in the hypertrophic chondrocytes either. This indicated that type IIA procollagen is processed, trafficked, and/or cleared from the hypertrophic zone by a different mechanism or at a different rate than type IIB procollagen.

It has been suggested that type IIB procollagen is processed outside the cell in the cartilage ECM where the N-propeptide is removed (von der Mark, 2006). We have not detected any signals from the matrix that would suggest the presence of the free N-propeptide in the matrix. One possible reason for this lack of detection could be that the N-propeptide, if present in the matrix, is somehow masked due to its small size in the milieu of the mature, fibrillar type II collagen domain and other ECM proteins. Yet using IIBN antibody we have not been able to see any signals by western blot analysis (expected molecular weight of approximately 9 kD after removal of the signal peptide, assuming no posttranslational modifications) when the cartilage preparations were processed by a reducing 15% SDS-PAGE, although an approximately 19 kD band was easily detected using the exon2-specific IIA antibody or the anti-IIE3-8 antibodies (not shown). This suggests that the type IIB procollagen-derived N-propeptide either has a very short half-life or is rapidly processed further that, at the very least, removes the exon 1-3 junction epitope. Incidentally, most of exon 1 codes for the signal sequence required for entry into the endoplasmic reticulum and is removed rapidly during translation and these sequences are not present on the fully synthesized procollagen. This makes the epitope recognized by IIBN present at the very N-terminus of type IIB procollagen, probably making it easier for further modification or degradation. Processing of the type IIA procollagen-derived free N-propeptide by matrix metalloproteinases (Fukui et al., 2002) has been observed suggesting that the type IIB-derived N-propeptide could be affected similarly in the matrix which would remove or mask the IIBN antibody epitope.

Another possibility for lack of IIBN antibody signals in the matrix is that the free, type IIB-derived N-propeptide is not secreted into the ECM and/or is rapidly absorbed back into the cell and processed. Alternatively, the free IIB-derived N-propeptide may be very soluble that prevents interactions with other cartilage ECM molecules required for detection by an antibody. To date, the mechanism and location of type II procollagen N-propeptide processing and type II collagen trafficking has not been fully established. The biological importance of the type IIB procollagen derived N-propeptide *in vivo* also remains to be explored, though the recombinant form has demonstrated an ability to kill tumor cells (Wang et al., 2010) and osteoclasts (Hayashi et al., 2011). However, the ability of IIBN antibody to detect type IIB procollagen in the hypertrophic chondrocytes of the murine growth plate demonstrates its potential to shed light on both normal and aberrant type II procollagen trafficking in chondrocytes.

4. Experimental Procedures

4.1 Antibody design, production, and purification

A polyclonal antibody termed IIBN, directed to the exon 1-3 protein junction in the murine type IIB procollagen was generated in rabbits. In mouse, the exon 1 coded amino acid

sequence is MIRLGAPQSLVLLTLLIAAVLRCQGQDA**Q** (Q, shown in bold, is formed with sequences contributed by both exons 1 and 2) and the exon 3 coded amino acid sequence is KLGP**K**. When exon 1 is spliced to exon 3 however, the splicing results in the sequence MIRLGAPQSLVLLTLLIAAVLRCQGQDARK**KLGP**K with arginine (R) (shown in bold) forming at the junction due to one nucleotide contributed by exon 1 (from the glutamine (Q) codon) and two contributed by exon 3 (underlined sequences denote exon 3 contributed amino acids), and hence the elimination of glutamine (Q) from the junction sequence. To design an antibody that would recognize this type IIB procollagen-specific epitope derived from the amino acids present at the exon 1-3 junction, we chose the smallest peptide possible for rabbit immunization which would also be reasonably big enough to be immunogenic. Therefore rabbits were immunized with the peptide C-QGQDARKLGP, with the cysteine added at the amino terminus of the peptide for efficient conjugation to KLH as a carrier for rabbit immunization.

Immune and non-immune serum from immunized rabbits were tested initially on chondrocyte cell lysates in western blots and a band at the approximate size for type II procollagen was detected on the blot, indicating appropriate immunization design and protocol. To allow for efficient characterization, IIBN antibody was affinity purified. For this, the C-QGQDARKLGP peptide was conjugated to a Sulfolink column (ThermoFisher) following the manufacturer's directions. To reduce non-specific antibody reactions, additional peptides with sequences derived from exon 1 (C-AAVLRCQGQ) and exon 3/4 (LGPKGQKGE-C), which border the exon 1-3 junction sequences were conjugated jointly to a separate Sulfolink column (subtracting column). For affinity purification of IIBN, the rabbit immune serum was first incubated with the subtracting column. The subtracted serum was then incubated with the immunizing peptide-conjugated Sulfolink column overnight at 4°C, washed several times with phosphate buffered saline (PBS) (pH 7.4), and IIBN eluted with 100 mM Glycine (pH 2.5), neutralized to pH 7 with 1 M Tris pH 8.0, and stored at -80°C. Concentration of IIBN antibody was determined colorimetrically by using Bio-Rad protein assay dye reagent and bovine serum albumin standards.

4.2 Western Blot Analysis

For western blot analyses, epiphyseal cartilage was harvested from embryonic (E) 17.5 and postnatal 14 day old (P14) wild type (+/+), *Col2a1*^{+ex2} homozygous (ki/ki) and heterozygous (ki/+) mice (Lewis et al., 2012). At E17.5, the chondroepiphyseal cartilages were harvested from one half of the embryo (i.e. one forelimb and one hindlimb), snap frozen in liquid nitrogen and stored at -80°C. The other half was formalin fixed and paraffin embedded for immunofluorescence and immunohistochemistry studies. After genotyping to identify the wild type, ki/ki, and ki/+ mice, the epiphyseal cartilage was ground over dry ice, suspended in a reducing (with dithiothreitol) SDS gel loading buffer, heated to 100°C for 5 min and loaded equally between four different lanes. For P14 mice, the epiphyseal cartilage was harvested from all limbs of a 14 day old mouse, snap-frozen in liquid nitrogen and stored at -80°C. After genotyping, the cartilage was ground over dry ice, suspended in a reducing SDS gel loading buffer, heated to 100°C for 5 min and approximately 1.5 mg of cartilage equivalent was loaded per lane in a 8% SDS-polyacrylamide gel (SDS PAGE). Following SDS-PAGE, western blot analyses using IIF, IIA and IIBN antibodies were

performed using standard protocols. To allow unequivocal identification of the fully processed $\alpha 1(\text{II})$ chains, pepsin-cleaved type II collagen was analyzed in the same gels and blots processed by the IIF antibody. The IIF (rat anti-type II collagen sera which recognizes epitopes on the triple helical collagenous domain (THD), see Figure 1) and IIA (rabbit serum specific to exon 2 sequences) antibodies were used at a dilution of 1:1000 and the affinity purified IIBN antibody was used at a dilution of 1 $\mu\text{g}/\text{ml}$; the corresponding species-specific secondary antibodies were used at dilutions of 1:2000, 1:3000, and 1:4000, respectively. Signals were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

4.3 Immunofluorescence and immunohistochemistry

For immunofluorescence studies, the sections were first rehydrated through a series of washes with decreasing ethanol concentrations and finally washed in PBS pH 7.4. Antigen retrieval was done using Proteinase K (10 $\mu\text{g}/\text{mL}$ in 10 mM Tris-HCl pH 7.5) treatment for 20 minutes at 37°C. After rinsing with PBS, the tissue was blocked with 10% normal goat serum followed by overnight incubation with the primary antibodies. For double-labeled immunofluorescence experiments, IIBN antibody was used at 5 $\mu\text{g}/\text{mL}$ diluted using 2% normal goat serum; the IIF antibody was used at a dilution of 1:100 and the IIA antibody at 1:400 in 2% normal goat serum. The next day, sections were rinsed with PBS and then incubated for 1 hour with fluorescent anti-rabbit or anti-rat secondary antibodies (diluted 1:250 using 2% normal goat serum). Following washings in PBS, the sections were coverslipped using Vectashield mounting media containing DAPI. The images were captured using a 60X, 1.4 NA oil immersion objective mounted on an Eclipse E800 microscope (Nikon) and QImaging Retiga 2000R Fast 1394 camera. Z stack images were captured and deconvolved to reduce extraneous glare using Metamorph software and the stacks compiled to a single image using Helicon Focus software.

For immunohistochemistry, following graded ethanol and PBS washes, the sections were treated with 3% hydrogen peroxidase in chilled methanol, washed with PBS, and antigen retrieval done with Proteinase K as above. Following washings with PBS, the sections were then treated with IIBN antibody (5 $\mu\text{g}/\text{mL}$ diluted in 2% normal goat serum) overnight followed by HRP-conjugated secondary antibody as above, and signals detected using DAB (3, 3'-diaminobenzidine tetrahydrochloride) substrate. Tartrazine yellow was used as the counter stain. Images were captured as above on an Eclipse E800 microscope.

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Highlights

1. Type II collagen is a major component of the cartilage extracellular matrix.
2. Type II collagen is expressed in two major isoforms: type IIA and type IIB procollagen.
3. Existing antibodies against type II collagen do not specifically detect the murine type IIB procollagen.
4. The IIBN antibody is directed to the exon1-3 protein junction in the murine type IIB procollagen.
5. IIBN antibody is specific to the murine type IIB procollagen in western blots, immunofluorescence and immunohistochemistry applications.

A. Type IIA procollagen **B. Type IIB procollagen** **C. Sequence of the exon 1-3 peptide junction in type IIB procollagen** **Figure 1.**

Type II procollagen protein isoforms and the antibodies that detect them. A schematic showing the primary structures of type IIA procollagen (**A**) and its splice variant, type IIB procollagen (**B**) is shown. The N-propeptide is coded by exons 1-8 which are sequentially spliced to each other to form the N-propeptide of the type IIA procollagen. (**B**) In type IIB procollagen, the exon 2-derived 69 amino acid sequence is removed due to the splicing of exon 1 to exon 3. (**C**) The amino acid sequence of the exon 1-3 protein junction in the type IIB procollagen is shown. Sequences specific to exon 1 and exon 3 are shown underlined. The arginine (R) forms at the new junction with sequences derived from both exons due to the nature of the splicing. This peptide sequence, with a cysteine added to the amino terminus for KLH conjugation, was used to raise antibodies in rabbits against the type IIB-specific splice variant. Below each protein isoform are given the names of the antibodies that detect the different structural domains of the type II procollagens. The IIA antibody generated against exon 2 is specific to the type IIA procollagen isoform (Oganesian et al., 1997), while the IIF (the fibrillar antibody against the type II collagen triple helical domain (THD)) and the anti-IIE3-8 (against protein sequences coded by the exons 3-8) (Fukui et al.,

2002) antibodies detect both the type IIA and IIB procollagen isoforms. The unique junction formed due to splicing of the exon 1 to exon 3 provides the only unique structural determinant, which is a potential epitope for specific type IIB procollagen detection.

Figure 2.

Analysis of the IIBN antibody in western blots. The chondroepiphyseal cartilage was harvested from the limbs of E17.5 wild type (+/+), the *Col2a1*^{+ex2} homozygous knock-in mouse (ki/ki), and the *Col2a1*^{+ex2} heterozygous (ki/+) mouse. Proteins were extracted by heating the ground cartilage with SDS loading buffer containing DTT, and a 8% SDS-PAGE followed by western blot analysis using the antibodies indicated was performed. The asterisks in panel C point to non-specific bands, which allowed for loading controls between the lanes. (THD: triple helical domain). See Figure 1 for a description of the antibodies.

Figure 3.

The IIBN antibody is specific to type IIB procollagen. The specificity of IIBN antibody was validated by western blot analysis of cartilage proteins extracted from P14 mice. The chondroepiphyseal cartilage was harvested from the limbs of P14 wild type (+/+) and *Col2a1^{ex2}* knock-in mouse (ki/ki), proteins extracted by heating the ground cartilage with SDS loading buffer containing DTT, and a 8% SDS-PAGE followed by western blot analysis was performed using the antibodies indicated. The asterisks in panel C point to non-specific bands.

Figure 4.

Analyses of collagen distributions using IIF, IIA, and IIBN antibodies by immunofluorescence. **(A, B)** Double-labeled immunofluorescence analyses with IIF and IIA antibodies in E17.5 wild type (+/+) femur shown either as a composite of IIF plus IIA signals **(A)** or with signals from IIA only **(B)**. Images are shown for the proliferating zone in the distal growth plate. **(C, D)** Double-labeled immunofluorescence analyses in the growth plates of the distal femur with IIF and IIBN antibodies in E17.5 wild type **(C)** and *ki/ki* **(D)** mice. Type II collagen distribution from the resting zone to the hypertrophic zone is shown. Higher magnification images with composite signals from IIF plus IIBN, or signals from IIBN only, are shown for the resting zone **(E, F)** and the proliferative zone **(G, H)** in the wild type femur. Higher magnification images of the hypertrophic zone in wild type **(I, J)** and *ki/ki* mice **(K, L)** with composite signals from IIF plus IIBN **(I, K)**, or signals from IIBN only **(J, L)**, are shown. Arrows in panels **I** and **J** indicate regions where signals from IIF and IIBN colocalize. Insets in panels **I** and **J** show a higher magnification of this colocalization indicated by the arrow marked with an asterisk. Notice the yellowish colocalization signals in the inset in panel **I** surrounded by the red signals from IIF; the corresponding inset in panel **J** shows the same image but with signals from IIBN (in green) only. Colors represent antibody localizations as follows: green = IIA antibody detecting type II collagen exon 2 coded sequences **(A, B)**, or IIBN antibody detecting the exon 1-3 protein junction of type IIB procollagen **(C-L)**; red = IIF antibody detecting the Col II triple helical domain; yellow = colocalization of IIF and IIA or IIF and IIBN; blue = DAPI-stained nuclei. Bar **(A, B, E-L)**: 10 μ m; **(C, D)**: 50 μ m; (insets): 5 μ m.

Figure 5.

Immunohistochemistry with IIBN antibody on E15.5 wild type femur to analyze specificity to cartilage regions. Panel B shows a higher magnification of the hypertrophic zone outlined in A, demonstrating the positive reactivity of IIBN to type IIB procollagen (some designated by arrows) in the hypertrophic zone. Positive staining for IIBN is seen as brown against a counterstain of tartrazine (yellow). Bar (A): 250 μ m; (B): 50 μ m.