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Presence of type IIB procollagen in mouse articular cartilage and growth plate is revealed by immunohistochemical analysis with a novel specific antibody

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Abstract

Type II collagen is the major fibrillar collagen in cartilage. It is synthesized in the form of precursors (procollagens) containing N- and C-terminal propeptides. The two main isoforms of type II procollagen protein are type IIA and type IIB procollagens, generated in a developmentally regulated manner by differential splicing of the primary gene transcript. Isoform IIA contains exon 2 and is produced mainly by chondroprogenitor cells while isoform IIB lacks exon 2 and is produced by differentiated chondrocytes. Thus, expression of IIA and IIB isoforms are reliable markers for identifying the differentiation status of chondrocytes but their biological function in the context of skeletal development is still not yet fully understood. Specific antibodies against IIA and IIB procollagen isoforms are already available. In this study, a synthetic peptide spanning the junction between exon 1 and exon 3 of the murine sequence was used as an immunogen to generate a novel rabbit polyclonal antibody directed against procollagen IIB. Characterization of this antibody by Western-blotting analysis of murine cartilage extracts and ELISA tests demonstrated its specificity to the type IIB isoform. Furthermore, by immunohistochemical studies, this antibody allowed the detection of procollagen IIB in embryonic cartilage as well as in articular cartilage and growth plate of young adult mice. Interestingly, this is the first antibody that has allowed the detection of procollagen IIB at both the intra- and extracellular level. This antibody therefore represents an interesting new tool for monitoring the spatial and temporal distribution of IIB isoforms in skeletal tissues of mouse models and for tracking the trafficking and processing of type IIB procollagen.

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Introduction

Type II collagen is a major component of the collagen fibrils in cartilage, providing the tissue with its tensile strength while the proteoglycans offer compressive properties. It is composed of three identical $\alpha 1(\text{II})$ chains synthesized as precursor forms (procollagens containing N- and C-terminal propeptides) by the chondrocytes. The gene coding for type II collagen (*Col2a1*)

undergoes alternative splicing of exon 2, which encodes a 69/70 amino acid long cysteine-rich (CR) domain that is present in the N-terminal part of procollagen IIA/IID isoforms but is absent in the procollagen IIB protein (Fig. 1A). In mouse, the IID isoform corresponds to an extra residue (an arginine) at the end of exon 2, when compared to the IIA isoform. Interestingly, an initial series of studies showed that the switch in expression of procollagen IIA to IIB mRNA is a sign of

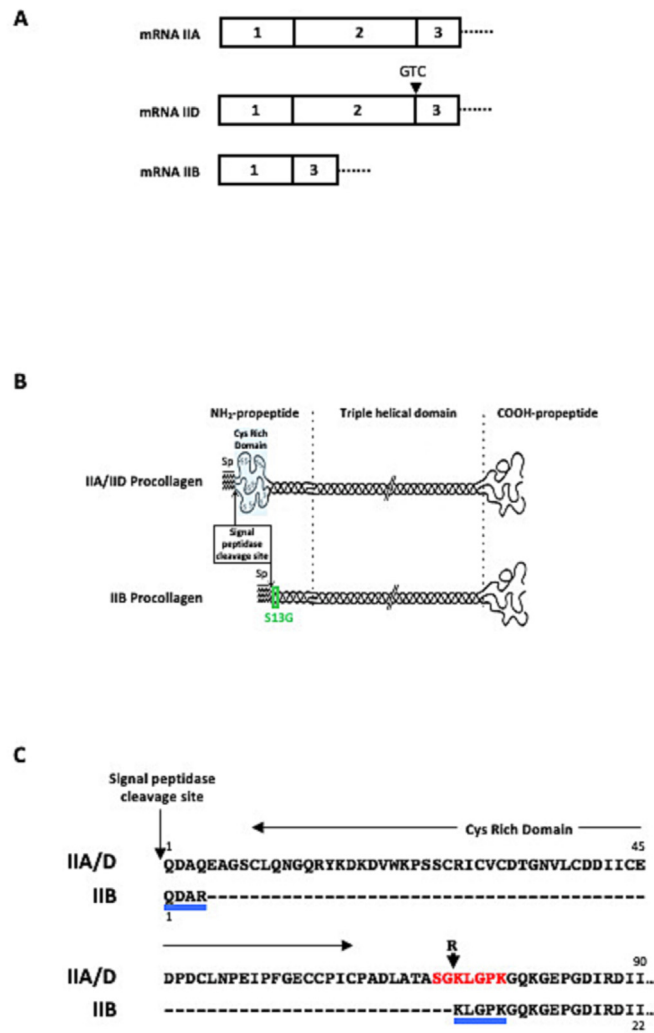


Fig. 1. Diagram to show the structure of type II procollagens A/D and B splice variants and amino acid sequences of their N-terminal parts. **(A)** Illustration of the IIA/D and IIB splice forms of Col2a1 mRNA. Exons 1, 2 and 3 are shown. The type IIA isoform contains exon 2 while type IIB isoform is devoid of exon 2. The IID splice form contains an additional 3 nucleotides at the end of exon 2 (down arrow), resulting in the insertion of an additional codon (arginine) at the end of translated exon 2. **(B)** Schematic illustration showing the different structural domains. The green square indicates the position of the immunizing peptide used to produce the S13G antibody. Sp represents the signal peptide. **(C)** Comparison between murine IIA/D and IIB N-terminal propeptides (NCBI Accession numbers NP_112440.2 and NP_001106987.2, respectively). The 25 first amino acids corresponding to the signal peptides are omitted together with the additional residues common to both propeptides at their C-terminal ends (...). The dashes (-) represent the CR domain missing in the IIB isoform. The small down arrow indicates the location of the extra arginine residue (R) present at the end of the exon 2 encoded domain of type IID procollagen. The sequence underlined in blue corresponds to the peptide used for boosting immunizations and affinity chromatography purification of the S13G antiserum. The sequence in red corresponds to the IIA-like peptide used in ELISA assays. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

engagement in chondrogenesis, or cartilage formation [1–5]. The IID isoform is expressed in human mesenchymal stem cells and ATDC5 cells undergoing chondrogenesis [6] but has not been detected in mouse cartilage [7]. While the indispensable role of type II collagen in cartilage was initially clearly demonstrated [8], the relative

contributions of IIA/D and IIB procollagen isoforms in cartilage biology are not yet completely understood, despite extensive research. *In vitro* studies have shown that recombinant IIA CR domain binds TGF-β1 and BMP-2 [9] and injection of full-length procollagen IIA mRNA in *Xenopus* embryos results in dorsalization, a sign of BMP inactivation [10].

However, a study using mouse mutant lacking exon 2 shows that there is no relationship between loss of IIA and BMP signaling activity [11]. Another mouse model was engineered to exclusively express exon 2 (*Col2a1^{+ex2}*) and mice appeared healthy with no overt skeletal phenotype [12], although electron microscopy analysis revealed that cartilage contains collagen fibrils of smaller diameter compared to those seen in wild-type cartilage matrix, suggesting that the exon 2-encoded protein may regulate collagen fibrillogenesis [13].

At the protein level, the detection of procollagen IIA and IIB in cartilage has been made possible by the use of polyclonal antibodies. An antibody to the IIA exon 2-encoded extra-domain allowed detection of the IIA/D N-propeptides in various normal or pathological situations [4,14]. Of note, the IID N-propeptide has never been visualized on its own due to the lack of specific antibodies, and translation of the IID mRNA remains hypothetical to this day. We previously reported the characterization of an antibody capable of specifically detecting human type IIB procollagen [15] while another study reported the characterization of an antibody specific for murine type IIB procollagen [16]. This antibody was used to investigate the distribution of type IIB procollagen in developing mouse cartilage by immunostaining and the positive signals were seen as small intracellular clusters, restricted to hypertrophic chondrocytes [16]. This observation was interpreted as a possible sign of rapid processing mechanism of procollagen IIB [16]. Here we report the characterization of a novel antibody specific for murine procollagen IIB, termed S13G, which reveals by immunostaining the presence of procollagen IIB in resting, proliferating and hypertrophic chondrocyte areas of embryonic cartilage, as well as in articular cartilage and growth plate of young adult mice. Of note, abundant type IIB procollagen was revealed with S13G in the cytoplasm of hypertrophic chondrocytes. These results suggest that S13G could be used to gain new insights into the dynamics of type II procollagen trafficking and processing in chondrocytes.

Results

The domain architecture of the IIA/D and IIB isoforms of procollagen II is represented in Fig. 1B. Procollagens IIA and IID differ from procollagen IIB by the presence of a CR domain encoded by exon 2. In order to obtain an antibody specific for procollagen IIB, the peptides used for immunization had to match to the sequence encoded by the exon 1/3 junction. This junction is very close to the N-terminal region of procollagen IIB (NCBI accession number NP_001106987.2) and, after removal of the signal peptide, the starting sequence of procollagen IIB is QDA (Fig. 1C). Therefore, the sequence of the peptide chosen for the first injections (called the

immunizing peptide) was QDARKLGPKGQK, where QDA is specific for exon 1 and KLGPKGQK for exon 3, with R forming at the splice junction from DNA sequences contributed by the two exons; the sequence of the peptide chosen for the booster injections (called the booster peptide) was QDARKLGPK and this very short peptide was also used for affinity purification of the S13G antibody from rabbit antiserum. A third peptide, SGKLGPK, corresponding to the junction of exons 2–3 of procollagen IIA (referred to as IIA-like peptide) was used in ELISAs to ensure that the S13G antibody did not cross-react with procollagen IIA (Fig. 1C and Fig. 2 D, E). All these controls were necessary owing to electrophoretic SDS-PAGE migrations too close to clearly discriminate between isoforms.

In Western blots of embryonic rib cage extracts, a major band (approximately 190 kDa) migrating as expected for procollagen IIB (and procollagens IIA/D as well) was revealed using the S13G antibody, [band indicated as $\text{pro}\alpha 1(\text{II})$] (Fig. 2A). The other band observed with a smaller molecular weight originates from a partial processing of procollagen IIB at its C-terminal part [band indicated as $\text{pN}\alpha 1(\text{II})$]. In comparison, staining of the membrane with a polyclonal antibody directed to the triple helical part of collagen II revealed, in addition to the band corresponding to the total unprocessed forms of procollagen II, lower and higher migrating bands corresponding to mature collagen II [band indicated as $\alpha 1(\text{II})$] and crosslinked mature forms [band indicated as β]. These clear stainings prompted us to further characterizations to exploit this antibody. In Western blots of chondrocyte extracts from mouse embryos, a single band (also about 190 kDa) was revealed using the S13G antibody whereas no band was observed if S13G was blocked by the booster peptide (Fig. 2B), attesting that the band revealed by S13G recognition concerns type IIB procollagen. The major other fibrillar collagen in its proform that could interfere with accurate analyses is collagen I (a marker of dedifferentiation of chondrocytes), whose electrophoretic migrations are very close to their collagen II counterparts). We took advantage of the fact that partially dedifferentiated chondrocytes produce both collagen I and collagen II, to examine a possible cross-reaction of the S13G antibody with procollagen I. Western-blotting analysis confirmed the production of type I and type II collagen. More precisely, extensive migration allowed a clear electrophoretic separation of procollagen IIB and procollagen I, indicating that the band revealed by S13G does not correspond to procollagen I.

Experiments were performed, with always in mind to rule out a recognition of the S13G antibody towards IIA. ELISA tests showed its immunoreactivity towards the booster peptide but

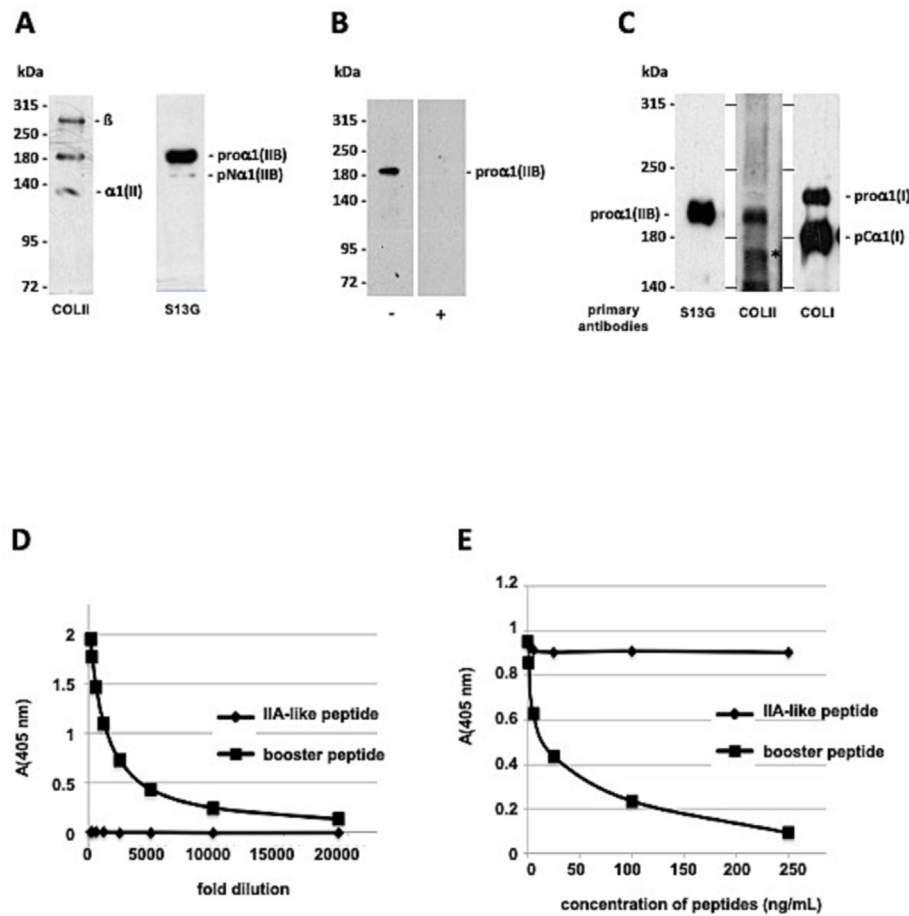


Fig. 2. Characterization of the S13G antibody by Western-blot and ELISA assays. **(A)** An extract of mouse embryonic E17.5 rib cage was run in two different lanes on a 6% acrylamide gel and after transfer the membrane was cut in 2 pieces. On the left, the membrane was probed with a commercial polyclonal antibody directed against mature molecules of mouse type II collagen (COLII), recognizing the triple helix domain and in consequence unprocessed and processed forms. Dimers of type II collagen molecules are also indicated (β). On the right, the membrane was probed with the S13G antibody. **(B)** Blocking of the S13G antibody with the booster peptide. P1 E17.5 mouse chondrocytes were treated for 3 days with BMP-2. Cell layer extracts were loaded in duplicate on a 6% polyacrylamide gel and after transfer the membrane was cut in 2 pieces. Before contact with the membrane and revelation, the S13G antibody was incubated alone (-) or in the presence of the booster peptide (+). **(C)** Western-blotting analysis of type II and type I collagen production by P1 E17.5 mouse chondrocytes treated for 3 days with BMP-2. Collagens in the cell culture medium were precipitated with 4.5 M NaCl and were loaded in triplicate on different lanes of a 6% acrylamide gel. After extensive migration (to allow discrimination between proteins of very similar apparent molecular masses, as described previously [15]) and transfer, the membrane was cut in 3 pieces, allowing analysis of the collagens with the S13G antibody or commercial antibodies recognizing type II (COLII) or type I (COLI) collagen, as indicated. As the anti-COLII antibody, the anti-COLI antibody is directed against mature molecules and thus allows detection of unprocessed and processed forms. The band indicated by an asterisk (*) most likely corresponds to pC $\alpha 1(\text{II})$ chains. For (A,B,C), the apparent molecular masses according to colored markers are indicated in the left. **(D)** ELISA assays showing S13G immunoreactivity towards the booster and IIA-like peptides. 300 ng of each peptide was used for coating and serial dilutions of S13G were performed, as indicated. **(E)** Competitive ELISA assays where 25 ng of the booster peptide was used for coating and incubated with a mixture of S13G antibody used at 1/750 dilution (70 ng/mL) and booster or IIA-like peptides, present at the indicated concentrations.

not towards the IIA-like peptide (Fig. 2D). Further, a competitive ELISA assay showed that the S13G signal was displaced by increasing the concentration of booster peptide but not by IIA-like

peptide (Fig. 2E). This lack of signal silencing by the IIA-like peptide indicates that the exon 3 specific KLGPK sequence common to the IIA/D and IIB procollagen is not involved, on its own, in

the recognition by the S13G antibody and that, consequently, it is the exon 1–3 junction, specific to procollagen IIB, that is targeted by S13G.

In parallel, we studied the immunohistochemical reactivity of S13G. The developing cartilage rudiments of E17.5 hind limb were intensely stained by the antibody (Fig. 3A). Specifically, staining was observed in areas of resting, proliferating and hypertrophic chondrocytes and excluded non-chondrogenic tissues such as perichondrium, loose connective tissue, and epidermis (Fig. 3A). Instead, those tissues were immunostained with an anti-collagen type I antibody (Fig. 3B). We then analyzed the distribution of type II collagen with the S13G antibody in the knee joint of 12-week-old mice. A panoramic view of the joint showed that S13G

staining was localized to the articular cartilage and growth plate (Fig. 3 D-F). Of note, S13G did not react with bone (Fig. 3D), confirming the specificity of S13G to cartilage. Next, we tested S13G in immunofluorescence applications, using the knee joint of 12-week-old mice (Fig. 3 H-K). We compared S13G signals with those observed with another antibody (6B3) recognizing an epitope localized in the triple helix of type II collagen (fibrillar domain), in double-labeling immunofluorescence experiments. High-magnification analysis of the growth plate showed the presence of 6B3 signals in the ECM (Fig. 3I) and S13G signals not only in the ECM but also in abundance within chondrocytes arranged in columns (Fig. 3J). All these data indicate that

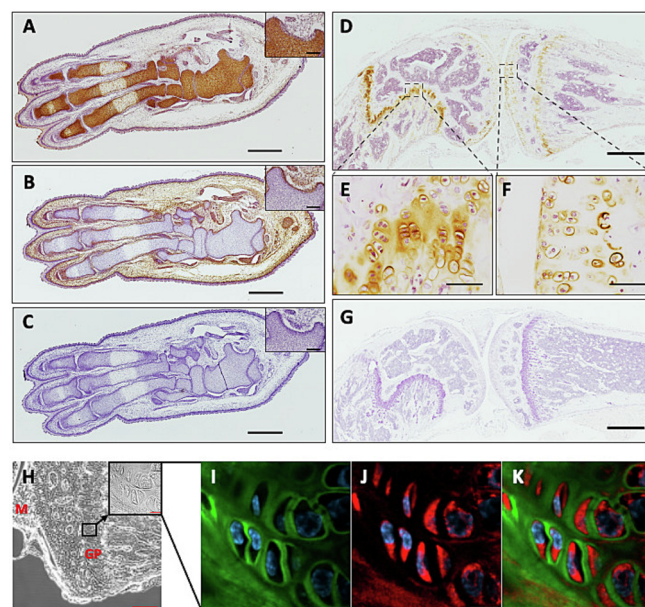


Fig. 3. Immunohistological localization of type IIB procollagen in mouse hind limbs. **(A)** Longitudinal section of the distal part of a E17.5 hind limb (showing the prospective tarsal, metatarsal and digital bones) immunostained with S13G. Type IIB procollagen protein is detected in all cartilage regions. High magnification of the inset shows that the perichondrium is not stained with S13G. **(B)** Section parallel to (A) and immunostained with anti-collagen type I antibody showing the presence of type I collagen in nonchondrogenic tissues. The high magnification in the inset highlights the staining of the perichondrium with anti-collagen type I antibody. **(C)** Parallel section to (A) and (B) where the primary antibody was replaced with 2.5% horse serum in PBS as a negative staining control. **(D)** Sagittal section of a knee joint from a 12-week-old mouse showing the femoral (left) and tibial (right) heads. Immunostaining with S13G shows the presence of type IIB procollagen in the growth plate and articular cartilage. **(E, F)** High magnifications of the areas surrounded by dotted lines in (D). **(G)** Parallel section to (D) where the primary antibody was replaced with 2.5% horse serum in PBS as a negative staining control. **(H)** Phase contrast microscopy observation of the tibial head from a histological section parallel to (D) and (G). The inset corresponds to the magnification of the boxed region and shows columns of prehypertrophic and hypertrophic chondrocytes in the growth plate (M: meniscus, GP: growth plate). **(I, J, K)** Double-labeled immunofluorescence analyses of the region boxed in (H), with S13G and 6B3 antibodies. Colors representing antibody localizations are green for 6B3 (detecting the type II collagen triple helical domain) and red for S13G (detecting the exon 1–3 protein junction of type IIB procollagen). The blue color corresponds to Hoechst-stained nuclei. **(I)** Immunofluorescence signals obtained from 6B3 only. **(J)** Immunofluorescence signals obtained from S13G only. **(K)** Composite Image with 6B3 and S13G signals. Bar (A-C): 500 μ m, inset: 100 μ m; Bar (D): 200 μ m; Bar (E, F): 50 μ m; Bar (G): 200 μ m; Bar (H): 200 μ m, inset: 10 μ m; Bar (I, J, K): 10 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

S13G can serve as an immunohistological probe for detecting procollagen IIB.

Discussion

We developed a new murine type IIB procollagen-specific antibody, designated S13G, which allows a very sensitive and specific detection of procollagen IIB by Western-blotting and immunohistochemistry. As expected from the nature of type IIB procollagen processing, S13G is specific to the unprocessed procollagen forms pro α 1(IIB) and pN α 1(IIB) which contain the amino-propeptide, but does not recognize the fully processed form α 1(IIB) (Fig. 2A). Of note, immunoblot analysis of mouse embryonic rib cage with S13G revealed no false-positive bands on full sized blotted gel (Fig. 2A), even after prolonged exposure times (data not shown). Moreover, in P1 chondrocytes, the band stained by S13G could be blocked by the booster peptide (Fig. 2B), implying that this band does correspond to type IIB procollagen. Additional evidence that S13G is specific for procollagen IIB isoform was provided by our ELISA tests. The S13G antibody was found to be highly specific for the selection (booster) peptide and, when tested in a competitive ELISA, there was no indication of cross-reactivity with the synthetically generated form of the epitope corresponding to procollagen IIA/IID. Besides, our Western blots indicated that S13G does not recognize procollagen I (Fig. 2C), which is consistent with our immunohistochemical analyses showing a lack of staining with S13G for various tissues containing collagen I and other fibrillar collagen types (collagens III and V).

While our Western blots clearly demonstrated the presence of type IIB procollagen in embryonic rib cage, our immunohistochemical analysis of embryonic limb sections revealed the presence of type IIB procollagen in the cartilaginous anlagen of the future long bones, encompassing areas of resting/proliferating and hypertrophic chondrocytes. Moreover, analysis of knee joint of young adult mice, stained with S13G, clearly revealed the presence of procollagen IIB in the articular cartilage and growth plate. Our results also indicate that S13G can be used in immunofluorescence studies. Interestingly, the simultaneous use of 6B3 and S13G antibodies in immunofluorescence studies reveals that 6B3 readily detects the mature form of collagen II in the ECM and S13G is particularly effective in detecting the intracellular presence of procollagen IIB. As an example, close examination by confocal microscopy of the growth plate revealed abundant procollagen IIB in the cytoplasm of hypertrophic chondrocytes, as well as in the ECM where the mature fibrillar domain of type II collagen was easily detected. Strikingly, the distribution of procollagen IIB observed with S13G appears different from the one reported with another

murine procollagen IIB polyclonal antibody (called IIBN) generated in a previous study [16]. In the latter study, immunofluorescence signals were detected in the hypertrophic zone of E17.5 femur, but not in the resting or proliferative zone [16]. Moreover, these signals were limited to small intracellular clusters of type IIB procollagen in hypertrophic chondrocytes and no IIBN signal was detected in the ECM [16]. Because type X collagen is produced primarily by hypertrophic chondrocytes [17], Patra et al. [16] proposed that the absence of intracellular IIBN staining in areas of resting and proliferative chondrocytes may be explained by rapid trafficking of procollagen IIB in these chondrocytes, whereas this trafficking would be slowed by a mechanism of excessive secretion of type X collagen in hypertrophic chondrocytes, thus allowing the detection of procollagen IIB. The general immunoperoxidase staining we observed with S13G in embryonic cartilage zones containing hypertrophic and non-hypertrophic chondrocytes, rather argues for a production of procollagen IIB that is not particularly influenced by that of type X collagen. This is also supported by the presence of procollagen IIB revealed by S13G in the growth plate and articular cartilage (tibia-femoral joint) of 12 week-old-mice, where type X collagen was detected with similar immunostaining patterns in the same regions at this age [18]. In addition, abundant procollagen IIB content was unequivocally visualized at the intracellular level using S13G and confocal fluorescence microscopy. We think that the discrepancy between our immunohistochemical data presented with S13G and those obtained with IIBN [16] results from the difference in peptide sequence chosen to raise the antibodies. As a matter of fact, it should be noted that the signal peptidase cleavage site was included at the N-terminus of the peptide sequence used to generate IIBN [16] whereas we were careful to exclude it from the peptide sequence used to generate S13G. It is therefore highly possible that the epitope recognized by IIBN encompasses this cleavage site and is rapidly removed during translation of procollagen IIB, as signal sequences are generally cleaved rapidly during translation. This would explain the extremely restricted IIBN signals observed at the intracellular level [16]. It was also suggested that the absence of IIBN signals in the ECM is due to rapid removal of the IIB-derived N-propeptide from the matrix or its masking by other ECM components [16]. Instead, our immunohistochemical and immunofluorescence analyses showing the presence of S13G signals in the ECM indicate that N-propeptide IIB is not removed as rapidly from the procollagen molecule and the ECM as originally thought. We can even add that by using another antibody (called anti-pNIIB52) specific to human/bovine N-propeptide IIB, we had also detected by immunostaining the presence of N-propeptide IIB in bovine articular cartilage ECM. It has been shown that ADAMTS-3 is the major pro-

collagen II N-propeptidase [19], but the exact location of the type II procollagen N-propeptide processing is still unknown to date. Thus, it remains to be determined whether the N-propeptide IIB detected in the ECM correspond to a free form of the N-propeptide or to an unprocessed form of procollagen IIB.

In conclusion, our study allowed the characterization of a novel murine procollagen type IIB-specific antibody, S13G. Immunohistochemical and immunofluorescence images of cartilage sections obtained with S13G showing not only intracellular but also extracellular matrix staining indicate that post-translational modifications do not prevent the detection of N-propeptide IIB in tissues. We therefore believe that this antibody represents a valuable new tool to study, and probably re-examine, the trafficking and processing of type IIB procollagen from the endoplasmic reticulum to the cartilage ECM using the mouse model.

Experimental procedures

Preparation and purification of antibodies specific for the N-propeptide of procollagen IIB

Partial design of peptide sequences, peptide synthesis, immunization of rabbits, ELISA assays and purification of the antiserum on immobilized boosting peptide (see Results for sequences) were performed by Covalab, France. For all the peptides, a C-terminal cysteine was added for conjugation purposes. The purified antibody (53 µg/mL) was designated as S13G and used at a dilution of 1:4000 for Western blot analyses or 1:100 for immunohistochemistry and immunofluorescence studies. ELISA assays using S13G as primary antibody followed standard protocols with peroxidase-linked secondary antibody and 2,2'-azino-bis(3-ethylbenzothiazole-6-sulphonic acid), ABTS, as chromogenic substrate.

Cell culture

Embryonic mouse chondrocytes were isolated from the costal cartilage of day 17.5 post-coitum mice. These chondrocytes were seeded on 90 mm Corning dishes with 1×10^6 cells/dish and cells at this stage were designated P0. P0 chondrocytes were cultivated with 1:1 Dulbecco's modified Eagle's medium/F-12 with GlutaMAX-I containing 10% fetal bovine serum (FBS) and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all products from Invitrogen), at 37 °C in 5% CO₂. Confluent cells were detached using 0.05% trypsin-EDTA, seeded in 6-well culture dishes with 1.5×10^5 cells/well and at this stage these cells were designated P1. P1 chondrocytes were cultivated for 3 days in medium containing 1% FBS supplemented with

50 ng/ml BMP-2 (Dibotermine- α , drug form of BMP-2 contained in InductOs kit, Wyeth), condition previously shown to allow type I and type II procollagen synthesis [20].

SDS-PAGE and Western blot analysis

For cell culture analysis, after several washings with phosphate buffered saline, cell layers were scraped in Laemmli sample buffer containing β -mercaptoethanol. For tissue analysis, embryonic rib cages were harvested from day 17.5 post-coitum mice, frozen and crushed with a mortar and pestle then were mixed with Laemmli sample buffer containing β -mercaptoethanol. Proteins were resolved in 6% polyacrylamide minigels and transferred to PVDF membranes (Millipore). After transfer, the membranes were probed with the appropriate primary antibodies and incubated with horseradish peroxidase (HRP)-linked antibodies. Commercial polyclonal rabbit antibodies to mouse collagen I and II were from Novotec, France (cat. 20,151 and 20251, respectively; used at 1:5000). Anti-rabbit IgG horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology (#7074: used at 1:1000). Detection on X-ray films was performed using Clarity ECL Western Blotting Substrates (Bio-Rad).

Immunohistochemistry and immunofluorescence

Hind limbs from embryonic (E) 17.5 and 12 week-old C57Bl/6 mice were fixed with 4% neutral buffered formalin, then demineralized, dehydrated, embedded in paraffin and cut in 5 µm sections. Deparaffinized and rehydrated tissue sections were digested with pepsin reagent (Sigma Aldrich) at 37 °C for 17 min, rinsed with phosphate buffered saline (PBS), and processed for immunohistochemistry or immunofluorescence studies.

For immunohistochemistry, incubation with S13G antibody or anti-collagen type I antibody (Novotec, cat. 20151, used at 1: 50) was performed using the ImmPress staining kit (Vector Laboratories) and the sections were counterstained with hematoxylin.

For double-labeled immunofluorescence experiments, tissue sections were blocked for 45 min with 10% donkey serum (Sigma) in PBS and then incubated overnight with the primary antibodies. S13G and a monoclonal antibody, clone 6B3 (Merck-Millipore) recognizing an epitope localized in the triple helix of type II collagen (diluted 1:100 in 10% donkey serum) were used. The next day, the sections were rinsed with PBS and then incubated for 1 h with secondary antibodies. Alexa Fluor 647-Donkey anti-rabbit IgG (BioLegend) and CF555 Donkey anti-mouse IgG (Biotium) secondary antibodies diluted 1:500 in 10% donkey serum were used.

After washing in PBS, nuclei were stained for 5 min with 1 × Hoechst (Fluka). Sections were then mounted in 50% glycerol solution for observation.

Images were captured using an ECLIPSE TI-E microscope (Nikon) coupled to a color (DS-Fi2) or monochrome (DSQ12-ND) camera using NIS-Elements imaging software (Nikon) or with an inverted confocal microscope (Zeiss LSM980 Airy scan2).

ELISA assays

A synthetic peptide (here called IIA-like peptide) SGKLGPKC was obtained from ProteoGenix. ELISA assays were performed using standard protocols with Nunc MaxiSorp 96-well plates. Coating of peptides was performed overnight at 4 °C. Blocking reaction was done with 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS) with 0.05% Tween-20 (PBST). For titration experiments, serial dilutions of S13G antibody were performed in 3% BSA in PBST and added for 1 hr in the wells coated with the booster or the IIA-like peptide, as indicated in Fig. 2D. For competition studies, increasing concentrations of booster or IIA-like peptides were mixed with the S13G antibody and were incubated in the wells coated with the booster peptide, as indicated in Fig. 2E.

CRedit authorship contribution statement

Emeline Perrier-Groult: Investigation, Visualization. **Shérine Moustaghfir:** Investigation. **Marielle Padeloup:** Investigation, Methodology. **Jean-Daniel Malcor:** Investigation. **Jérôme Lafont:** Investigation, Visualization. **Frédéric Mallein-Gerin:** Writing – review & editing.

DATA AVAILABILITY

Data will be made available on request.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- [1]. Sandell, L.J., Morris, N., Robbins, J.R., Goldring, M.B., (1991). Alternatively spliced type II procollagen mRNAs define distinct populations of cells during vertebral development: differential expression of the amino-propeptide. *J. Cell. Biol.*, **114** (6), 1307–1319. <https://doi.org/10.1083/jcb.114.6.1307>.
- [2]. Ng, L.J., Tam, P.P., Cheah, K.S., (1993). Preferential expression of alternatively spliced mRNAs encoding type II procollagen with a cysteine-rich amino-propeptide in differentiating cartilage and nonchondrogenic tissues during early mouse development. *Dev. Biol.*, **159** (2), 403–417. <https://doi.org/10.1006/dbio.1993.1251>.
- [3]. Sandell, L.J., Nalin, A.M., Reife, R.A., (1994). Alternative splice form of type II procollagen mRNA (IIA) is predominant in skeletal precursors and non-cartilaginous tissues during early mouse development. *Dev. Dyn.*, **199** (2), 129–140. <https://doi.org/10.1002/aja.1001990206>.
- [4]. Lui, V.C., Ng, L.J., Nicholls, J., Tam, P.P., Cheah, K.S., (1995). Tissue-specific and differential expression of alternatively spliced alpha 1(II) collagen mRNAs in early human embryos. *Dev. Dyn.*, **203** (2), 198–211. <https://doi.org/10.1002/aja.1002030208>.
- [5]. Oganessian, A., Zhu, Y., Sandell, L.J., (1997). Type IIA procollagen amino propeptide is localized in human embryonic tissues. *J. Histochem. Cytochem.*, **45** (11), 1469–1480. <https://doi.org/10.1177/002215549704501104>.
- [6]. McAlinden, A., Johnstone, B., Kollar, J., Kazmi, N., Hering, T.M., (2008). Expression of two novel alternatively spliced COL2A1 isoforms during chondrocyte differentiation. *Matrix Biol.*, **27** (3), 254–266. <https://doi.org/10.1016/j.matbio.2007.10.002>.
- [7]. McAlinden, A., Shim, K.H., Wirthlin, L., Ravindran, S., Hering, T.M., (2012). Quantification of type II procollagen splice forms using alternative transcript-qPCR (AT-qPCR). *Matrix Biol.*, **31** (7–8), 412–420. <https://doi.org/10.1016/j.matbio.2012.08.002>.
- [8]. Li, S.W., Prockop, D.J., Helminen, H., Fässler, R., Lapveteläinen, T., Kiraly, K., Peltari, A., Arokoski, J., Lui, H., Arita, M., et al., (1995). Transgenic mice with

- targeted inactivation of the Col2 alpha 1 gene for collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. *Genes Dev.*, **9** (22), 2821–2830. <https://doi.org/10.1101/gad.9.22.2821>.
- [9]. Zhu, Y., Oganessian, A., Keene, D.R., Sandell, L.J., (1999). Type IIA procollagen containing the cysteine-rich amino propeptide is deposited in the extracellular matrix of prechondrogenic tissue and binds to TGF-beta1 and BMP-2. *J. Cell. Biol.*, **144** (5), 1069–1080. <https://doi.org/10.1083/jcb.144.5.1069>.
- [10]. Larraín, J., Bachiller, D., Lu, B., Agius, E., Piccolo, S., De Robertis, E.M., (2000). BMP-binding modules in chordin: a model for signalling regulation in the extracellular space. *Development*, **127** (4), 821–830.
- [11]. Leung, A.W., Wong, S.Y., Chan, D., Tam, P.P., Cheah, K. S., (2010). Loss of procollagen IIA from the anterior mesendoderm disrupts the development of mouse embryonic forebrain. *Dev. Dyn.*, **239** (9), 2319–2329. <https://doi.org/10.1002/dvdy.22366>.
- [12]. Lewis, R., Ravindran, S., Wirthlin, L., Traeger, G., Fernandes, R.J., McAlinden, A., (2012). Disruption of the developmentally-regulated Col2a1 pre-mRNA alternative splicing switch in a transgenic knock-in mouse model. *Matrix Biol.*, **31** (3), 214–226. <https://doi.org/10.1016/j.matbio.2011.12.004>.
- [13]. McAlinden, A., Traeger, G., Hansen, U., Weis, M.A., Ravindran, S., Wirthlin, L., Eyre, D.R., Fernandes, R.J., (2014). Molecular properties and fibril ultrastructure of types II and XI collagens in cartilage of mice expressing exclusively the $\alpha 1$ (IIA) collagen isoform. *Matrix Biol.*, **34**, 105–113. <https://doi.org/10.1016/j.matbio.2013.09.006>.
- [14]. Aigner, T., Zhu, Y., Chansky, H.H., Matsen, F.A., Maloney, W.J., Sandell, L.J., (1999). Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. *Arthritis Rheum.*, **42** (7), 1443–1450. [https://doi.org/10.1002/1529-0131\(199907\)42:7<1443::AID-ANR18>3.0.CO;2-A](https://doi.org/10.1002/1529-0131(199907)42:7<1443::AID-ANR18>3.0.CO;2-A).
- [15]. Aubert-Foucher, E., Mayer, N., Padeloup, M., Pagnon, A., Hartmann, D., Mallein-Gerin, F., (2014). A unique tool to selectively detect the chondrogenic IIB form of human type II procollagen protein. *Matrix Biol.*, **34**, 80–88. <https://doi.org/10.1016/j.matbio.2013.09.001>.
- [16]. Patra, D., DeLassus, E., McAlinden, A., Sandell, L.J., (2014). Characterization of a murine type IIB procollagen-specific antibody. *Matrix Biol.*, **34**, 154–160. <https://doi.org/10.1016/j.matbio.2013.10.014>.
- [17]. Kirsch, T., von der Mark, K., (1991). Isolation of human type X collagen and immunolocalization in fetal human cartilage. *Eur. J. Biochem.*, **196** (3), 575–580. <https://doi.org/10.1111/j.1432-1033.1991.tb15852.x>.
- [18]. der Kraan, V., Stoop, R., Meijers, T.H.M., Poole, A.R., van den Berg, W.B., (2001). Expression of type X collagen in young and old C57Bl/6 and Balb/c mice. Relation with articular cartilage degeneration. *Osteoarthritis Cartilage*, **9**, 92–100. <https://doi.org/10.1053/joca.2000.0364>.
- [19]. Fernandes, R.J., Hirohata, S., Engle, J.M., Colige, A., Cohn, D.H., Eyre, D.R., Apte, S.S., (2001). Procollagen II amino propeptide processing by ADAMTS-3. Insights on dermatosparaxis. *J. Biol. Chem.*, **276** (34), 31502–31509. <https://doi.org/10.1074/jbc.M103466200>.
- [20]. Gouttenoire, J., Bougault, C., Aubert-Foucher, E., Perrier, E., Ronzière, M.C., Sandell, L., Lundgren-Akerlund, E., Mallein-Gerin, F., (2010). BMP-2 and TGF-beta1 differentially control expression of type II procollagen and alpha 10 and alpha 11 integrins in mouse chondrocytes. *Eur. J. Cell. Biol.*, **89** (4), 307–314. <https://doi.org/10.1016/j.ejcb.2009.10.018>.