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Molecular properties and fibril ultrastructure of types II and XI collagens in cartilage of mice expressing exclusively the $\alpha 1(\text{IIA})$ collagen isoform

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Abstract

Until now, no biological tools have been available to determine if a cross-linked collagen fibrillar network derived entirely from type IIA procollagen isoforms, can form in the extracellular matrix (ECM) of cartilage. Recently, homozygous knock-in transgenic mice (*Col2a1*^{+ex2}, ki/ki) were generated that exclusively express the IIA procollagen isoform during post-natal development while type IIB procollagen, normally present in the ECM of wild type mice, is absent. The difference between these *Col2a1* isoforms is the inclusion (IIA) or exclusion (IIB) of exon 2 that is alternatively spliced in a developmentally regulated manner. Specifically, chondroprogenitor cells synthesize predominantly IIA mRNA isoforms while differentiated chondrocytes produce mainly IIB mRNA isoforms. Recent characterization of the *Col2a1*^{+ex2} mice has surprisingly shown that disruption of alternative splicing does not affect overt cartilage formation. In the present study, biochemical analyses showed that type IIA collagen extracted from ki/ki mouse rib cartilage can form homopolymers that are stabilized predominantly by hydroxylysyl pyridinoline (HP) cross-links at levels that differed from wild type rib cartilage. The findings indicate that mature type II collagen derived exclusively from type IIA procollagen molecules can form heterofibrils with type XI collagen and contribute to cartilage structure and function. Heteropolymers with type XI collagen also formed. Electron microscopy revealed mainly thin type IIA collagen fibrils in ki/ki mouse rib cartilage. Immunoprecipitation and mass spectrometry of purified type XI collagen revealed a heterotrimeric molecular composition of $\alpha 1(\text{XI})\alpha 2(\text{XI})\alpha 1(\text{IIA})$ chains where the $\alpha 1(\text{IIA})$ chain is the IIA form of the $\alpha 3(\text{XI})$ chain. Since the N-propeptide of type XI collagen regulates type II collagen fibril diameter in cartilage, the retention of the exon 2-encoded IIA

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globular domain would structurally alter the N-propeptide of type XI collagen. This structural change may subsequently affect the regulatory function of type XI collagen resulting in the collagen fibril and cross-linking differences observed in this study.

Keywords

Type IIA procollagen; alternative splicing; cartilage; collagen cross-links; heteropolymer; collagen fibrils; extracellular matrix; post-translational modifications

1. Introduction

Collagens are structural proteins present in extracellular matrices of all tissues. Type II collagen the most abundant fibril-forming collagen in cartilage, is synthesized as a homotrimeric procollagen molecule [$\alpha 1(\text{II})$]₃ consisting of a rod-like triple-helical domain flanked by an N- propeptide and a C- propeptide (Uitto, 1977). The propeptides are proteolytically removed in cartilage by specific proteases to generate native triple-helical collagen molecules before or during fibril assembly (Canty and Kadler, 2005; Fernandes et al., 1997; Fertala et al., 1996; Fertala et al., 1994; Hojima et al., 1989; Kadler et al., 1987). Type XI collagen, a heterotrimeric collagen molecule [$\alpha 1(\text{XI})\alpha 2(\text{XI})\alpha 3(\text{XI})$] (Morris and Bachinger, 1987), polymerizes to form the core of the type II collagen fibril and it is becoming increasingly clear that it serves as a template for type II collagen fibrillogenesis and regulates type II fibril diameters in cartilage (Blaschke et al., 2000; Eyre and Wu, 1987). The $\alpha 3(\text{XI})$ chain of type XI procollagen and the $\alpha 1(\text{II})$ chain of type II procollagen are encoded by the same gene (Burgeson and Hollister, 1979; Reese and Mayne, 1981) but the $\alpha 3(\text{XI})$ chain is post-translationally over-glycosylated and retains the amino propeptide in the native molecule (Eyre and Wu, 1987; Thom and Morris, 1991). In cartilage, type II and type XI collagen co-polymerize with type IX collagen and are cross-linked by lysyl oxidase mediated bonds to form a heteropolymeric fibrillar framework that gives cartilage its tensile strength (Eyre et al., 2002). The prevalent mature cross-link is the trivalent hydroxylysyl pyridinoline (HP) residue, which links at two sites (from N-telopeptide to helix and from C-telopeptide to helix) between head-to-tail overlapping type II collagen molecules packed in fibrils. Pyridinolines and divalent cross-links (keto-amines) covalently bond type IX collagen molecules to N- and C- telopeptides on the surface of type II collagen fibrils. Divalent cross-links also link type XI collagen molecules to each other and to C-telopeptides of type II collagen within the heteropolymer (Wu and Eyre, 1984, 1995).

Type II collagen is expressed, synthesized and secreted into the extracellular matrix as two isoforms. The type II procollagen gene, *Col2a1* (Ala-Kokko and Prockop, 1990; Metsaranta et al., 1991), is widely expressed during early development and is found in non-chondrogenic as well as chondrogenic tissues (Cheah et al., 1991). *Col2a1* is alternatively spliced during chondrogenesis whereby chondroprogenitor cells express predominantly exon 2-containing IIA spliced mRNA isoforms while differentiated chondrocytes express mainly exon 2-lacking IIB isoforms (McAlinden et al., 2008; Ryan and Sandell, 1990; Sandell et al., 1991; Sandell et al., 1994). Exon 2 encodes a globular 69 amino acid cysteine-rich (CR) von Willebrand Factor C-like domain in the N-propeptide that is highly conserved between species (Bornstein, 2002). Developmentally-regulated alternative splicing of exon 2 is

unique to chondrocytes and it has been postulated that this isoform switching event is critical for proper cartilage development (Zhu et al., 1999). However, our studies have shown apparent normal cartilage and skeletal formation in a mouse model where *Col2a1* pre-mRNA alternative splicing is inhibited by introduction of an exon 2 splice site knock-in (ki) mutation. The homozygous knock-in mice (*Col2a1*^{+ex2}, ki/ki) exclusively express the IIA procollagen isoform while the IIB procollagen isoform is absent (Lewis et al., 2012). Further, the majority of type IIA procollagen synthesized in ki/ki epiphyseal cartilage tissue was found to be processed normally, as occurs for type IIB procollagen in wild type cartilage (Lewis et al., 2012). Subsequently, processed homotrimeric triple-helical type II collagen molecules may be the same whether they are derived from type IIA or IIB procollagen. For type XI collagen however, since the $\alpha 3(\text{XI})$ chain has the same primary sequence and structure as the $\alpha 1(\text{II})$ chain but retains the N-propeptide in the native type XI collagen molecule (Thom and Morris, 1991; Wu and Eyre, 1995), it is conceivable that $\alpha 3(\text{XI})$ procollagen chains containing the extra amino acids encoded by exon-2 could subtly affect assembly and/or diameter control of type II-type XI collagen hetero-fibrils.

A post-translational modification that may have an influence on type IIA/IIB collagen molecular assembly and fibrillogenesis is the 3-hydroxylation of specific proline residues (3Hyp) in $\alpha 1(\text{II})$ chains (Weis et al., 2010). Pro-986 has been shown to be highly 3-hydroxylated in mouse cartilage and rat chondrosarcoma $\alpha 1(\text{IIB})$ chains. Pro-944 and Pro-707 are also 3-hydroxylated and positioned within 3 residues of the collagen D-period molecular stagger (234 amino acids) suggesting a supplementary role in collagen fibril assembly (Fernandes et al., 2011; Hudson et al., 2012; Weis et al., 2010). Mutations in prolyl 3-hydroxylase isoenzymes that affect proline 3-hydroxylation (Baldridge et al., 2008; Cabral et al., 2012; Marini et al., 2010; Takagi et al., 2012) and mutations that alter the primary structure of the $\alpha 1(\text{II})$ collagen chains can disrupt fibrillogenesis and result in a disorganized cartilage extracellular matrix during development and growth (Fernandes et al., 1998; Prockop et al., 1979a, b).

Since sufficient amounts of type IIA (pro)collagen protein have been difficult to isolate and purify from embryonic or fetal cartilage, it is not known whether type IIA (pro)collagen molecules are normally post-translationally modified, cross-linked and form functional fibrils in the matrix. The viable, *Col2a1*^{+ex2} knock-in mouse (Lewis et al., 2012) that expresses only the type IIA procollagen isoform in cartilage has enabled us to address these questions. In this study, we sought to establish the quality and composition of assembled type IIA collagen fibrils in the homozygous type IIA knock-in mouse cartilage. We show that newly synthesized $\alpha 1(\text{XI})$ and $\alpha 2(\text{XI})$ chains can trimerize with the $\alpha 1(\text{IIA})$ chain isoform of $\alpha 3(\text{XI})$ to form type XI collagen molecules which are deposited in the matrix of the homozygous mouse cartilage. Using a Western blot based method of screening for collagen heteropolymer formation in cartilage we show that in ki/ki mouse cartilage, molecules of type IIA collagen are covalently cross-linked to each other and to type XI collagen molecules to form a heteropolymer of type IIA and type XI collagen analogous to normal cartilage which uses $\alpha 1(\text{IIB})$ chains. We also show that the type IIA collagen molecules are substrates for the prolyl 3-hydroxylase isoenzymes and although they can assemble into fibrils that are stabilized by trivalent pyridinoline cross-links, these fibrils are

thinner and cross-links were less prevalent in the Col2a1^{+ex2} ki/ki and heterozygous (ki/+) cartilage than in +/+ cartilage. The data suggest that the retention of an extra 69 amino acids cysteine-rich globular domain in the $\alpha 1$ (IIA) chain would structurally alter the N-propeptide of type XI collagen resulting in changes in the regulation of the type II–XI collagen fibril diameter and the cross-linked collagen network.

2. Results

2.1 Collagen electrophoretic and post-translational analyses

Electrophoresis of pepsin extracted and purified collagen from the P8 mouse rib cartilages revealed several Coomassie blue stained pepsin resistant bands (Figure 1A). Mass spectrometry was used to identify the bands and representative tryptic peptides from the bands are tabulated in Figure 1B. Purified type II collagen revealed component $\alpha 1$ (II) chains which in ki/ki cartilage had assembled solely from type IIA procollagen molecules (Figure 1A, lane: ki/ki 0.7M NaCl precipitate; ppte).

The post-translational 3-hydroxylation of proline residues in the $\alpha 1$ (IIA) collagen chains from ki/ki cartilage was comparable to that in $\alpha 1$ (II) chains from +/+ cartilage (Figure 1B, %3Hyp occupancy in bands 1 and 2). Mass spectrometry showed 89%, 58% and 15% 3-hydroxylation at Pro-986, Pro-944 and P-707 respectively in the $\alpha 1$ (IIA) chain from ki/ki cartilage and 92%, 61% and 18% in $\alpha 1$ (IIB) chain from +/+ cartilage. Purified type XI collagen from the ki/ki cartilage (Figure 1A, lane: ki/ki 2.2M ppte) clearly showed $\alpha 1$ (XI): $\alpha 2$ (XI): $\alpha 3$ (XI) chains in a 1:1:1 ratio confirming a heterotrimeric type XI collagen molecule had formed. Mass spectrometry of the $\alpha 3$ (XI) band from the ki/ki mouse cartilage (Figure 1A, band 3), identified peptides originating from $\alpha 1$ (IIA) protein, indicating that the $\alpha 3$ (XI) is the product of the *Col2a1* gene (Figure 1B). Although not as intensely stained, type XI collagen bands were also observed in the heterozygous mouse cartilage (ki/+ 2.2M ppte). In wild type cartilage (+/+ 2.2M ppte), residual type II collagen was observed under the same precipitating conditions indicating only partial purification of type XI collagen, as has sometimes been observed when collagen concentrations in extracts are high (Fernandes et al., 2007b). Comparable 3Hyp occupancy was also observed at all three proline residues in $\alpha 3$ (XI) chains from +/+ and ki/ki cartilage (Figure 1B, bands 3 and 6). Comparing 3Hyp occupancies of $\alpha 1$ (II) and $\alpha 3$ (XI) (same chain but assembled in different molecules) prepared from +/+ cartilage and ki/ki cartilage showed increases in $\alpha 3$ (XI) in both at P-986 (8% increase), P-944 (12%), P-707 (33%) and P-986 (11% increase), P-944 (12%), P-707 (22%) respectively.

To determine if type IIA procollagen chains were incorporated as $\alpha 3$ (XI) chains in collagen XI heterotrimers, immunoprecipitation of newly synthesized type XI collagen molecules from RIPA buffer extracts of P8 rib cartilage using an anti- $\alpha 2$ (XI)-N-propeptide antibody (Fernandes et al., 2007a) followed by western blotting with anti-type IIA Exon 2 antibody (Lewis et al., 2012; Reardon et al., 2000) was carried out. Clearly as seen in Figure 1C pro $\alpha 1$ (IIA) and pN $\alpha 1$ (IIA) chains in ki/ki cartilage were detected. No pro $\alpha 1$ (IIA) or pN $\alpha 1$ (IIA) bands were detected when the immunoprecipitating antibody was left out (Figure 1C, lane: ki/ki no primary Ab; 1°). The anti-type IIA antibody also detected similar bands in ki/+ and +/+ cartilages, indicating that some type IIA procollagen was incorporated in type

XI collagen in +/+ and ki/+ rib cartilage. For the ki/ki cartilage this conclusively proved that $\alpha 1(\text{IIA})$ procollagen chains were incorporated in the type XI collagen molecule having a molecular chain composition of $\alpha 1(\text{XI})\alpha 2(\text{XI})\alpha 1(\text{IIA})$.

2.2 Collagen heteropolymer analysis

Pepsin- extracted collagen from ki/ki, ki/+, +/+ rib cartilage run on SDS-PAGE stained by Coomassie Blue are shown in Figure 2A. Western blot of a similar gel separation using a monoclonal antibody (1C10) specific to residues 934–945 in the $\alpha 1(\text{II})$ chain in Figure 2B (Fernandes et al., 2003b) identified the $\alpha 1(\text{II})$ chains in extracts of ki/ki, ki/+ and +/+ cartilages. The Western blot in Figure 2C unequivocally shows that processed type IIA collagen in ki/ki cartilage was cross-linked in a polymer. The monoclonal antibody (10F2) recognizes a pepsin-generated epitope in the C-telopeptide domain of type II collagen when it is attached to triple-helical domain cross-linking sites as illustrated in Figure 3A (Fernandes et al., 2003a). As seen in Figure 2C, it recognizes $\alpha 1(\text{II})$ collagen chains in pepsin-extracts of ki/ki, ki/+ and +/+ cartilages.

To determine if a type XI-to-type II collagen cross-links had formed, we used the antibody 5890 (Fernandes et al., 2007b) which recognizes an epitope in the N-telopeptide of the $\alpha 1(\text{XI})$ chain even when cleaved from $\alpha 1(\text{XI})$ but remaining cross-linked to another chain, illustrated in Figure 3B. Figure 2D, shows that pAb 5890 recognizes $\alpha 1(\text{II})$ chains from ki/ki, ki/+ and +/+ cartilages and a pepsin-solubilized bovine control $\alpha 1(\text{II})$ chain (lane 4). A second band migrating slightly slower than $\alpha 1(\text{II})$ chain is evident in ki/ki, ki/+ and +/+ lanes. This band migrates in the position of a standard bovine $\alpha 3(\text{XI})$ chain (lane 5), and which pAb 5890 also recognizes. The results indicate that type XI molecules can be linked to each other and to type II collagen molecules through their N-telopeptide domains in ki/ki and ki/+ cartilage as in +/+ cartilage.

2.3 Collagen cross-link analysis

Table 1 shows that pyridinoline cross-links are present in P7 and P28 day mouse rib cartilage collagen. Hydroxylysyl pyridinoline is the predominant cross-link in ki/ki and ki/+ as in +/+ cartilage. The total pyridinoline content and HP/LP ratio showed trends to lower levels in ki/+ and ki/ki cartilage at P7 and P28 days when compared to wild type.

2.4 Transmission electron microscopy

To visualize the ultrastructure of assembled type IIA collagen fibrillar network, rib cartilage from ki/ki, ki/+ and +/+ mice were analyzed by transmission electron microscopy. High magnification electron micrographs clearly showed banded collagen fibrils in P28 rib cartilages of all three phenotypes. (Figure 4). Fibrils that formed exclusively from type IIA procollagen (ki/ki) had smaller mean diameters (asterisk, 26.5 ± 6.8 nm) when compared to +/+ (arrowhead, 41.2 ± 9.8 nm) where thick fibrils were predominant. In the ki/+ cartilage (36.8 ± 15.7 nm) where both type IIA and type IIB procollagen is synthesized, thin fibrils (asterisks) with diameters similar to those seen in ki/ki cartilage were also seen, but larger diameter fibrils (arrow) and aggregations reminiscent of fibril fusions (double arrows) were also detected in some areas of the ki/+ cartilage matrix. The observations are representative of all animals examined for the respective phenotype.

3. Discussion

Type IIA collagen fibril

Although the 'embryonic' type IIA procollagen isoform was first identified around two decades ago (Ryan and Sandell, 1990), a structural role for this protein in polymers has been difficult to prove since the processed molecules lacking N-propeptides are identical in sequence. Evidence that type IIA procollagen molecules are incorporated and persist in structural fibrils in certain tissues naturally, have come from studies on the vitreous humor of the adult eye; immunogold electron microscopy has shown type II procollagen fibrils labeled with anti-type IIA exon 2 antibody (Reardon et al., 2000). The type IIA procollagen knock-in mouse persistently expresses only the type IIA procollagen isoform in cartilage. Here we conclusively show that the type IIA procollagen isoform can indeed be processed and cross-linked to form heteropolymeric fibrils and become a structural component in rib cartilage.

Electron microscopy (Figure 4) and biochemical analyses (Figures 1, 2) indicate that type IIA collagen fibrils are deposited in the extracellular matrix of *ki/ki* rib cartilage. Clearly, in the absence of type IIB procollagen, type IIA procollagen molecules containing all $\alpha 1(\text{IIA})$ chains, can be processed as we have previously shown (Lewis et al., 2012) and can primarily assemble into fibrils of smaller diameters compared to those seen in wild-type cartilage matrix. It is known that cartilage typically has two distinct populations of collagen fibrils; one thin (~20 nm in diameter) and the other thick (>40 nm diameter) (Hagg et al., 1998; Holmes and Kadler, 2006; Parry, 1984). For *ki/+* rib cartilage, although banded collagen fibrils are seen, it is apparent that individual fibrils are thinner than in *+/+* cartilage but fibril fusions were also apparent. One possible explanation for this is the partial cleavage of N-propeptides from type II collagen molecules having one or more $\alpha 1(\text{IIA})$ chains. Theoretically in heterozygous cartilage, 1/8 of the trimeric type II procollagen molecules can have all pro $\alpha 1(\text{IIA})$ chains, 1/8 can have all pro $\alpha 1(\text{IIB})$ chains, 3/8 of the type II procollagen molecules can have one pro $\alpha 1(\text{IIA})$ chain and 3/8 can have 2 pro $\alpha 1(\text{IIA})$ chains. In *ki/+* epiphyseal cartilage, both pro $\alpha 1(\text{IIA})$ and pro $\alpha 1(\text{IIB})$ chains as well as fully processed $\alpha 1(\text{II})$ chains are present (Lewis et al., 2012) and this is also apparent in rib cartilage (data not shown). Whether the polymeric type II collagen in *ki/+* rib cartilage includes heterotrimeric molecules containing $\alpha 1(\text{IIA})$ and $\alpha 1(\text{IIB})$ chains or only homotrimeric molecules of $\alpha 1(\text{IIA})$ or $\alpha 1(\text{IIB})$ is unknown. If the N-propeptides are retained in molecules that have at least one or two $\alpha 1(\text{IIA})$ chains, it is possible that the presence of an extra 69 amino acids in the $\alpha 1(\text{IIA})$ N-propeptide could alter the structure sufficiently to disturb cleavage by ADAMTS-2 or ADAMTS-3, the known type II collagen N-propeptidases (Fernandes et al., 2001; Le Goff et al., 2006) resulting in problems with lateral size control during fibrillogenesis. It is noteworthy that when the N-propeptides are retained on type IIB collagen molecules, extremely thin fibrils have been observed in the extracellular matrix by transmission electron microscopy (Fernandes et al., 2003a). Also when the N-propeptides of $\alpha 1(\text{I})$ collagen chains are retained within type I collagen molecules, fibril fusions are present in skin from Ehlers Danlos Syndrome VII patients and in dermatosparactic cattle (Colige et al., 1999; Fernandes et al., 2001; Holmes et al., 1993; Smith et al., 1992; Watson et al., 1998; Watson et al., 1992). More recently, mutations in the

N-propeptide of $\alpha 1(V)$ collagen chain have been resulted in type I/type V fibril fusions in the skin of patients with a rare form of Ehlers Danlos Syndrome (with kyphoscoliosis) when observed by electron microscopy (Symoens et al., 2011). Interestingly, type III collagen, which has an N-propeptide domain homologous in length and sequence to that of $\alpha 1(IIA)$, is known to retain its N-propeptides in polymeric fibrils in various tissues (Wu et al., 2010).

Studies on the *ki/ki* mouse cartilage also revealed that the type IIA collagen molecules formed cross-linked polymers (Figure 2C) where the C-telopeptide in one type IIA collagen molecule is cross-linked to the helical region in another type II collagen molecule. The results in Figure 2D further showed that the $\alpha 1$ N-telopeptide in one type XI collagen molecule is cross-linked to the helical region in the $\alpha 1(II)$ chain in a type IIA collagen molecule and therefore a heteropolymeric fibril of type IIA and type XI collagen had also formed in the *ki/ki* cartilage. These results are consistent with the known cross-linking profile and chain-specific interactions of $\alpha 1(IIIB)$ C-telopeptide and $\alpha 1(XI)$ N-telopeptide domain with type II collagen (Fernandes et al., 2003a; Wu and Eyre, 1984, 1995).

Post-translational modification of type IIA collagen: Lysyl oxidase based cross-links and prolyl 3 hydroxylations

In the *ki/ki* rib cartilage, fibrillar collagen is cross-linked by the lysyl oxidase mechanism resulting in mature pyridinoline cross-links (Table 1). Clearly, type IIA collagen molecules are able to assemble into microfibrils with the precise molecular overlap and proximities required for the trivalent cross-links to form. Hydroxylysyl pyridinoline is the predominant cross-link, indicating that the lysines at residues 87 and 930 in $\alpha 1(IIA)$ triple helix and in the N-telopeptides and C-telopeptides can be post-translationally hydroxylated by lysyl hydroxylases, *Plod1* and *Plod 2* respectively (Fernandes et al., 2007a; Hautala et al., 1992; van der Slot et al., 2003; Yeowell and Walker, 1999). Since total pyridinoline content expressed as moles/mole of collagen was reduced in P7 and P28 day *ki/ki* and *ki/+* when compared to *+/+* rib cartilage (Table 1), the conversion of these specific hydroxylysines to hydroxylysine aldehydes by the enzyme lysyl oxidase (Eyre et al., 2008) was likely compromised. Whether this relates to the prevalence of smaller diameter collagen fibrils in the P28 day rib cartilage or if the lower pyridinoline content confers altered mechanical properties to type IIA collagen fibrils in rib cartilage as the mice get older is not known but no obvious respiratory problems due to thoracic insufficiency were noticed in mice that lived for a year. These biochemical observations on rib cartilage and previous immunohistochemical observations on epiphyseal and articular cartilage have stimulated further studies where biomechanical properties and susceptibility of the ECM to mechanical and enzyme degradation are being examined.

The importance of post-translational prolyl 3-hydroxylation for the assembly and structure of fibril forming collagens is only beginning to be explored (Hudson et al., 2012). It notable that prolyl 3-hydroxylase 1 null mice display abnormalities in the collagen-rich tissues such as tendon, skin, and bone (Vranka et al., 2010) and a knock out of *Crtap* (a gene encoding a P3H1 associated protein originally found in cartilage), results in a generalized connective tissue disorder with disturbed collagen fibrillogenesis in bone and cartilage and lack of 3Hyp at Pro-986 in $\alpha 1(I)$ and $\alpha 1(II)$ chains (Baldrige et al., 2010; Morello et al., 2006). Mass

spectrometry showed that the degree of post-translational 3-hydroxylation of proline residues in the $\alpha 1(\text{IIA})$ chains from ki/ki cartilage is similar to that found in the $\alpha 1(\text{IIB})$ chains from +/+ cartilage (Figure 1B) and so the probability that the assembly of thin type IIA collagen fibrils in the ki/ki cartilage was influenced by a variation in 3Hyp occupancy was low. However, since the prolyl 3-hydroxylase 1 and prolyl 3-hydroxylase 2 (enzymes encoded by the *lepre1* and *lepre11* genes) are responsible for the 3-hydroxylation of P-986 (Weis et al., 2010) and P-944 (Fernandes et al., 2011) respectively, it is clear that that even the ‘embryonic’ type IIA collagen chain is a substrate for these isoenzymes.

Incorporation of $\alpha 1(\text{IIA})$ form of $\alpha 3(\text{XI})$ in type XI collagen

Col2a1 encodes not only the $\alpha 1$ chain of homotrimeric type II procollagen but also the $\alpha 3$ chain of type XI procollagen; these chains only differ with respect to increased glycosylation of the $\alpha 3$ chain (Burgeson and Hollister, 1979; Eyre and Wu, 1987; Furuto and Miller, 1983). Type XI collagen functions as a template for type II collagen fibrillogenesis and retained type XI collagen N-propeptides limit type II collagen fibril diameter (Blaschke et al., 2000). By purifying type XI collagen from ki/ki rib cartilage and confirming by mass spectrometry that the $\alpha 3(\text{XI})$ chain was $\alpha 1(\text{II})$ in primary sequence, we have shown that the $\alpha 1(\text{IIA})$ chain is incorporated into the type XI collagen molecule. Processing of type XI collagen N-propeptides is complex. The entire $\alpha 1(\text{XI})$ N-propeptide is initially retained, but later only the globular region N-terminal to the minor helix is cleaved; the homologous globular region in the $\alpha 2(\text{XI})$ N-propeptide is rapidly removed after synthesis while the $\alpha 3(\text{XI})$ N-propeptide (normally the $\alpha 1(\text{IIB})$ isoform) is not removed (Thom and Morris, 1991; Wu and Eyre, 1995). Therefore, it was important to determine if the $\alpha 1(\text{IIA})$ procollagen chain was incorporated (as pro $\alpha 3(\text{XI})$ chain) in the type XI heterotrimer and retained its IIA N-propeptide. Clues to the existence of unprocessed $\alpha 3(\text{XI})/\alpha 1(\text{IIA})$ chains came from the persistence of type IIA collagen localization pericellular regions of P28 and P70 articular cartilage and growth plate cartilage from ki/ki and ki/+ mice (Lewis et al., 2012). As seen in Figure 1C, immunoprecipitation of nascent type XI collagen from RIPA buffer extracts of cartilage, followed by western blotting with anti-type IIA Exon 2 antibody showed (albeit qualitatively) that pro $\alpha 1(\text{IIA})$ chains are incorporated in type XI collagen molecules found in ki/ki cartilage as well as in the ki/+ and +/+ cartilage. Since type XI collagen appears to be concentrated in pericellular regions of normal cartilage where the type II collagen fibrils are thin on electron microscopy (Keene et al., 1995), it is tempting to speculate that the cysteine rich (CR) N-propeptide in pro $\alpha 3(\text{XI})/\text{pro}\alpha 1(\text{IIA})$ chains may in part be responsible for the mainly thin fibrils observed throughout ki/ki rib cartilage by electron microscopy (Figure 4). Interestingly, pro $\alpha 1(\text{IIA})$ chains were also found incorporated in the type XI collagen molecules from +/+ and ki/+ cartilage. Indeed, using a rat chondrocyte cell line expressing low levels of type II collagen mRNA, it was shown that incorporation of pro $\alpha 1(\text{II})$ chains with pro $\alpha 1(\text{XI})$ and/or pro $\alpha 2(\text{XI})$ chains into type XI collagen heterotrimers took preference over assembly of pro $\alpha 1(\text{II})$ chains into homotrimers of type II collagen (Oxford et al., 1994).

The question of whether the IIA CR N-propeptide domain encoded by exon 2 plays a distinct role in regulating skeletal development can be answered to some extent from the present study. Per se, it does not seem likely that the CR domain interferes with type IIA

procollagen fibrillogenesis, and this may be expected as homologous domains exist in other procollagens including type I and type III procollagens. However, our findings suggest that the mis-expression of the IIA domain in the N-propeptide of type XI collagen will change the structure of its N-propeptide and thereby alter its function in regulating type II–XI collagen fibril diameter. Previous *in vitro* studies have shown that the CR domain can bind to TGF- β and BMP-2 (Zhu et al., 1999). Subsequent studies in *Xenopus* embryos also suggested a potential role of IIA procollagen in binding to and regulating BMPs (Larrain et al., 2000). However, data from transgenic mice that either lack exon 2 (Leung et al., 2010) or persistently express exon 2 (Lewis et al., 2012) does not suggest an apparent function for the IIA domain in regulating growth factor activity. No cartilage phenotype has been reported in exon 2 knock-out mice, although forebrain development in some of these mutant embryos was found to be affected (Leung et al., 2010). Similarly, no overt skeletal phenotype was observed in the *Col2a1^{+ex2}* mice (Lewis et al., 2012) further suggesting that the IIA CR domain does not regulate growth factor activity during chondrogenesis. However, with persistent expression of IIA domain in the trabeculae of the metaphyseal bone in *Col2a1^{+ex2}* mice (Lewis et al., 2012) and altered bone mass (unpublished observations), the potential of the CR domain regulating BMP activity in post-natal skeletal tissues has still to be properly addressed.

So why is exon 2 spliced out as cartilage develops? It has been proposed that the thin fibrils of cartilage constitute a 10+4 microfibrillar arrangement in which a core of four microfibrils (two of type II collagen and two of type XI collagen) is surrounded by a ring of ten (type II collagen) microfibrils (Holmes and Kadler, 2006). Further, mutations in the N-propeptide of $\alpha 1(V)$ chain of type V collagen - a collagen that regulates the diameter of type I collagen fibrils in non-cartilage tissues - results in altered type I–V collagen fibril diameters as determined by electron microscopy (Symoens et al., 2011). Based on this and our observations on the presence of thinner than normal fibrils in *ki/ki* cartilage, a reasonable explanation is that the retention of the 69 amino acid IIA globular domain in the N-propeptide of type XI collagen $\alpha 1(\text{IIA})/\alpha 3(\text{XI})$ chains (diameter controlling domain) may partly contribute to the thin collagen fibrils by further limiting fibril diameter. Consequently, in *ki/ki* cartilage and perhaps in pre-chondrogenic mesenchyme where type IIA collagen isoforms are predominant and a specific type of collagenous extracellular matrix is required to support developing tissue, the $\alpha 1(\text{IIA})$ chain isoform will be incorporated as the $\alpha 3(\text{XI})$ chain in type XI collagen molecules (Figure 1C), and thin collagen fibrils would assemble. As cartilage matures, a developmentally regulated splicing machinery is initiated to remove exon 2 from $\alpha 1(\text{IIA})/\alpha 3(\text{XI})$ chains (McAlinden et al., 2005; McAlinden et al., 2007) and thicker collagen fibrils, that are perhaps better suited for cartilage biomechanical function, can assemble.

4. Materials and Methods

4.1. Tissue acquisition

Ribs were obtained from *Col2a1^{+ex2}* homozygous knock-in (*ki/ki*), heterozygous knock-in (*ki/+*) and wild type (WT) littermates at post-natal day 7, 8, and 28 (P7, P8, P28). Generation of *Col2a1^{+ex2}* knock-in animals has been described recently (Lewis et al., 2012).

These mice contain a 5' splice site mutation that results in inhibition of *Col2a1* exon 2 alternative splicing. Homozygous ki/ki mice exclusively express the IIA procollagen isoform and heterozygous ki/+ mice express both IIA and IIB procollagen isoforms. WT mice exclusively express the IIB procollagen isoform post-natally. Ribs were dissected free of soft tissues and immediately frozen until further use.

4.2. Collagen Extraction

Ribcages were thawed and the cartilaginous areas of the rib plates from +/+, ki/ki and ki/+ mice were dissected and intercostal muscles removed. Following extraction with 4M guanidine hydrochloride (GuHCl) to remove proteoglycans, the cross-linked collagen network in the residue was depolymerized and extracted using pepsin (Fernandes et al., 2003a). Type II and type XI collagen molecules were precipitated from the pepsin digest at 0.7M and 2.2M NaCl respectively, and harvested by centrifugation (Fernandes et al., 2007b).

4.3. Total Collagen and Collagen cross-link analysis

Rib cartilage was drained of excess liquid, lyophilized, weighed and then hydrolyzed in 6M HCl at 110°C for 24 hrs. An aliquot of the hydrolysate was colorimetrically assayed for hydroxyproline. Collagen content was expressed as % collagen/dry weight. Pyridinoline cross-links were quantified in these samples by C-18 reverse-phase HPLC and fluorometry and expressed as moles per mole of collagen (Fernandes and Eyre, 1999; Fernandes et al., 1998).

4.4. Immunoprecipitation of type XI procollagen

Mouse rib cartilage was extracted using RIPA buffer (Sigma) and cleared using Milipore PureProteome Protein A Magnetic Beads to eliminate non-specific interactions. Immune complexes were formed by incubating the cleared RIPA extracts with anti- $\alpha 2(XI)$ N-propeptide antibody (Fernandes et al., 2007a) for two hours at 4°C. Immune complexes were then incubated with Protein A Magnetic Beads for 15 minutes at room temperature under gentle vortexing. Beads and bound complexes were captured with a magnet (Millipore) and washed 3 times with RIPA buffer. Unbound material was discarded. Bound complexes were released from beads by boiling in Laemmli sample buffer under reducing conditions (Laemmli, 1970) and the type XI procollagen in the immunoprecipitate was probed with anti-type IIA Exon 2 procollagen antibody by western blotting (Lewis et al., 2012).

4.5. Electrophoresis and Western blotting

Pepsin solubilized collagen chains were resolved by Laemmli SDS-PAGE and, when necessary stained with Coomassie Blue. Collagen chains transferred to PVDF membrane were probed with monoclonal antibody 1C10 that recognizes type II collagen chains (Fernandes et al., 2003b). To fingerprint type II collagen polymer formation, the collagen chains were transferred to PVDF and probed with monoclonal antibody (mAb) 10F2 which recognizes a cleavage site (neo-epitope) in a sequence in the C-telopeptide cross-linking domain of type II collagen (Fernandes et al., 2003a). Thus this antibody detects the $\alpha 1(II)$ chain or any other collagen chain cross-linked to the $\alpha 1(II)$ C-telopeptide. Blots were also

probed with polyclonal antibody (pAb) 5890 which specifically recognizes $\alpha 1(\text{XI})$ N-telopeptide stubs that are normally cross-linked to the triple helical region $\alpha 1(\text{II})/\alpha 3(\text{XI})$ chain (Fernandes et al., 2007b). Hence this antibody detects the $\alpha 1(\text{II})$ and $\alpha 3(\text{XI})$ collagen chain cross-linked to the $\alpha 1(\text{XI})$ N-telopeptide indicating a heteropolymer of type II-type XI and a homopolymer of type XI-type XI had formed. Pepsin solubilized and purified bovine type II and type XI collagen were used as standards.

4.6 Mass Spectrometry

Collagen chains were resolved by SDS-PAGE gel electrophoresis under reducing conditions and identified by staining with Coomassie Blue. Individual collagen α -chains were cut out and subjected to in-gel trypsin digestion (Fernandes et al., 2007b). Electrospray MS was performed on the tryptic peptides using an LCQ Deca XP ion trap mass spectrometer equipped with in-line liquid chromatography (LC) (ThermoFinnigan) using a C8 capillary column (300 $\times$ 150 mm; Grace Vydac 208MS5.315) as we have described before (Weis et al., 2010). Sequest search software (ThermoFinnigan) was used for peptide identification using the NCBI protein database. Collagenous peptides not found by Sequest had to be identified manually by calculating the possible MS/MS ions and matching these to the actual MS/MS. The percentage 3-hydroxylation of proline (3Hyp) at a particular site was determined from the abundance of 3Hyp-containing peptide ions as a fraction of the sum of both 3Hyp and Pro versions of the same tryptic peptide (Fernandes et al., 2011).

4.7. Electron microscopy

Rib cartilage from ki/ki, ki/+ and +/+ mice were fixed in with 4% paraformaldehyde and 0.25% glutaraldehyde in 100mM sodium cacodylate buffer, pH 7.4, at 4°C for 24 hours and decalcified in Tris-buffer containing 10% (w/v) EDTA (pH 7.4). The samples were rinsed in phosphate-buffered saline (PBS), dehydrated in an ascending series of ethanol from 30% to 70%, and embedded in the acrylic resin LR White (London Resin Company, London, UK) as we have done before (Hansen et al., 2012; Hjorten et al., 2007). Ultrathin sections were cut with an ultramicrotome and collected on Formvar/carbon-coated nickel grids. Finally, all sections were rinsed with water and negatively stained with 2% uranyl acetate for 7 minutes and analyzed using an EM 410 electron microscope (Philips, Amsterdam, The Netherlands). Fibril diameters were measured and calculated using NIH ImageJ software. 160 – 250 fibrils for each phenotype were evaluated.

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Figure 1. Molecular composition of type II and type XI collagen from P8 ki/ki rib cartilage

A. SDS-PAGE of pepsin extracted collagen from ki/ki, ki/+ and +/+ rib cartilage revealed Coomassie blue stained pepsin resistant collagen chains of type II and type XI collagen in sequential precipitate (ppt) at 0.7M NaCl and 2.2M NaCl respectively.

B. Mass spectrometry of peptides from trypsin digests of type II collagen and type XI collagen (bands 1 – 6) identified masses matching sequences of α1(II) chains in bands 1 and 2 (0.7M ppt) and α3(XI) chains in bands 3 and 6 from +/+ and ki/ki rib cartilage. The percentage (%) of 3-hydroxylation of proline residues (P[#]₉₈₆, P[#]₉₄₄, P[#]₇₀₇) in α1(II) chains from type II and α1(II)/ α3(XI) chains from type XI collagen are also shown. 3Hyp occupancy was comparable for α1(II) chains (bands 1, 2) and α3(XI) chains (bands 3, 6) from ki/ki and +/+ rib cartilage.

C. Immunoprecipitation of newly synthesized type XI collagen from RIPA buffer extracts of ki/ki, ki/+ and +/+ rib cartilage using anti-α2(XI) N-propeptide antibody followed by western blot using anti-type IIA Exon 2 antibody detected pro α1(IIA) and pN-α1(IIA) collagen chains incorporated in type XI collagen from ki/ki as well as ki/+ and +/+ rib cartilage.

Figure 2. Type II and type XI collagen heteropolymer formation in ki/ki P8 rib cartilage

A. Pepsin-solubilized collagen from ki/ki, ki/+, +/+ rib cartilage stained with Coomassie Blue following SDS-PAGE showing $\alpha 1(II)$, $\alpha 1(XI)$, $\alpha 2(XI)$ and $\alpha 3(XI)$ chains.

B. Western blot of samples equivalent to those in **A** (above) and probed with anti-type II collagen antibody (mAb 1C10) confirmed type II collagen chains in all three phenotypes and in bovine type II collagen standard. Partial reactivity was observed for the bovine $\alpha 3(XI)$ chain (also a product of *Col2a1* gene), indicating post-translational differences.

C. Western blot of samples identical to those electrophoresed in **B** (above) and probed with mAb 10F2. This antibody specifically recognizes the C-telopeptide domain of type II collagen when it is cross-linked to $\alpha 1(II)$ collagen chains as observed for ki/ki, ki/+, +/+ and bovine type II collagen. The antibody also detected the bovine $\alpha 1(XI)$ chain and the $\alpha 3(XI)$ chain (migrating slightly slower than bovine $\alpha 1(II)$ chain) in purified type XI collagen standard as we have shown before (Fernandes et al., 2003a).

D. Western blot of samples identical to those electrophoresed in **B** (above) and probed with antibody 5890. This antibody specifically recognizes N-telopeptide domain of $\alpha 1(XI)$ collagen when cross-linked to chains of $\alpha 1(II)$ in ki/ki, ki/+, and +/+ rib cartilage. The antibody also recognized the slower migrating $\alpha 3(XI)$ chains. In bovine standards, the antibody recognized $\alpha 1(II)$ and $\alpha 3(XI)$ as expected (Fernandes et al., 2003b).

Figure 3. Molecular interpretations of collagen heteropolymer assembly from Western blot analysis

A. Antibody 10F2 reacted with the α 1(II) chains, showing that the C-telopeptide domains of α 1(II) had become cross-linked to type II collagen chains from a different molecule (upper panel) and thus a homopolymer of type II collagen (type IIA collagen in ki/ki) had formed (lower panel).

B. Antibody 5890 reacted with the α 1(II) chains and the α 3(XI) chains, indicating that the N-telopeptide of α 1(XI) had become cross-linked to α 3(XI) chains or α 1(II) chains from a different molecule (upper panel) and thus a homopolymer of type XI collagen and a heteropolymer of type XI and type II (type IIA collagen in ki/ki) had formed (lower panel). These heterotypic cross-linking reactions have been demonstrated for type IIB collagen heteropolymers that are present in the matrix of developing cartilage (Fernandes et al., 2007b; Wu and Eyre, 1984, 1995)

Figure 4. Transmission electron microscopy of matrix in P28 ki/ki rib cartilage extracellular matrix

High magnification micrographs of ki/ki rib cartilage (at areas near the cell) showed that type IIA collagen molecules could assemble into thin-banded fibrils (asterisk). The diameters of these fibrils were smaller than the majority of fibrils in the +/+ cartilage (arrowheads) although thin fibrils were also observed in +/+ cartilage (asterisk). In ki/+ cartilage both thin fibrils (asterisk) and thick fibrils (arrow) were seen. Fibril fusions (double arrows) were also observed in the ki/+ cartilage but the mean diameters were still less than the fibrils in +/+ cartilage. Bar = 500nm.

Table 1

Hydroxylysyl (HP) and Lysyl (LP) pyridinoline content in rib cartilage

Mouse	% Collagen dry weight	mole HP mole collagen	mole LP mole collagen	mole total Pyr mole collagen	HP LP
P7 +/-	8.6	0.58	0.12	0.70	4.8
P7 ki/+	9.4	0.49	0.10	0.60	4.9
P7 ki/ki	10.4	0.41	0.10	0.50	4.1
P28 +/-	9.5	0.37	0.05	0.42	7.4
P28 ki/+	10.9	0.29	0.05	0.34	5.8
P28 ki/ki	9.0	0.27	0.07	0.34	3.8

Values represent an average of 3 estimations