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Abstract

Background: Collagen XIα1, encoded by the *COL11A1* gene, is a minor fibrillar collagen that is overexpressed in various human cancers, in which its presence correlates with tumor aggressiveness and progression. **Methods:** In this study, we developed two novel mouse monoclonal antibodies (mAbs), Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9, that target the putative C-telopeptide of human collagen XIα1. The antibodies were raised to the RRHTEGMQA sequence, a unique nine-amino acid stretch within the putative C-telopeptide of human collagen XIα1. **Results:** Corresponding to nearly identical V(D)J gene segments and complementarity-determining regions (CDRs), the antibodies specifically bound the RRHTEGMQA epitope in ELISAs but did not react with the C-propeptide. This specificity was further confirmed with the purified Anti-colXIα1 clone 9 mAb, which demonstrated strong reactivity to recombinant proteins containing the RRHTEGMQA sequence in both ELISAs and Western blot assays. This sequence seems to behave as a linear B-cell neoepitope, in which the RRHT motif is crucial for epitope recognition. Otherwise, no immunodetections were observed either in cultures and lysates from the *COL11A1*-highly expressing A204 human cell line or on tissue sections from specimens of human pancreatic ductal adenocarcinoma (PDAC), with strong desmoplastic reactions, **Conclusions:** Lacking a precise knowledge of the characteristics of the putative C-telopeptide of human collagen XIα1, these antibodies could enhance our understanding of the processing of human procollagen XIα1 and contribute to a better characterization of the tumor microenvironment of *COL11A1*-expressing cancers.

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Keywords: mouse monoclonal antibodies; *COL11A1;* human collagen XIα1; putative C-telopeptide

1. Introduction

The *COL11A1* human gene codes for the α1 chain of human procollagen XI and mature collagen XI, an extracellular minor fibrillar collagen. (Pro)collagen XIα1 is highly synthesized in diverse human cancers, and this expression is correlated with tumor aggressiveness and progression [1–6]. Together with the *INHBA* and *THBS2* genes, the *COL11A1* gene was initially correlated with a multicancer invasion- and metastasis-associated gene expression signature [1]. Later on, this gene was also identified as a member of a pancancer signature present in late-stage aggressive cancers [4]. As components of the extracellular matrix, collagens remodel the tumor microenvironment. Matrix stiffening and collagen fiber alignment, in which collagen XIα1 is thought to be involved, have been shown to promote cancer cell migration [5]. Conversely, knockdown of the *COL11A1* gene significantly diminishes the invasive potential of cancer cells [6].

COL11A1/(pro)collagen XIα1 is mainly expressed by a subset of myofibroblastic cancer-associated fibroblasts (CAFs), which are also specifically characterized by their surface expression of Leucine-Rich Repeat-Containing 15 (LRRC15) protein and integrin alpha-11 (ITGA11) as well as their secretion of Matrix Metalloproteinase-11 (MMP-11, stromelysin 3). These *COL11A1*+ myofibroblasts are prominent in the desmoplastic reaction of human invasive carcinomas [7–11].

Some well-established human cancer cell lines also express high levels of *COL11A1* mRNA/(pro)collagen XIα1 [12] and are useful as models to track the expression and biological significance of this extracellular matrix component.

The canonical sequence of the P12107-1 Name A isoform of human procollagen XIα1 consists of 1806 amino acid residues and has an estimated molecular weight of 181 kDa [13]. Based on comparison with other better-characterized collagens, this procollagen, upon secretion, is expected to be processed by extracellular proteinases excising the N- and C-terminal propeptides [14]. The 21 C-terminal residues of the processed mature extracellular collagen XIα1 -(1543) IQPLPILSSKKTRRHTEGMQA (1563)- would then comprise the putative nonhelical C-telopeptide [13].

Monoclonal antibodies to the C-telopeptide of human collagen XIα1 could help shed light on the actual processing of human procollagen XIα1 and lead to a better characterization of *COL11A1*-expressing tumors and the extracellular matrix microenvironment. In addition, the tuned derivatives of these antibodies, naked, as antibody–drug conjugates (ADCs) or as components of targeted immunovesicles (liposomes, exosomes)/immunonanoparticles, could be employed as therapeutic weapons to combat the progression of *COL11A1*-expressing tumors. However, to date, no antibodies have been generated to this region.

In this study, we developed two novel mouse monoclonal antibodies (mAbs)—Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9—to the putative C-telopeptide of human collagen XIα1 and assessed their reactivity and genetic features.

2. Materials and Methods

2.1. Human Collagen XIα1 and C-Propeptide Recombinant Forms

The putative extracellular processed form of human collagen XIα1 (Figure 1), with an added 6xHis head, recombinantly expressed in CHO cells, was provided by GenScript (Piscataway, NJ 08854, USA). It has a theoretical molecular weight of 98.37 kDa.

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U9642210G0-1_Collagen XIalfa1

Protein sequence: 1077 aa

MGWSCIILFLVATATGVHSHHHHHHQEAQAQAILQQARIALRGPPGPMGLTGRPGPVGGPGSSGAKGESGDPGPQGPRGVQGPPGPTGKPGKRGR
PGADGGRGMPGEPGAKGDRGFDGLPGLPGDKGHRGERGPQGPPGPPGDDGMRGEDGEIGPRGLPGEAGPRGLLGPRGTPGAPGQPGMAGVD
GPPGPKGNMGPQGEPGPPGQQGNPGPQGLPGPQGPIGPPGEKGPQGKPGLAGLPGADGPPGHPGKEGQSGEKGALGPPGPQGPIGYPGPRGV
KGADGVRGLKGSKGEKGEDGFPGFKGDMGLKGDRGEVGQIGPRGEDGPEGPKGRAGPTGDPGPSGQAGEKGKLGVPGLPGYPGRQGPKGSTGF
PGFPGANGEKGARGVAGKPGPRGQRGPTGPRGSRGARGPTGKPGPKGTSGGDGPPGPPGERGPQGPQGPVGFPGPKGPPGPPGKDGLPGHPG
QRGETGFQGKTGPPGPGGVVGPQGPTGETGPIGERGHPGPPGPPGEQGLPGAAGKEGAKGDPGPQGISGKDGPAGLRGFPGERGLPGAQGAPG
LKGGEGPQGPPGPVGSPGERGSAGTAGPIGLPGRPGPQGPPGPAGEKGAPGEKGPQGPAGRDGVQGPVGLPGPAGPAGSPGEDGDKGEIGEPG
QKGSKGDKGENGPPGPPGLQGPVGAPGIAGGDGEPGPRGQQGMFGQKGDEGARGFPGPPGPIGLQGLPGPPGEKGENGDVGPMGPPGPPGPR
GPQGPNGADGPQGPPGSVGSVGGVGEKGEPGEAGNPGPPGEAGVGGPKGERGEKGEAGPPGAAGPPGAKGPPGDDGPKGNPGPVGFPGDPG
PPGEPGPAGQDGVGGDKGEDGDPGQPGPPGPSGEAGPPGPPGKRGPPGAAGAEGRQGEKGAKGEAGAEGPPGKTGPVGPQGPAGKPGPEGLR
GIPGPVGEQGLPGAAGQDGPPGPMGPPGLPGLKGDPGSKGEKGHPGLIGLIGPPGEQGEKGDRGLPGTQGSPGAKGDGGIPGPAGPLGPPGPPGL
PGPQGPKGNKGSTGPAGQKGDSGLPGPPGSPGPPGEVIQPLPILSSKKTRRHTEGMQA*

Figure 1. The amino acid sequence of the putative extracellular processed form of human collagen XIα1 (residues 512 to 1563 of the P12107-1 A isoform), recombinantly expressed in CHO cells, provided by GenScript. It encompasses the C-terminal telopeptide -(1543) IQPLPILSSKKTRRHTEGMQA (1563)- sequence. It is preceded by the MGWSCIILFLVATATGVHS signal peptide and by a 6xHis head.

Similarly, the putative C-propeptide, of 243 amino acid residues (Figure 2), with a theoretical molecular weight of 27.45 kDa, was also recombinantly expressed in CHO cells using GenScript.

Expression Sequence:

MGWSCIILFLVATATGVHSDADDNILDYSDGMEEIFGSLNSLKQDIEHMKFPMGTQTNPARTCKDLQLSHP
DFPDGEYWIDPNQGCSGDSFKVYCNFTSGGETCIYPDKKSEGVRISSWPKEKPGSWFSEFKRGKLLSYLDVE
GNSINMVQMTFLKLLTASARQNFTYHCHQSAAWYDVSSGSYDKALRFLGSNDEEMSYDNNPFIKTLYDGC
ASRKGYEKTVIEINTPKIDQVPIVDVM INDFGDQNQKFGFEVGPVCFLG*

Figure 2. The amino acid sequence of the putative C-propeptide, from human procollagen XIα1 (residues 1564 to 1806 of the P12107-1 A isoform), recombinantly expressed in CHO cells, provided by GenScript. It is preceded by the MGWSCIILFLVATATGVHS signal peptide.

The COL11A1 Fusion Protein, with an N-terminal GST tag, from ChromoTek GmbH & Proteintech, Germany (Cat. No. Ag16509), was recombinantly expressed in *Escherichia coli*. After removal of the GST tag (Ag37791), the first 19 N-terminal amino acid residues PLPILSSKKTRRHTEGMQA corresponded to the putative C-telopeptide (Figure 3). This product has a theoretical molecular weight of 29.6 kDa.

Peptide Sequence:

PLPILSSKKTRRHTEGMQADADDNILDYSDGMEEIFGSLNSLKQDIEHMKFPM
GTQTNPARTCKDLQLSHPDFPDGEYWIDPNQGCSGDSFKVYCNFTSGGETCIYP
DKKSEGVRISSWPKEKPGSWFSEFKRGKLLSYLDVEGNSINMVQMTFLKLLTAS
ARQNFTYHCHQSAAWYDVSSGSYDKALRFLGSNDEEMSYDNNPFIKTLYDGC
ASRKGYEKTVIEINTPKIDQVPIVDVMINDFGDQNQKFGFEVGPVCFLG

Figure 3. The amino acid sequence of the COL11A1 Fusion Protein (residues 1545 to 1806 of the P12107-1 A isoform), recombinantly expressed in *Escherichia coli*, after the removal of an N-terminal GST tag, provided by Proteintech. The first 19 N-terminal (1545) PLPILSSKKTRRHTEGMQA (1563) amino acid residues are part of the putative C-telopeptide. The next 243 amino acid residues (1564 to 1806) constitute the C-propeptide.

2.2. Mouse Immunization and Hybridoma Generation

Peptide conjugates KLH-Cys-GG-RRHTEGMQA and KLH-Cys-GG-EGMQADADD, corresponding to different amino acid stretches of the human procollagen XIα1 around the C-telopeptide, were provided by Abyntek Biopharma S.L. (Zamudio, Bizkaia, Spain). Cysteine and

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glycine residues were added to ensure correct linking to the carrier protein, as previously conducted [15]. Upon reconstitution in sterile water B Braun and in saline for injections, an emulsion was made with the Sigma Adjuvant System (Sigma-Aldrich, Cat. No. S6322) for immunization purposes, following the manufacturer's instructions.

Female 6-week-old DBA/1J mice were obtained from The Jackson Laboratory through Charles River Laboratories S.A., Spain, and female 4-week-old BALB/c mice were obtained from Charles River Laboratories S.A., Spain. The mice were housed in the facilities of the Bioterio of the University of Oviedo under conventional conditions. They were handled following the Guidelines of the Experimental Animal Ethics Committee of the University of Oviedo and after the approval of animal experimental procedures by the Government of the Principality of Asturias, Spain, Resolutions *PROAE 62/2019* and *PROAE 4/2022.*

Mice were i.p. injected with 200 µg of conjugate/500 µl of emulsion, at least 15 days apart. Once hyperimmunized, for hybridoma generation, they were euthanized via cervical dislocation. Spleens were aseptically removed and gently homogenized for fusion with Sp2/0 mouse myeloma cells using standard methods [16].

Hybridoma supernatants, in RPMI Medium/Hypoxanthine–Aminopterin–Thymidine (HAT)/20% Fetal Bovine Serum (FBS), were primarily screened using an indirect ELISA against KLH, mechanical lysates from A204 and A549 cell cultures, and the human collagen XIα1 recombinant form (Figure 1).

A 1:1000 dilution of anti-mouse γ-chain-HRPO (Sigma-Aldrich, Cat, No. A3673) in 10 mM Phosphate-Buffered Saline (PBS)–1% Bovine Serum Albumin (BSA)–0.1% Tween 20 was applied to identify mouse IgG-containing supernatants.

The selected antibody-secreting hybridomas were subcloned twice to ensure antibody monoclonality. Antibody subisotyping was conducted by means of the IsoStrip™ Mouse Monoclonal Antibody Isotyping Kit (11493027001 Roche).

For purified antibody preparations, subclones were progressively adapted to grow in RPMI Medium–20% FBS, subsequently in protein-free and serum-free CD Hybridoma Gibco™ Medium (ThermoFisher Scientific, Cat. No. 11279023)–20% FBS, and finally weaned from FBS.

Cultures were assessed to be free of *Mycoplasm*a by means of the MycoStrip™ Mycoplasma Detection Kit (InvivoGen, Cat. No. rep-mys-10), following the manufacturer´s instructions.

Monoclonal antibodies were purified using an Amersham Biosciences ÄKTA Purifier 10 FPLC System w/ UPC-900 and 1 mL MabCaptureC™ MiniChrom Columns (ThermoFisher Scientific, Cat. No. 5943662001), following the manufacturer´s instructions; dialyzed against 10 mM of PBS, pH 7.4; concentrated via centrifugation in 10K concentrators; and finally filtered through sterile 0.25 µm filters.

2.3. Isotype-Matched Negative Control mAb

Anti-pneumolysin IgG1, kappa PLY-7 mAb [17] served as the isotype-matched negative control.

2.4. Cell Cultures and Lysates

Human rhabdomyosarcoma A204 (no. HTB-82), large cell lung carcinoma NCI-H661 (no. HTB-183), and alveolar lung carcinoma A549 (no. CCL-185) cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM supplemented with sodium pyruvate, L-glutamine, non-essential amino acids, 10% FBS, ascorbate 2-phosphate (37.5 µg/mL) (Sigma-Aldrich, Cat. No. A8960), and 10 ng/mL of recombinant TGF-β1 (PeproTech, Cat. No. 100-21C), in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Cells were cultured for at least 15 days, in T-25 and T-75 flasks (Sarstedt, Cat. No. 83.3910.302 and 83.3911.302, respectively). Passages and cell collections were conducted using trypsinization. Different harvests from each cell culture condition were pooled.

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For immunocytochemistry (ICC), cells were cultured in 4-well culture slides (BD Falcon™, ref. 354114 or Nunc™ Lab-Tek™ II CC2™ (ThermoFisher Scientific, Cat. No. 154917PK or Sarstedt, Cat. No. 94.6170.402) for three–five days.

To generate cell lysates, after the removal of spent media, the flasks were washed with 10 mM of cold PBS–0.1% sodium azide (PBS-SA). Cell cultures were then vigorously collected in cold PBS-SA or a hot SDS-PAGE sample buffer with the help of cell scrapers. Cell lysates were frozen and thawed several times.

2.5. ELISA for Antibody Reactivity Screening and Assessment

Flat-bottom 96-well MaxiSorp Nunc-Immuno plates (ThermoFisher Scientific, Cat. No. 439454) were coated with 1 µg/100 µl well of the human collagen XIα1 recombinant form, the human collagen XIα1 C-propeptide recombinant form, the COL11A1 Fusion Protein with no GST tag (Ag37791), the peptide conjugates used as immunogens, or with 10 µg/100 µl well of mechanical cell lysates, in PBS-SA, pH 7.3, for 6 h at 37 ºC, and they were then blocked with 200 µl/well of PBS-1% BSA-SA for 1 h at 37 ºC and left overnight at 4 ºC. After being washed, the wells were successively incubated with 100 µl/well of crude hybridoma supernatants or with 1 µg/100 µl well of purified monoclonal antibody in PBS-1% BSA-SA (2 h at 37º C); 100 µl well of a 1:1000 dilution of anti-mouse γ-chain-HRPO (Sigma-Aldrich, Cat, No. A3673) in PBS-1% BSA-0.1% Tween 20 (1 h at 37 ºC); and 100 µl well of ready-to-use supersensitive TMB solution for the ELISA (Sigma-Aldrich, Cat, No. T4444) (5 min at 37 ºC). Color development was stopped by adding 100 µl well of 2 M H_2SO_4. Plates were read at 450 nm on a Synergy LX Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). All determinations were made in triplicate and averaged. Data were analyzed using the BioTek Gen5 software. Optical density data were blanked by subtracting blanks (culture media or PBS-1% BSA-SA) from the readings.

Plates were washed three times with 200 µl/well of PBS–0.1% Tween-20 between incubations.

2.6. ELISA Competition Tests with Soluble Peptides

ELISA competition tests with soluble peptides were performed essentially as already described [18]. Immunograde, ≥ 95% pure, synthetic N-acetylated RRHTEGMQA, EGMQADADD, and (450)KRTISIWGT(458) from pneumolysin -as a scrambled control- peptides were supplied by Abyntek Biopharma S.L. (Zamudio, Bizkaia, Spain).

ELISA plates were coated with the human collagen XIα1 recombinant form, as above. The reactivity of each supernatant was titered in ELISAs; an assessed dilution of each supernatant, which rendered a reliable absorbance signal, was chosen as a positive control.

For competition tests, the supernatant dilutions were mixed, at equal volumes, with 1:10 serial dilutions of the synthetic peptide in PBS-1% BSA-SA, and preincubated for 2 h at 37 ºC and overnight at 4 ºC. The next day, the mixtures were then added to the ELISA coated wells and incubated for 2 h at 37 ºC. The rest of the ELISA continued as described above. No peptide was added in the blanks. The inhibitory activity of any blocking peptide sample was estimated from its color reading in relation to the absorbance of the positive controls.

2.7. SDS-PAGE and Western Blot Assays

Human Immunization-Grade Type XI Collagen was purchased from Chondrex (Catalog # 1085).

Purified protein concentrations were estimated using absorbance at 280 nm with a UV-1280 UV-VIS Spectrophotometer (Shimadzu).

A total of 15 µg/lane of recombinant protein or 50 µg/lane or 40 µl/lane of whole cell lysates and 10 µl/lane of PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoFisher Scientific, Cat. No. 26619), was subjected to 12, 10, or 6% polyacrylamide SDS-PAGE under reducing conditions and, subsequently, electrotransferred onto an Immobilon®-NC Transfer Membrane, with a 0.45 µm

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pore size (Sigma-Aldrich, Cat, No. HATF00010), using the Mini-PROTEAN® Tetra Cell (2-gel) and Tetra Blotting Module (BIO-RAD).

As already stated, some cell lysates were also generated by scraping cell cultures in the presence of hot sample buffer.

After blocking overnight at 4 °C in PBS-3% BSA-SA, the membranes were then probed with 10 mL of crude hybridoma supernatants or with 10 μg of purified mAb in 10 mL of PBS-1% BSA-SA with gentle rocking for 2 h at room temperature. After several washing steps with PBS–0.1% Tween-20 and PBS, the blots were incubated with 10 mL of a 1:4000 dilution of anti-mouse γ chain-specific HRPO conjugated (Sigma-Aldrich, Cat, No. A3673) in PBS–1% BSA–0.1% Tween-20, for 2 h as above, and finally developed with 1-Step™ Ultra TMB Blotting Solution (ThermoFisher Scientific, Cat. No. 37574).

2.8. PEP-FOLD4 Peptide Structure Predictions

Peptide sequences were submitted to the PEP-FOLD4 *on-line* server under the current default conditions [19].

2.9. V Gene Sequencing of Hybridomas

Sequencing of the whole heavy and light chains of mAbs was performed using whole transcriptome shotgun sequencing (RNA-Seq), Absolute Antibody Ltd., United Kingdom.

Nucleotide sequences were analyzed using IMGT/V-QUEST (http://imgt.cines.fr) [20].

2.10. Immunocytochemistry (ICC)

ICC procedures were performed by personnel of the Molecular Histopathology Unit in Animal Models of Cancer (IUOPA).

Cells were fixed in 4 % formaldehyde for 15 min in the chamber slide. Primary antibodies were applied, as described in Table 1

Table 1. Antibodies used in ICC analyses.

Primary antibody (species)	Protein recognized	Source	Dilution	Incubation conditions
Anti-colXIα1 clone 9 (mAb)	Human collagen XIα1	In-house	1:300 from 1 mg/mL	48 h at 4 ºC
PLY-7 (mAb)	Pneumococcal pneumolysin	In-house	1:300 from 1 mg/mL	48 h at 4 ºC
Vimentin Antibody (V9) (mAb)	Human vimentin	Santa Cruz Biotechnology, Inc. SC-6260	1:500	48 h at 4 ºC

The primary antibody was omitted in the negative controls. Subsequently, slides were incubated with the EnVision FLEX HRP (DAKO K8000) system for 30 min at room temperature. The samples were then visualized with diaminobenzidine (DAB) for 30 sec. Finally, the immunostained slides were dehydrated, mounted, studied under an Olympus BX61 Automatic Microscope, and photographed using the DP Controller/Manager software, with the assistance of personnel of the Photonic Microscopy and Image Processing Unit, of the Scientific and Technical Services (SCTs) of the Universidad de Oviedo.

2.11. Immunohistochemistry (IHC)

For immunohistochemical techniques, specimens of pancreatic ductal adenocarcinoma (PDAC), with strong desmoplastic reactions, and normal pancreas counterparts from the same patients were provided by the Principality of Asturias BioBank (PT23/077). The specimens were processed

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following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees (Comité de Ética de la Investigación con Medicamentos del Principado de Asturias, reference CEImPA 2022.462).

IHC procedures were performed by personnel of the BioBank. The DAKO Autostainer system was used.

The samples were fixed with 10% formaldehyde for 24 h and embedded in paraffin.

Tissue sections (3 µm) were deparaffinized, rehydrated, and subjected to epitope retrieval using heat induction (HIER) at 95 ºC for 20 min, at pH 9 and pH 6 (Agilent-DAKO), in the Pre-Treatment Module, PT-LINK (DAKO). Endogenous peroxidase activity was blocked with the EnVision™ FLEX Peroxidase-Blocking Reagent (DM821) for 5 min. Subsequently, sections were first incubated with Anti-colXIα1 clone 9 and PLY-7 (dilution 1:100 from 1 mg/mL, both) for 30 min. Subsequently, the Dako EnVision® + Dual Link System-HRP (Agilent-DAKO) was applied. The samples were then stained using DAB chromogen as a substrate in Dako EnVision™ FLEX/HRP (Agilent-DAKO), counterstained with hematoxylin, dehydrated, and then mounted with permanent medium (Agilent-DAKO Mounting Medium, CS703). The sections were studied under a light microscope (Nikon - ECLIPSE C*i*).

The negative controls were processed by omitting the primary antibody

3. Results

3.1. mAb Reactivity

Upon immunization with the two designed immunogens, KLH-Cys-GG-RRHTEGMQA and KLH-Cys-GG- EGMQADADD, we sought to generate mAbs with the following abilities: besides interfering with proteinases, mAbs targeting the RRHTEGMQA sequence that could recognize the extracellular C-telopeptide and those targeting the EGMQADADD sequence that could even recognize procollagen before its processing. However, we were only successful by rescuing hybridomas from BALB/c mice after immunization with the KLH-Cys-GG-RRHTEGMQA immunogen.

The two mAbs described herein were generated from the same BALB/c mouse; both are IgG1 kappa subtype.

Table 2 shows the immunoreactivity of initial crude supernatants, in RPMI-HAT-20% FBS, from the two hybridomas against the components of the conjugates which were used as immunogens.

Table 2. Immunoreactivity against components of the immunogens.

	ELISA[a]		
	Antigen		
Supernatant/mAb	**Immunogen 1 KLH- C - GG- EGMQADADD**	**Immunogen 2 KLH- C - GG- RRHTEGMQA**	**KLH**
Anti-colXIα1 clone 3 #14/08/2023	-	3+	-
Anti-colXIα1 clone 9 #14/08/2023	2+	3+	-
Medium RPMI-HAT-20% FBS	-	-	-

[a] Color development was estimated by eye. # From supernatants in RPMI-HAT-20% FBS, with indication of collection date.

According to these results, the Anti-colXIα1 clone 3 mAb seems to exclusively recognize the KLH-C-GG-RRHTEGMQA conjugate, while the Anti-colXIα1 clone 9 mAb seems to also recognize the KLH-C-GG-EGMQADADD sequence, although with lower intensity.

Table 3 shows some other reactivity characteristics against different antigen preparations.

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Table 3. Some other immunoreactivity characteristics.

	ELISA[a]				Conventional denaturing and reducing Western blot			
					6% gel	12% gel	10% gel	
Supernatant/ mAb	Recombinant collagen XIα1	Recombinant C-propeptide	A204 cell lysate	A549 cell lysate	Purified human collagen XI	Recombinant C-propeptide	A204 cell lysate	A549 cell lysate
Anti-colXIα1 clone 3	3.259	0.000	0.105	0.029	-	-	-	-
Anti-colXIα1 clone 9	3.519	0.000	0.055	0.064	-	-	-	-

[a] ELISA optical density scale of blanked data from 0 to 4.

In the ELISA, the Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9 supernatants showed strong reactivity against the recombinant human collagen XIα1 but not against the recombinant C-propeptide or with cell lysates from the A204 and A549 cell lines. The human rhabdomyosarcoma A204 cell line, which expresses the highest recorded levels of *COL11A1* mRNA [12], was used as a positive control, while alveolar lung carcinoma A549 cells expressing very low levels of *COL11A1* mRNA served as a quasi-negative control. All the Western blots indicated in Table 3 were negative.

To confirm the fine specificity of the two mAbs, blocking assays with soluble free N-acetylated peptides were performed. As shown in Figure 4, there was a clear concentration-dependent inhibition of the recognition of collagen XIα1 by the RRHTEGMQA peptide but not by the EGMQADADD peptide or by the KRTISIWGT scrambled control peptide.

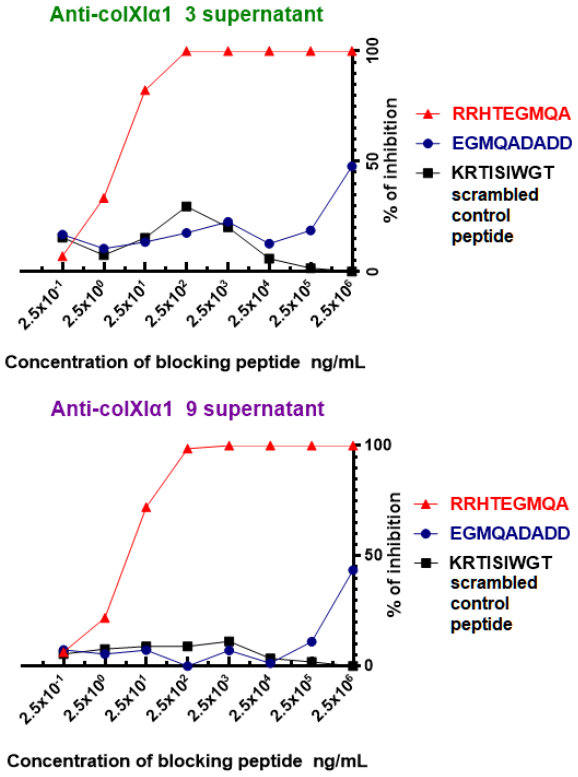

Figure 4. ELISA blocking of recognition of recombinant collagen XIα1, with soluble free N-acetylated peptides. Figure drawn using GraphPad Prism Version 10.0.3 (275) for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

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These results show that both the Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9 supernatants specifically recognize the RRHTEGMQA sequence of the human collagen XIα1 putative C-telopeptide. The lack of inhibition of this recognition by the EGMQADADD peptide points to a prominent role of the RRHT amino acid residues in the recognition of the epitope by these two mAbs.

As the genetic characterization of these two clones showed that they were almost identical (see below), the following observations were made only with the Anti-colXIα1 clone 9 mAb.

The recombinant COL11A1 Fusion Protein from Proteintech was also assayed in an ELISA and Western blot with already finally purified preparations of the Anti-colXIα1 clone 9 and PLY-7 mAbs. Table 4 shows their ELISA immunoreactivity characteristics. The Anti-colXIα1 clone 9 mAb was reactive with collagen XIα1 and, to a lesser extent, with the COL11A1 Fusion Protein but not with the C-propeptide, which lacks the RRHTEGMQA sequence and served as a negative control.

Table 4. The ELISA immunoreactivity of the finally purified preparations of the Anti-colXIα1 clone 9 and PLY-7 mAbs.

	ELISA[a] Antigen (1 µg/ 100 µl well)		
Purified mAb (1 µg/ 100 µl well)	**Recombinant COL11A1 Fusion Protein (Proteintech)**	**Recombinant collagen XIα1 (GenScript)**	**Recombinant C-propeptide (GenScript)**
Anti-colXIα1 clone 9	0.699	2.228	0.012
Irrelevant PLY-7	0.250	0.116	0.109

[a] ELISA optical density scale of blanked data from 0 to 4.

In Western blots, from 12 % SDS-PAGE gels and 15 µg/lane of purified proteins, as shown in Figure 5, the anti-ColXIa1 clone 9 mAb recognized recombinant collagen XIa1 protein (containing degradation products), as well as the COL11A1 Fusion Protein, but not the C-propeptide (see also Supplementary Material S1).

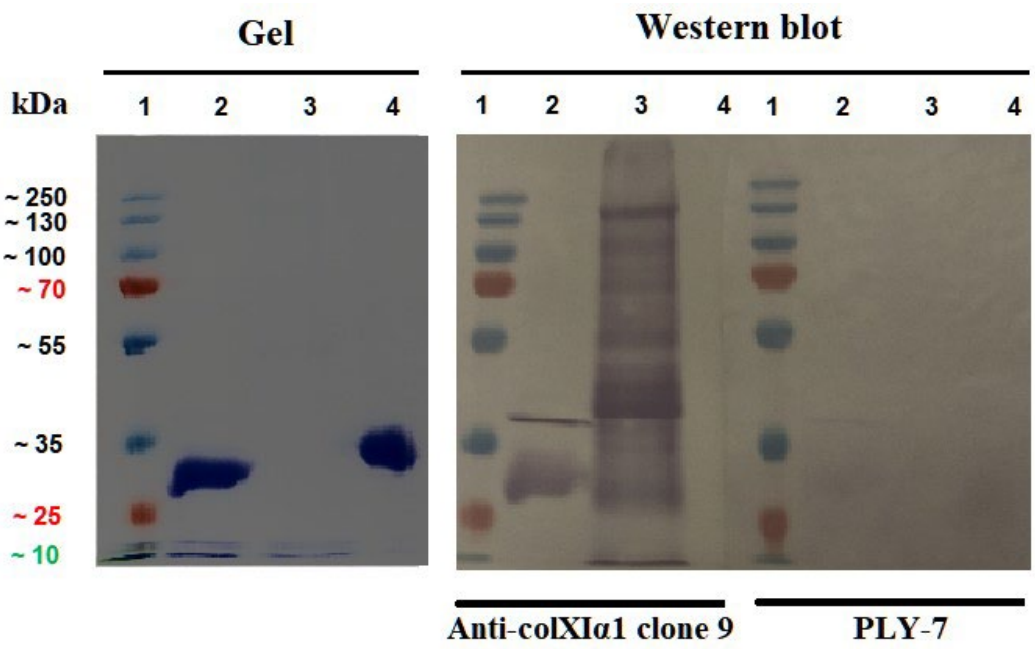

Figure 5. SDS-PAGE gel staining and Western blot of recombinant antigens with finally purified preparations of Anti-colXIα1 clone 9 and PLY-7 mAbs. Lane 1: PageRuler™ Plus Prestained Protein Ladder. Lane 2: COL11A1 Fusion Protein (Proteintech). Lane 3: Collagen XIα1 (GenScript). Lane 4: C-propeptide (GenScript). Full-length blots/gels are presented in Supplementary Figure S5.

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These results confirm that the Anti-colXIα1 clone 9 mAb specifically recognizes, by both ELISA and Western blot, the RRHTEGMQA amino acid stretch which is contained in the sequence of the putative C-telopeptide of human collagen XIα1.

3.2. Peptide Structure Predictions

To gain more insight into the nature of the epitope recognized by the Anti-colXIα1 clone 9 mAb, the structures of peptide sequences corresponding to some of the assayed antigens were predicted using the PEP-FOLD4 server.

Figure 6A shows the structural prediction of the 50 N-terminal amino acid sequence PLPILSSKKTRRHTEGMQADADDNILDYSDGMEEIFGSLNSLKQDIEHMK of the COL11A1 Fusion Protein from Proteintech, with the first 19 N-terminal PLPILSSKKTRRHTEGMQA amino acid residues of the putative C-telopeptide. In this model, the RRH amino acid residues are part of a disordered stretch preceding an α-helix. The prediction for the free RRHTEGMQA peptide is a frank α-helix (Panel B). The structural prediction (Panel C) for the C-terminal RRHTEGMQA stretch is very similar to the one in Panel A above for the last 50 amino acid sequence NKGSTGPAGQKGDSGLPGPPGSPGPPGEVIQPLPILSSKKTRRHTEGMQA of the GenScript's recombinant collagen XIα1 form, whose last 21 C-terminal IQPLPILSSKKTRRHTEGMQA amino acid residues correspond to the putative C-telopeptide. Thus, according to these predictions and the abovementioned ELISA and Western blot analyses, the epitope recognized by the Anti-colXIα1 clone 9 mAb behaves like a linear epitope, with essential recognition of the RRHT amino acid residues.

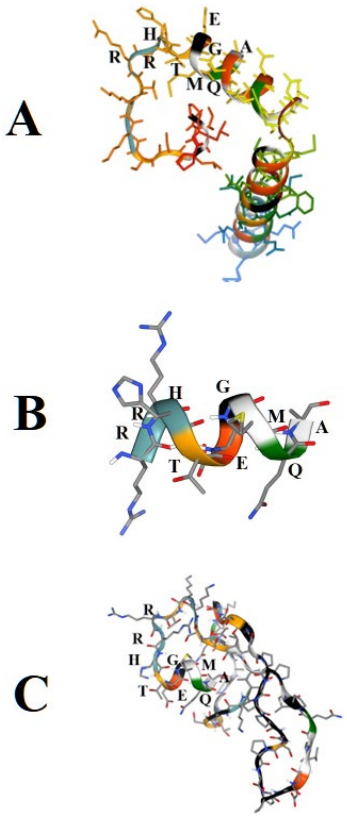

Figure 6. The PEP-FOLD4-derived structural predictions of peptides related to the putative C-telopeptide. Panel A: The 50 N-terminal amino acid sequence of the COL11A1 Fusion Protein from Proteintech, with the first 19 N-terminal PLPILSSKKTRRHTEGMQA amino acid residues of the putative C-telopeptide. Panel B: A free RRHTEGMQA peptide. Panel C: The 50 C-terminal amino acid sequence of the GenScript's recombinant collagen XIα1 form, whose last 21 C-terminal IQPLPILSSKKTRRHTEGMQA amino acid residues correspond to the putative C-telopeptide. In Panels A and B, the N-terminus of the peptides is on the left, but in Panel C, it is on the right.

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3.3. Genetic Characterization of the Two mAbs

Sequencing of the whole heavy and light chains of the two mAbs confirmed that both were IgG1, kappa.

Table 5 shows the most probable usage of germinal V(D)J gene segments by the two mAbs according to the IMGT/V-QUEST (https://www.imgt.org/IMGTindex/V-QUEST.php) analysis. The V_H and V_L domains of the two mAbs were most likely made from the same germinal gene segments.

Table 5. The most probable germinal V(D)J gene segment usage by the two mAbs.

mAb	V_H	D_H	J_H	V_{kappa}	J_{kappa}
Anti-colXIα1 clone 3	**IGHV9-2-1*01 F**	**IGHD2-10*01 F**	**IGHJ3*01 F**	**IGKV8-30*01 F**	**IGKJ4*01 F**
Anti-colXIα1 clone 9	**IGHV9-2-1*01 F**	**IGHD2-10*01 F**	**IGHJ3*01 F**	**IGKV8-30*01 F**	**IGKJ4*01 F**

Table 6 shows the amino acid sequences of the CDRs of the two mAbs, as identified by IMGT/V-QUEST. With very limited nucleotide differences, the CDRs of the V_H domains of the two mAbs are almost identical, as well as the CDRs of their V_L domains, which explains their very similar monoclonal reactivity.

Table 6. Deduced IMGT CDRs.

mAb	CDRH1	CDRH2	CDRH3	CDRL1	CDRL2	CDRL3
Anti-colXIα1 clone 3	GYTFTDYS	INTETG**E**P	**V**RRANYGNAWF**V**Y	QS**L**LYRSN**Q**KNY	WAS	QQYYDYPFT
Anti-colXIα1 clone 9	GYTFTDYS	INTETG**Q**P	**I**RRANYGNAWF**A**Y	QN**L**LYRSN**H**KNY	WAS	QQYYDYPFT

Amino acids that are not identical between the mAbs are in bold.

When the sequences of these CDRs were compared to sequence databases, some individual CDRs were found to be present in different immunoglobulins, but neither the combination of the three CDRs of each V domain nor the combination of the six CDRs of each of the two mAbs were identified in any immunoglobulin reported so far. Thus, this may affirm that they are two novel mAbs.

For patent purposes, in accordance with The Budapest Treaty of 1977, the Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9 mouse hybridomas were deposited in the European Collection of Authenticated Cell Cultures (ECACC) repository with accession numbers 23112901 and 23112902, respectively, given by the International Depositary Authority. They were determined to be free of mycoplasma contamination.

The V_H and V_L nucleotide sequences of the Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9 mAbs were deposited in the GenBank Nucleotide Sequence Database [21] with accession numbers PP150425 and PP150426 and PP150423 and PP150424, respectively.

3.4. Immunocytochemistry (ICC) and Immunohistochemistry (IHC) Analyses

Immunocytochemistry and immunohistochemistry analyses were performed with the finally purified preparations of the Anti-colXIα1 clone 9 and PLY-7 mAbs.

As shown in Figure 7, the Anti-colXIα1 clone 9 mAb developed a rather faint immunostaining in the well-known *COL11A1*-positive A204 and NCI-H661 cell lines but not in the *COL11A1*-quasi-negative A549 cell line. These three cell lines are known to express the cytoskeletal intermediate filament vimentin; their anti-vimentin staining served as an ICC positive control.

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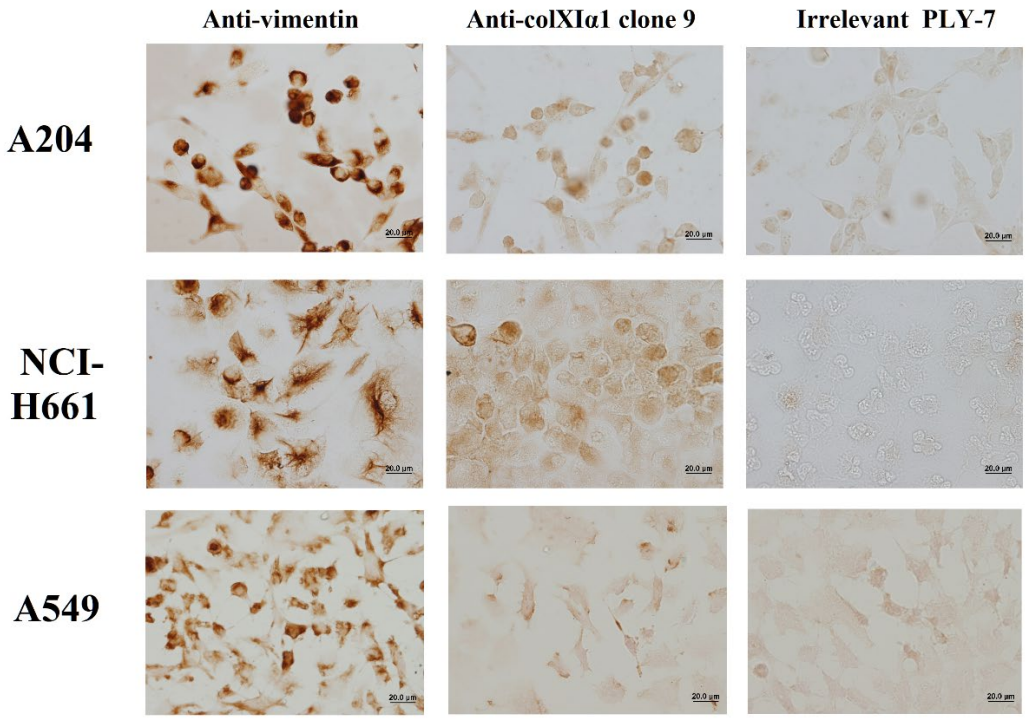

Figure 7. Representative images of immunostaining of cultured cancer cell lines with the purified Anti-colXIα1 clone 9 and irrelevant PLY-7 mAbs. Original magnification: 400x; scale bar: 20.0 µm.

No clear immunostaining patterns were observed in any of the IHC pancreatic ductal adenocarcinoma (PDAC) specimens studied.

4. Discussion

The molecular biology underlying the expression of human procollagen XIα1 is far from being understood. In particular, the function and structural characteristics of (pro)collagen XIα1 have been studied in tendons, vitreous humor, and cartilages from mice, rats, cows, and chickens, among other origins and body locations [22,23], but not in humans. No detailed molecular studies have been conducted on human cancer either. A purified recombinant form of human procollagen XIα1 is not available, and no study has been carried out on its C-terminal enzymatic processing by furin or BMP-1, as in the case of human procollagen Vα1 [24].

Based on similarity with other collagens, it is assumed that the terminal C-telopeptide of the mature human collagen XIα1 corresponds to the (1543) IQPLPILSSKKTRRHTEGMQA (1563) amino acid sequence of procollagen XIα1. According to diverse studies, the C-telopeptide would be involved in intrafibrillar cross-linking with triple-helical regions of adjacent molecules. Minor collagen XIα1 would act as a nucleator in the assembling of extracellular collagen fibrils [14,22,23], and being massively surrounded by major I, II, and III fibrillar collagens [14], this C-telopeptide could remain buried and not exposed on the surface of fibrils, thus hindering its recognition by antibodies. Nevertheless, it could be exposed at the time of enzymatic cleavage by extracellular proteinases and of further processing steps.

In the case of human collagens Iα1, IIα1, and IIIα1, soluble C-terminal cross-linked telopeptides (CTXs) are released and may be detected in biological fluids for diagnostic purposes [25–28] and serve as biomarkers of bone resorption or osteoarthritis. Different antibody preparations, used in immunoassays, are available for detecting and measuring these CTXs and for the immunostaining of extracellular collagens [29,30].

The aim of this study was to rescue and partially characterize two mAbs—Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9— raised to the putative C-telopeptide amino acid stretch of the mature

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human collagen XIα1. They recognize the RRHTEGMQA amino acid sequence but not the EGMQADADD sequence, which includes the DADD amino acids of the N-terminus of the C-propeptide, or the recombinant C-propeptide form. In humans, this RRHTEGMQA nine-amino acid sequence is only present as such in (pro)collagen XIα1 and partially found in some other unrelated proteins.

These two mAbs used the same set of V, D, and J gene segments for the confection of their V domains, and their CDRs are almost identical. Their reactivity is also very similar.

Based on structural predictions and on the results obtained from the ELISA and Western blot assay, the Anti-colXIα1 clone 9 mAb recognizes a linear epitope within the C-telopeptide, in which the RRHT amino acid residues are central to mAb recognition .

Altogether, bearing in mind the low-intensity immunostaining of cultured cells by the Anti-colXIα1 clone 9 mAb, in comparison with its reactivity with the free collagen XIα1 recombinant form and in agreement with the putative C-terminal processing of procollagen XIα1, the RRHTEGMQA epitope sequence should be mostly envisaged as a linear B-cell neoepitope as it is not identified as such in procollagen.

A plethora of antibodies reactive with diverse collagens has been reported and described. A few recognize B-cell neoepitopes, which become exposed after enzymatic processing [31–36]. Thus, the epitope recognized by the Anti-colXIα1 clone 9 mAb may be added to the list of collagen B-cell neoepitopes.

On tissue sections from human PDAC samples, the purified Anti-colXIα1 clone 9 mAb did not stain fibroblast-like cells associated with carcinoma cell niches, as the 1E8.33 mAb does [37]; additionally, no staining of extracellular matrix components was observed in A204 cell cultures. Thus, these observations seem to corroborate the suggested buried status of the C-telopeptide of human collagen XIα1 in the extracellular matrix of the tumor microenvironment; alternatively, the conformation assumed by the RRHTEGMQA stretch in vivo is not recognized by the mAbs.

A human collagen Iα2 C-telopeptide binding antibody has been shown to reduce the rate of cleavage of the C-terminal propeptide by BMP-1 and to limit extracellular fibril formation both in vitro and in organo-typic keloid-like constructs [31,38]. Blocking antibodies to secretory components of myofibroblastic CAFs have been shown to affect carcinoma progression. For instance, an antibody to the microfibrillar-associated protein 5 (MFAP5) suppressed tumor growth in vivo, with a concurrent reduction in the expression of the *COL11A1* gene [39]. Similarly, an antibody to MMP-11 showed significant anti-tumoral effects in animal models [40], as did a humanized antibody to secretory Collagen triple helix repeat containing-1 (CTHRC1) protein [41]. Therefore, antibodies to the C-telopeptide of the mature human collagen XIα1, by interfering with (pro)collagen processing steps, could show a progression restraining capacity of tumors expressing the *COL11A1* gene, thus pointing to therapeutic potential as well.

One main limitation of this study is that there was not available a purified recombinant form of human procollagen XIα1 and that it was not assayed its experimental enzymatic processing. Therefore, it remains to be precisely determined the actual nature and characteristics of the putative C-telopeptide of human collagen XIα1 and if our mAbs do actually recognize the product(s) resulting from in vitro and in vivo processing.

5. Conclusions

To our knowledge, this is the first time that highly specific mAbs to a unique B-cell neoepitope of the putative C-telopeptide of human collagen XIα1 have been generated. These mAbs may be of help to researchers in analyzing the cell biology of human (pro)collagen XIα1 and for a better characterization of *COL11A1*-positive tumors, and, if able to interfere with *COL11A1*-expressing stromal and cancer cells, they could have in vivo therapeutic potential as well

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical and Scientific Committees of the Principality of Asturias, Project no 42/12 and reference CEImPA 2022.462 (February 6, 2023). Human specimens, currently obtained after informed consent to participate, were from the Principality of Asturias BioBank (PT23/077). Mice were handled following the Guidelines of the Experimental Animal Ethics Committee of the University of Oviedo and after the approval of animal experimental procedures by the Government of the Principality of Asturias, Spain, Resolutions *PROAE 62/2019* (December 19, 2019) and *PROAE 4/2022* (March 21, 2022).

Data Availability Statement: The V_H and V_L nucleotide sequences of the Anti-colXIα1 clone 3 and Anti-colXIα1 clone 9 mAbs were deposited in the GenBank Nucleotide Sequence Database [21] with accession numbers PP150425 and PP150426 and PP150423 and PP150424, respectively. Anti-colXIα1 clone 9 and PLY-7 mAbs may be sent upon request, depending on the available stocks.

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Conflicts of Interest: Marcos Ladreda-Mochales is a shareholder of Startquake, S.L.

Abbreviations

The following abbreviations are used in this manuscript:

ADCs	Antibody–Drug Conjugates
ATCC	American Type Culture Collection
BMP-1	Bone Morphogenetic Protein-1
BSA	Bovine Serum Albumin
CAFs	Cancer-Associated Fibroblasts
CDRs	Complentarity-Determining Regions
CHO cells	Chinese Hamster Ovary cells
COL11A1	Collagen Type XI Alpha 1 Chain gene
ColXIα1	Collagen XIα1
CTHRC1	Collagen Triple Helix Repeat Containing-1
CTXs	Cross-linked Telopeptides
DAB	DiAminoBenzidine
ELISA	Enzyme-Linked ImmunoSorbent Assay
ECACC	European Collection of Authenticated Cell Cultures
FBS	Fetal Bovine Serum
GST	Glutathione S-Transferase
HAT	Hypoxanthine–Aminopterin–Thymidine
HRPO	HorseRadish PerOxidase
ICC	ImmunoCytoChemistry

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IHC	ImmunoHistoChemistry
INHBA	Inhibin Subunit Beta A gene
ITGA11	Jntegrin Alpha-11
KLH	Keyhole Limpet Hemocyanin
LRRC15	Leucine-Rich Repeat-Containing 15
mAbs	Mouse Monoclonal Antibodies
MFAP5	MicroFibrillar-Associated Protein 5
MMP-11	Matrix MetalloProteinase-11
PDAC	Pancreatic Ductal AdenoCarcinoma
PBS	Phosphate-Buffered Saline
SA	Sodium Azide
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
TGF-β1	Transforming Growth Factor Beta1
THBS2	Thrombospondin 2 gene
TMB	TetraMethylBenzidine

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