Biochimica et Biophysica Acta 1820 (2012) 1009–1019

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Changes in glycosaminoglycans and proteoglycans of normal breast and fibroadenoma during the menstrual cycle

Cilene Rebouças de Lima [a], José de Arimatéa dos Santos Junior [b], Afonso Celso Pinto Nazário [b], Yara M. Michelacci [a,*]

[a] Disciplina de Biologia Molecular, Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo—UNIFESP, São Paulo, SP, Brazil
[b] Departamento de Ginecologia, Escola Paulista de Medicina, Universidade Federal de São Paulo—UNIFESP, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 29 November 2011
Received in revised form 10 April 2012
Accepted 11 April 2012
Available online 20 April 2012

Keywords:
Fibroadenoma
Proteoglycan
Menstrual cycle
Normal breast
Decorin
Versican

ABSTRACT

Background: Fibroadenoma is the most common breast tumor in young women, and its growth and metabolism may be under hormonal control. In the present paper we described the proteoglycan (PG) composition and synthesis rate of normal breast and fibroadenoma during the menstrual cycle.
Methods: Samples of fibroadenoma and adjacent normal breast tissue were obtained at surgery. PGs were characterized by agarose gel electrophoresis and enzymatic degradation with glycosaminoglycan (GAG) lyases, and immunolocalized by confocal microscopy. To assess the synthesis rate, PGs were metabolic labeled by ^{35}S-sulfate.
Results: The concentration of PGs in normal breast was higher during the secretory phase. Fibroadenoma contained and synthesized more PGs than their paired controls, but the PG concentrations varied less with the menstrual cycle and, in contrast to normal tissue, peaked in the proliferative phase. The main mammary GAGs are heparan sulfate (HS, 71%–74%) and dermatan sulfate (DS, 26%–29%). The concentrations of both increased in fibroadenoma, but DS increased more, becoming 35%–37% of total. The DS chains contained more β-D-glucuronic acid (IdoUA/GlcUA ratios were >10 in normal breast and 2–7 in fibroadenoma). The ^{35}S-sulfate incorporation rate revealed that the *in vitro* synthesis rate of DS was higher than HS. Decorin was present in both tissues, while versican was found only in fibroadenoma.
Conclusions: In normal breast, the PG concentration varied with the menstrual cycle. It was increased in fibroadenoma, especially DS.
General significance
PGs are increased in fibroadenoma, but their concentrations may be less sensitive to hormonal control.

1. Introduction

Fibroadenoma, a benign tumor composed by stromal and glandular tissues, is the most common benign tumor among young women in reproductive phase. Although 25% of affected women are asymptomatic, multiple lesions may occur in 13–20% of them [1,2].

It is well known that the steroid hormones estrogen and progesterone regulate the development of mammary gland and play a role in the initiation and progression of breast cancer [3,4]. The estrogen receptor (ER) and the progesterone receptor (PR) belong to the steroid hormone receptor family of ligand inducible transcription factors [5]. These hormones play important roles in the control of proliferation and differentiation of breast cells, but other tissue components and growth factors may also be involved, underlining the complexity of these control mechanisms [6–8].

In all animal tissues, the cells are located in a microenvironment composed of a dynamic and complex array of collagens, glycoproteins, proteoglycans (PGs) and glycosaminoglycans (GAGs)—the extracellular matrix. Although most animal cells can only grow *in vitro* when they are attached to surfaces through extracellular matrix, which is also the substrate for cell migration, the extracellular matrix provides much more than just mechanical and structural support. The extracellular matrix has implications in developmental patterning, stem cell niches and cancer. It also imparts spatial context for signaling events through cell surface growth factor receptors and adhesion molecules. Extracellular matrix macromolecules can determine the cell behavior, proliferation, differentiation, migration, polarity, and survival by communicating with the intracellular cytoskeleton and playing a part in growth factor signaling.

Abbreviations: PG, proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; BSA, bovine serum albumin; PBS, phosphate buffered saline

* Corresponding author at: Disciplina de Biologia Molecular, Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo—UNIFESP, Rua Três de Maio, 100, 04044-020, São Paulo, SP, Brazil. Tel.: +55 11 5576 4438x1187; fax: +55 11 5573 6407.
E-mail address: yara.bioq@epm.br (Y.M. Michelacci).

0304-4165
doi:10.1016/j.bbagen.2012.04.010

PGs are important components of extracellular matrix and cell surface, and individual PGs interact specifically with other matrix components, such as collagen, laminin and fibronectin, as well as with growth factors and cytokines [9–12]. These interactions may affect cell growth, migration, adhesiveness, and differentiation. Some of these functions are dependent on the protein core, while other rely on the GAG side chains [13–15]. In breast tumors, as well as in a wide variety of tumors, a significant increase in the expression of PGs and GAGs has been described, and the amount of extracellular chondroitin sulfate (CS) in many tumors is high in comparison to the normal tissue of origin [16,17]. However, the magnitude of this increase and the PG structure vary widely depending on the size and type of tumor [18–20].

On the other hand, the synthesis and degradation of GAGs and PGs may be regulated by factors that control the development and function of mammary glands [21,22]. Although hormonal influence has been reported upon breast tissue and malignant tumors [3,23], their effects upon breast tissue GAGs and PGs have not been investigated.

Regarding benign tumors such as fibroadenoma, the number of studies is very low. The changes that occur in the extracellular matrix as a consequence of hormonal fluctuations during the menstrual cycle are poorly understood. Concerning GAGs and PGs, there are even less studies.

The aim of the present paper was to investigate the changes that occur in fibroadenoma GAGs and PGs in comparison to the normal breast tissue during the menstrual cycle.

2. Material and methods

2.1. Patient selection

Patients attending the Department of Gynecology, Division of Mastology, Section of Benign Mammary Diseases of Escola Paulista de Medicina, UNIFESP, were initially selected if they had benign lesions. Only patients with ages ranging from 15 to 35 years and without family history of breast cancer were included. Patients were excluded from the study if they were using oral contraceptives, had endocrine diseases, were pregnant or lactating during the last 12 months, or if the benign lesion turned out to be a manifestation of a more serious breast condition.

Altogether 28 patients were included (Table 1). To better characterize the phase of menstrual cycle, clinical and laboratory parameters were used. Blood was collected 30 min before surgery to determine the serum progesterone levels by chemiluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA), with an assay sensitivity of 0.2 ng/ml [24]. The phase was considered secretory (or luteal) when values were superior to 3 ng/ml. The study protocol was previously approved by the Ethics Committee (Comitê de Ética em Pesquisa—CEP) of Escola Paulista de Medicina, UNIFESP (CEP 1090/09).

2.2. Tissue samples

Samples of fibroadenoma and adjacent normal breast tissue, which were used as matched controls, were obtained at surgery performed at Hospital São Paulo, Sector of Breast Benign Disease, Depto. de Ginecologia (Gynecology), Escola Paulista de Medicina—UNIFESP. Shortly after surgical excision, the fibroadenoma and normal breast tissues were transferred to Depto. de Bioquímica (Biochemistry), Escola Paulista de Medicina—UNIFESP, where all analyses were performed. The tissue samples were dissected, weighed, carefully cut in small pieces, and stored at −80 °C until use for PG extraction or immunofluorescence. For metabolic labeling of PGs, small tissue samples (200–500 mg. wet weight) were collected shortly after surgical excision and, under sterile conditions, were transferred to tissue culture flasks as described in Section 2.4.

2.3. PG extraction

Each sample (350–550 mg, wet weight) was grinded in liquid nitrogen, and incubated with 10 volumes (3.5–5.5 ml) of 4 M guanidine hydrochloride (GuHCl, Aldrich Chemical Co. Inc, Milwaukee, WI, USA) in 0.05 M sodium acetate buffer, pH 6.5, containing protease inhibitors (0.1 M -aminocaproic acid, 6.5 mM benzamidine, 5.5 mM iodocetamide and 0.1 M phenylmethylsulfonyl fluoride), as previously described [17,25]. After overnight incubation at 4 °C

Table 1
Characterization of menstrual cycle and tissue wet weight.

Patient #	Age (years)	Day of cycle	Progesterone (ng/ml)	Phase of the menstrual cycle	Fibroadenoma wet weight (mg)	Normal wet weight (mg)
9	18	2	1.7	Proliferative	398.1	490.5
27	27	2	1.8	Proliferative	1471.5	3286.7
16	21	4	1.0	Proliferative	216.8	265.0
22	20	4	0.9	Proliferative	190.7	241.5
23	27	4	0.3	Proliferative	251.1	375.4
15	22	6	1.1	Proliferative	195.4	465.6
11	17	8	0.2	Proliferative	296.0	376.0
19	35	8	0.7	Proliferative	339.1	332.3
10	22	9	0.9	Proliferative	525.8	655.0
4	22	11	0.7	Proliferative	394.0	681.0
6	28	12	0.7	Proliferative	103.2	213.1
18	16	12	0.5	Proliferative	595.6	122.0
5	19	13	0.5	Proliferative	1470.0	532.0
13	16	14	2.2	Proliferative	547.0	533.6
25	24	14	0.6	Proliferative	360.0	533.7
7	18	16	28.3	Secretory	385.0	639.4
14	29	16	8.6	Secretory	340.2	330.4
20	27	17	10.1	Secretory	146.1	189.6
2	19	18	4.5	Secretory	431.5	282.1
21	17	19	4.1	Secretory	223.6	302.9
1	20	20	5.9	Secretory	298.5	190.2
8	30	23	4.9	Secretory	396.1	435.3
3	33	24	6.6	Secretory	141.3	499.7
12	19	25	7.2	Secretory	288.0	229.0
28	22	25	30.0	Secretory	1052.0	725.0
26	24	27	12.8	Secretory	505.5	1150.0
24	33	28	4.9	Secretory	342.0	552.0
17	30	30	3.3	Secretory	237.7	338.9

under agitation, debris was removed by centrifugation, and the PGs were precipitated by slow addition of methanol (3 volumes) to the supernatant. Subsequently, the precipitates formed after 18 h at −20 °C were collected by centrifugation (1300×*g*, 15 min), washed with 80% methanol, and vacuum-dried. The dried material was resuspended in water (350–550 μl, 1 μl/mg of initial wet weight), and the solutions were stored at −20 °C.

To assess the efficiency of the extraction procedure, the debris (residual tissue not solubilized by GuHCl) were submitted to proteolysis (2 mg/ml papain in 0.05 M phosphate-cysteine buffer pH 6.5, containing 20 mM EDTA, 1 ml per 100 mg of initial wet tissue), as previously described [26]. After overnight incubation at 50 °C, nucleic acids and proteins were removed by precipitation with 10% TCA in 1 M NaCl (10 min, ice bath), and the GAGs were precipitated from the supernatant by the slow addition of 3 volumes of methanol, under agitation. After overnight at −20 °C, the precipitate formed was collected by centrifugation, vacuum-dried, resuspended in 100 μl of water, and stored at −20 °C. The PG and the GAG samples were analyzed by a combination of agarose gel electrophoresis and enzymatic degradation with specific GAG lyases, as described in Section 2.5.

2.4. Metabolic labeling with ^{35}S-sulfate

For metabolic labeling of PGs, small tissue samples (200–300 mg, wet weight) were collected immediately after surgery and, under sterile conditions, washed with 5 ml of phosphate buffered saline (PBS) containing gentamicin (4 mg/ml), and transferred to tissue culture flasks containing 10 ml of F12 medium, antibiotics (penicillin, 10,000 U; streptomycin 100 mg), and ^{35}S-sulfate (50 μCi/ml, IPEN-CNEN, São Paulo, SP, Brazil). The tissue explants were maintained at 37 °C in a 2.5% CO_2 atmosphere, and after 24 h, the medium and the tissue explants were collected and processed separately. The PGs were precipitated from the conditioned culture medium by careful and slow addition of methanol (2 volumes), and after 18 h at −20 °C, the precipitate formed was collected by centrifugation, dried and resuspended in 1 ml of water for analysis. The PGs were extracted from the tissue explants as described in Section 2.3.

2.5. Characterization of PGs and GAGs

GAGs were released from the core proteins by proteolysis with papain (2 mg/ml in 0.06 M phosphate-cysteine buffer pH 6.5 and 20 mM EDTA) as previously described [17]. Aliquots of the intact PGs or GAGs (5 μl containing 0.5–5 μg) were submitted to agarose gel electrophoresis in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9 (PDA) [27]. After fixation with cetyltrimethylammonium bromide and Toluidine Blue staining, PGs and GAGs were quantified by densitometry of the gel slabs (Scanner CS-9000, Shimadzu). These compounds were further characterized by enzymatic degradation with bacterial GAG lyases (chondroitin AC lyase, chondroitin B lyase and heparitinase II from *Flavobacterium heparinum*), prepared [28,29] and incubated [30] as previously described. Chondroitin AC lyase (EC 4.2.2.5) acts only on β-D-glucuronic acid-containing regions of CS and dermatan sulfate (DS), while chondroitin B lyase (EC 4.2.2.19) is specific for α-L-iduronic acid-containing regions of DS [31]. Heparan sulfate (HS) is resistant to both chondroitinases, but is depolymerized by heparitinase II. Briefly, aliquots of either PGs or GAG free chains (50–100 μg) were incubated with 2×10^{-4} units of enzymes in 0.05 M ethylenediamine-acetate buffer pH 8.0 (for chondroitin lyases) or 7.0 (for heparitinase II), in a final volume of 20 μl). After 6 h incubations at the optimum temperature of each enzyme (37 °C for chondroitin AC lyase, 20 °C for chondroitin B lyase and 30 °C for heparitinase II), aliquots of the incubation mixtures were submitted to agarose gel electrophoresis (5 μl) and paper chromatography (15 μl, Whatman No. 1 paper, isobutyric acid/1.25 M NH_3, 5:3, v/v, 24 h). The degradation products were visualized in the paper chromatograms by alkaline silver nitrate staining. The iduronic acid (IdoUA) and glucuronic acid (GlcUA) contents of the galactosaminoglycans (CS and DS) were estimated from the amounts of IdoUA-GalNAc(SO_4) and GlcUA-GalNAc(SO_4) disaccharide repeating units formed by chondroitinases B and AC, respectively.

Polyacrylamide gel electrophoresis of the PGs and GAGs was performed in a Bio-Rad Mini-Protean System. Samples were mixed with sample buffer containing 3% SDS and heated for 10 min at 100 °C. Aliquots (5–10 μl) were applied to gradient gels (3–20%) and run for 4–5 h at 5 V/cm. Gels were stained with 0.2% silver nitrate as previously described [32].

Further identification of decorin and versican was performed by immunoblotting, as described by Sambrook et al. [33]. After either agarose or polyacrylamide gel electrophoresis, intact PGs and their core proteins were transferred to nitrocellulose membranes. After blocking, membranes were probed with either rabbit polyclonal anti-human decorin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, Decorin H-80, sc-22753) or mouse monoclonal anti-versican (Seikagaku Kogyo Company Ltd., Tokyo, Japan, clone 2-B-1, catalog number 270428) (1:3000) in 1% bovine serum albumin (BSA) in PBS. Then, the membranes were washed with PBS and incubated with the appropriate peroxidase-conjugated secondary antibody (Millipore, Temecula, CA, USA, catalog numbers AP304P and 12-349). The antibody binding was visualized through either enhanced chemiluminescence substrate (Pierce ECL Thermo Scientific, Rockford, IL, catalog number 32209) or 3-3′ diaminobenzidine (DAB, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, sc-209686).

The ^{35}S-sulfate labeled compounds were visualized by exposure of the gel slabs or paper chromatograms to a Packard Cyclone TM Storage Phosphor System by 24 h. For quantification, the bands containing radiolabeled compounds were counted in a liquid scintillation spectrophotometer, using Ultima Gold LSC-Cocktail (Packard Instruments Company Inc., Downers Grove, IL, USA). The quantitative results were always corrected for ^{35}S-decay.

2.6. Histology and confocal microscopy

The confocal microscopy was performed as already described [17], with a few modifications. In brief, tissues were fixed in 4% paraformaldehyde in PBS. After 18 h, they were washed in PBS and maintained in 30% sucrose in PBS, at room temperature. After 24 h, the tissues were frozen in Tissue Freezing Medium (Leica Instruments G., Nussloch, Germany) and stored at −20 °C. They were cut in 20 μm sections in cryostat, washed 10 times in PBS, incubated with 0.1 M glycine in PBS for 1 min, washed 5 times with PBS, incubated with 1% BSA in PBS for 10 min, and washed with PBS 10 times. For double immunofluorescence labeling studies, cryosections were incubated for 1 h with two primary antibodies: first anti-decorin and then anti-versican (both diluted 1:50 in 1% BSA-PBS). The cryosections were washed 10 times in PBS (30 s each), and subsequently Alexa-Fluor-conjugated appropriate secondary antibodies (1:50 dilution in PBS) were applied for 1 h. Alexa-Fluor 488 goat anti-rabbit IgG conjugate (green) and Alexa-Fluor 594 goat anti-mouse IgG conjugate (red, Molecular Probes, Eugene, OR, USA) were used. Alternatively, proteins of the cytoskeleton (actin filaments) were labeled by incubation with Alexa-Fluor 586-Phalloidin (1:150 dilution in PBS, 1 h). DNA was labeled by incubation with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride, Molecular Probes, Eugene, OR, USA, 1:1000 dilution in PBS, 5 min). The cryosections were washed 10 times in PBS and a final rinse in distilled water, coverslips were mounted with Fluoromount G/PBS (2:1) (Electron Microscopy Sciences, Fort, Washington, PA, USA), and observed in a Nikon Eclipse E600 microscope equipped with CF160 epi-fluorescence optics. Confocal images were obtained with a Carl Zeiss LSM510 Confocal Microscope.

For histology images, the tissues were cut in 12 μm sections in cryostat, transferred to gelatin-coated slides, dehydrated, rehydrated, and stained by hematoxylin and eosin (H&E). Images were obtained

with an Olympus Q-Color5 U-TVO.5XC-3- Japan microscope and the Image Pro-Plus 5.1 software.

2.7. Statistical analyses

Bartlett's test was used to test if groups are homoscedastic. One-way ANOVA with Dunnett' spot test and Student's *t*-test were performed using JMP Statistical Discovery 8. Significance was defined by P values less than 0.05.

3. Results

3.1. PGs and GAGs from fibroadenoma and adjacent normal breast tissue

Fig. 1A shows a representative agarose gel electrophoresis of PGs extracted from fibroadenomas (F) and their respective adjacent normal tissues (N). PGs from both normal and tumoral tissues migrated as a single broad band, and were quantified by densitometry of the agarose gel slabs. Means ± standard errors of three measurements for each sample (56 samples, 28 fibroadenomas and their respective matched controls) are shown in Fig. 1B. The yield of the extraction procedure was always high (>90%), as assessed by the quantification of the residual GAGs after total solubilization of tissues by proteolysis. In normal breast, the mean PG concentration was higher in the secretory phase of the menstrual cycle ($P<0.01$), and in fibroadenoma the PG concentration was always increased in comparison to normal. Nevertheless, in contrast to normal tissues, the mean PG concentration in fibroadenoma did not significantly vary with the phases of the menstrual cycle. The magnitude of PG increase in fibroadenoma varied from 1.5 to 6 times in different cases, and the differences between fibroadenoma and normal breast were statistically significant in both phases of the menstrual cycle. Fig. 1C that shows that, in normal breast, the PG concentration fluctuates with the menstrual cycle, exhibiting a broad and high peak during the secretory phase. In contrast, although a fluctuation also occurred in fibroadenoma, the highest peak occurred during the proliferative phase.

To further characterize the PGs, the GAG chains were released by proteolysis and submitted to agarose gel electrophoresis in PDA buffer. Two main bands appeared in both tissues (Fig. 2A), migrating as DS and HS, although the DS from fibroadenoma migrated as a more polydisperse band. The identification of these GAGs was confirmed by incubation with specific GAG lyases, and the iduronic acid- and glucuronic acid-contents of DS were estimated by the action of chondroitin B and AC lyases, respectively. Fig. 2B shows that, during the

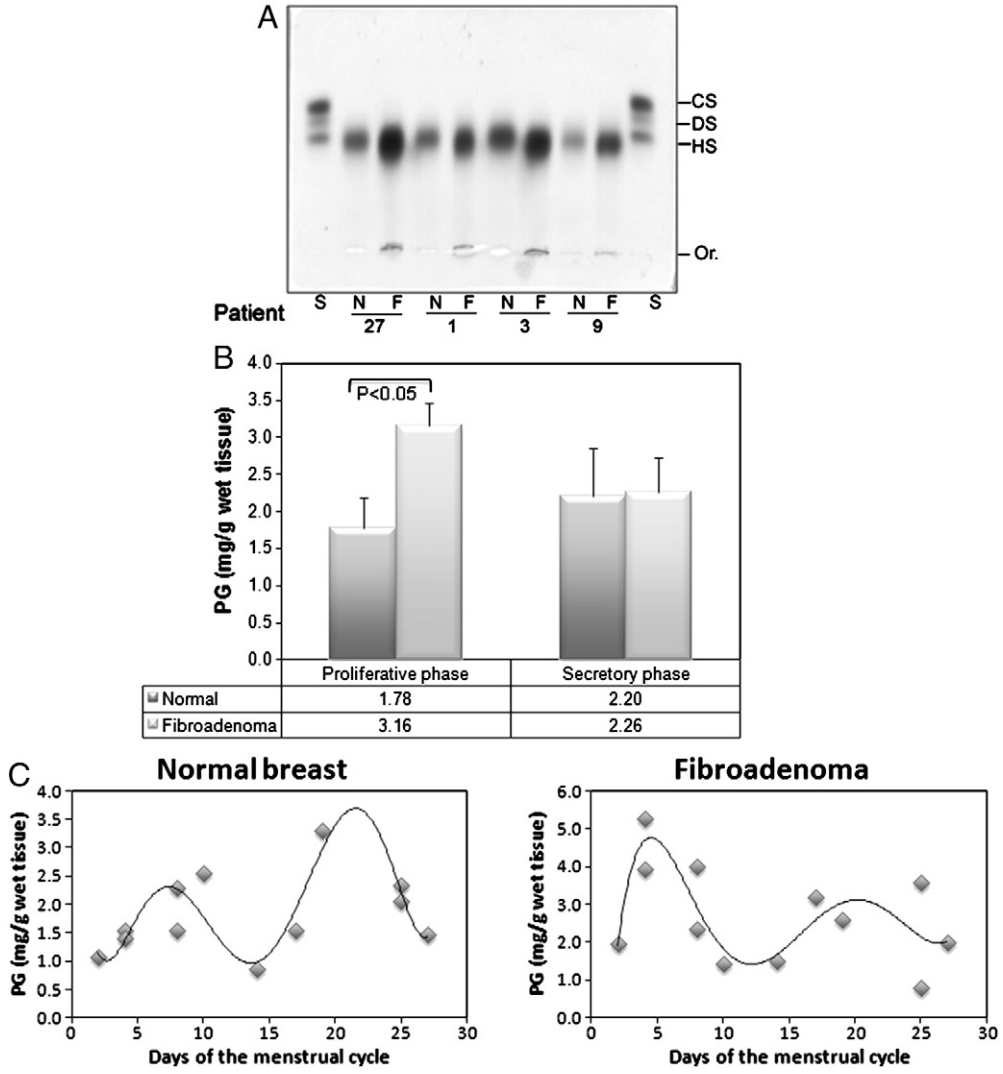
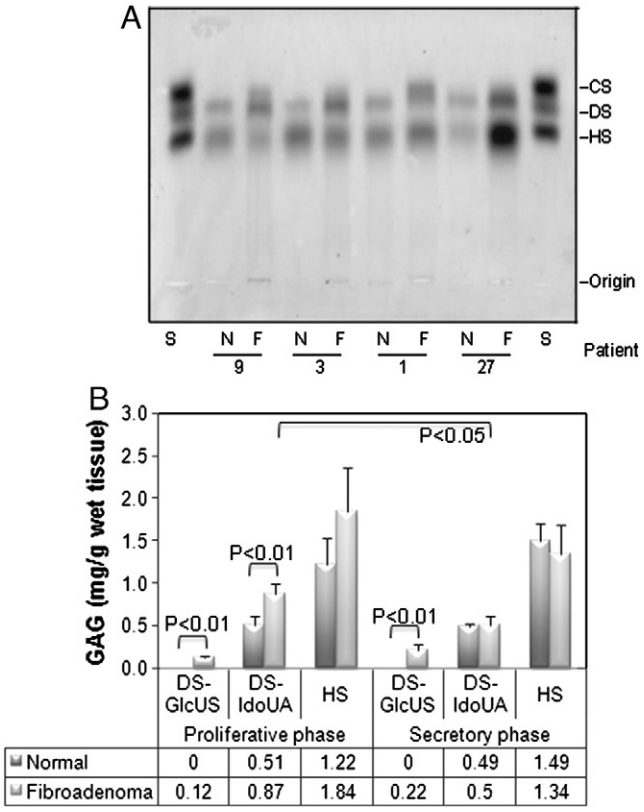


Fig. 1. PGs from human normal breast and fibroadenoma in the proliferative and secretory phases of the menstrual cycle. PGs were extracted from fibroadenoma (F) and normal adjacent breast tissue (N) as described in Section 2.3. Aliquots (5 μl) were submitted to agarose gel electrophoresis in PDA buffer and stained by Toluidine Blue, as described in Section 2.5. (A) Representative agarose gel electrophoresis of PGs extracted from patients 27, 1, 3 and 9 (see Table 1). PGs were quantified by densitometry of the agarose gel slabs, and (B) shows quantitative results (mean ± standard error, brackets indicate differences that were statistically significant). (C) Shows individual data. Each point represents mean of three measurements for each sample. S, mixture of standard GAGs; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate, Or., origin.



Normal	0	0.51	1.22	0	0.49	1.49
Fibroadenoma	0.12	0.87	1.84	0.22	0.5	1.34

Fig. 2. GAGs from human normal breast (N) and fibroadenoma (F) in proliferative and secretory phases of the menstrual cycle. (A) GAGs released by proteolysis from PGs were analyzed by agarose gel electrophoresis in PDA buffer, which separates chondroitin sulfate (CS) dermatan sulfate (DS) and heparan sulfate (HS). The identification of these compounds was confirmed by incubation with specific GAG lyases, and the β-D-glucuronic acid (GlcUA) and α-L-iduronic acid (IdoUA) contents of DS were estimated by degradation with chondroitin AC and B lyases. The average concentration of GAGs ± standard error is shown in (B). Brackets indicate differences that were statistically significant.

secretory phase, both DS and HS were increased in normal breast, and the glucuronic acid-contents of DS was very low (IdoUA/GlcUA ratios >10). In fibroadenoma, the contents of glucuronic acid were increased, and the iduronic acid contents decreased in the secretory phase (IdoUA/GlcUA ratios 7.58 and 2.19 in proliferative and secretory phases, respectively). In both phases, the fibroadenoma DS was increased in comparison to normal breast, going from 26%–29% of total GAGs to 35%–37%, especially concerning its glucuronic acid-containing regions.

3.2. Metabolic labeling of PGs

To investigate the PG synthesis rate, 50 tissue samples (25 fibroadenoma and 25 normal adjacent breast tissue, used as paired controls), obtained immediately after surgery, were transferred to tissue culture flasks containing F12 medium and ^{35}S-sulfate. After 24 h at 37 °C, both the conditioned medium and the tissue explants were collected and analyzed for ^{35}S-PGs, as described in Section 2.4.

Fig. 3 shows a representative agarose gel electrophoresis of PGs extracted from fibroadenoma and normal breast, analyzed by Toluidine Blue staining (Fig. 3A) and radioautography (Fig. 3B). Upon Toluidine Blue staining, only the standard GAGs were visualized due to the low amounts of PGs, while radioautography showed two main bands, both in conditioned culture medium (M) and tissue explants (PG). Small amounts of ^{35}S-GAGs were isolated from the tissue residues (after PG extraction by GuHCl), migrating as sharp bands (GAG). Fig. 3C shows that the ^{35}S incorporation rate in PGs was higher in almost all fibroadenoma samples (except for one case in each phase), in comparison to their respective matched controls. The magnitude of this increase varied among different samples, but the differences between normal and tumoral tissues were significant for both tissue and medium ^{35}S-PGs (Fig. 3D).

To further characterize these compounds, the GAG chains were released from the ^{35}S-PGs by proteolysis, and characterized. Fig. 4A shows that the main GAGs from both normal breast and fibroadenoma migrated as ^{35}S-DS and ^{35}S-HS. Nevertheless, the incorporation of ^{35}S-sulfate in DS was higher than in HS, possibly due to a higher turnover rate of this GAG. The identification of these compounds was confirmed by degradation with chondroitin AC lyase, chondroitin B lyase and heparitinase II. Both ^{35}S-DS and ^{35}S-HS were increased in fibroadenoma, in comparison to normal breast (Fig. 4B).

Fig. 5 shows that the fibroadenoma ^{35}S-DS contained increased amounts of GlcUA in comparison to normal breast, both 4-sulfated and 6-sulfated disaccharide units (Di4S-GlcUA and Di6S-GlcUA, respectively). The fibroadenoma/normal breast ratios were lower for 4-sulfated iduronic acid-containing disaccharides (Di4S-IdoUA), and no 6-sulfated iduronic acid-containing disaccharides (Di6S-IdoUA) were found.

3.3. Immunoblotting of extracellular matrix PGs

The core protein of extracellular matrix PGs was identified by immunoblotting. Decorin was detected in both normal breast tissue and fibroadenoma, during all the menstrual cycle, at higher concentrations in fibroadenoma (Fig. 6). In contrast, versican was detected only in fibroadenoma, at higher concentrations during the secretory phase of the menstrual cycle. The decorin core protein was also detected after incubation of the PGs with chondroitin AC and B lyases (Fig. 6).

3.4. Histology and confocal microscopy

Fig. 7 shows representative images of normal breast and fibroadenoma obtained during the proliferative and the secretory phases of the menstrual cycle.

The distribution of decorin and versican in normal breast and fibroadenoma was analyzed by confocal microscopy, and Fig. 8 shows that decorin (green) occurs in both normal tissue and fibroadenoma, especially in the intra-lobular stroma, in both phases of the menstrual cycle, while versican (red) was detected only in fibroadenoma.

4. Discussion and conclusions

4.1. Discussion

Fibroadenomas are benign tumors of the breast typically composed of stromal and epithelial cells. Since they arise from lobules, it is not surprising that they occur predominantly in women in the 15–25 age group, when the main feature of breast development is the addition of lobular structures to the already developing duct system.

Fibroadenomas comprise about 50% of all breast biopsies, and this rate rises to 75% for biopsies in women under the age of 20 years [2,34]. The tumors are round or ovoid, elastic, nodular, and have a smooth surface. Approximately 90% of fibroadenomas are less than 3 cm in diameter, indicating that its growth is usually self-limited, but despite their prevalence, the mechanisms controlling fibroadenoma development and growth are poorly understood.

Analyses of the cellular components of fibroadenomas demonstrated that both the stromal and the epithelial cells are polyclonal

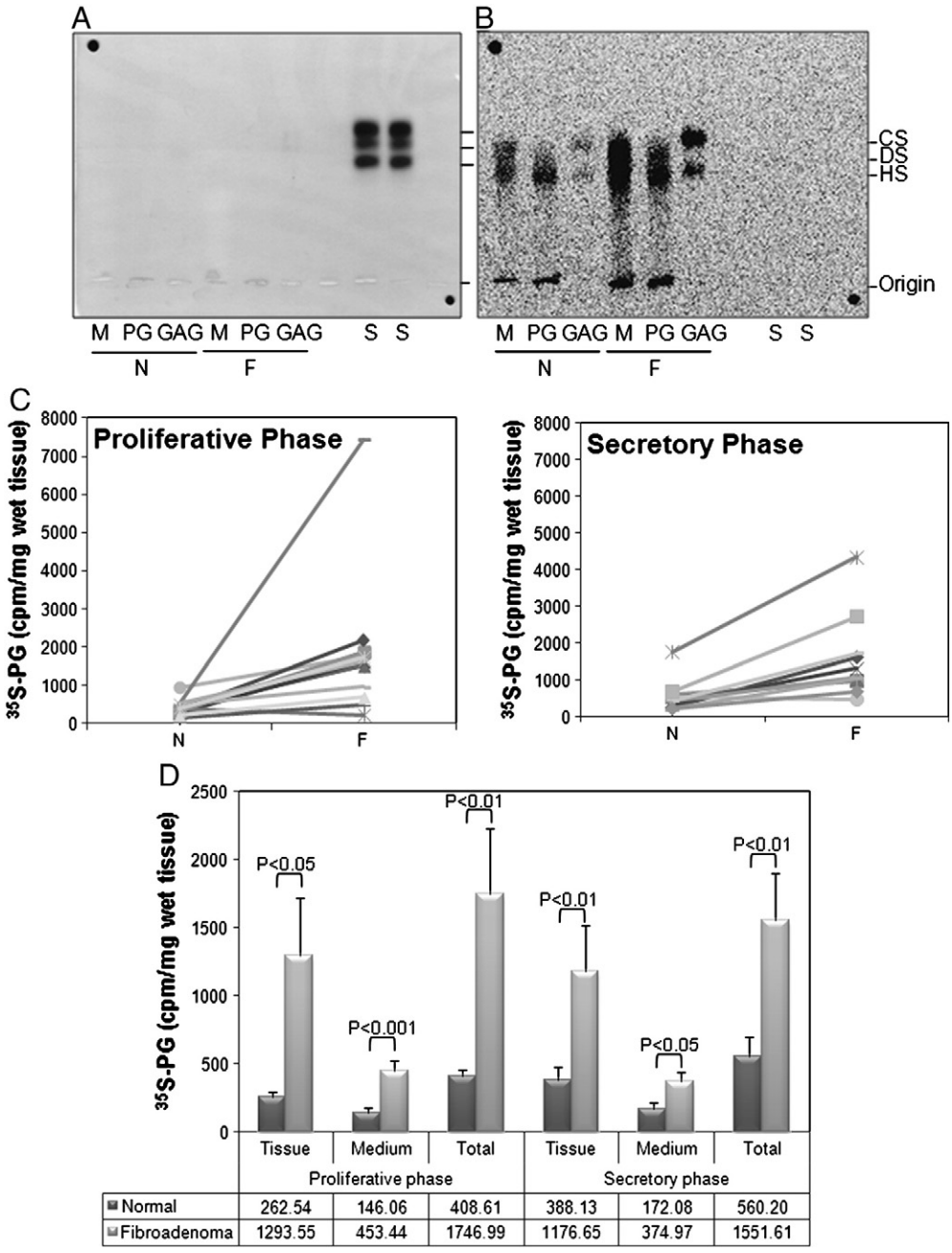
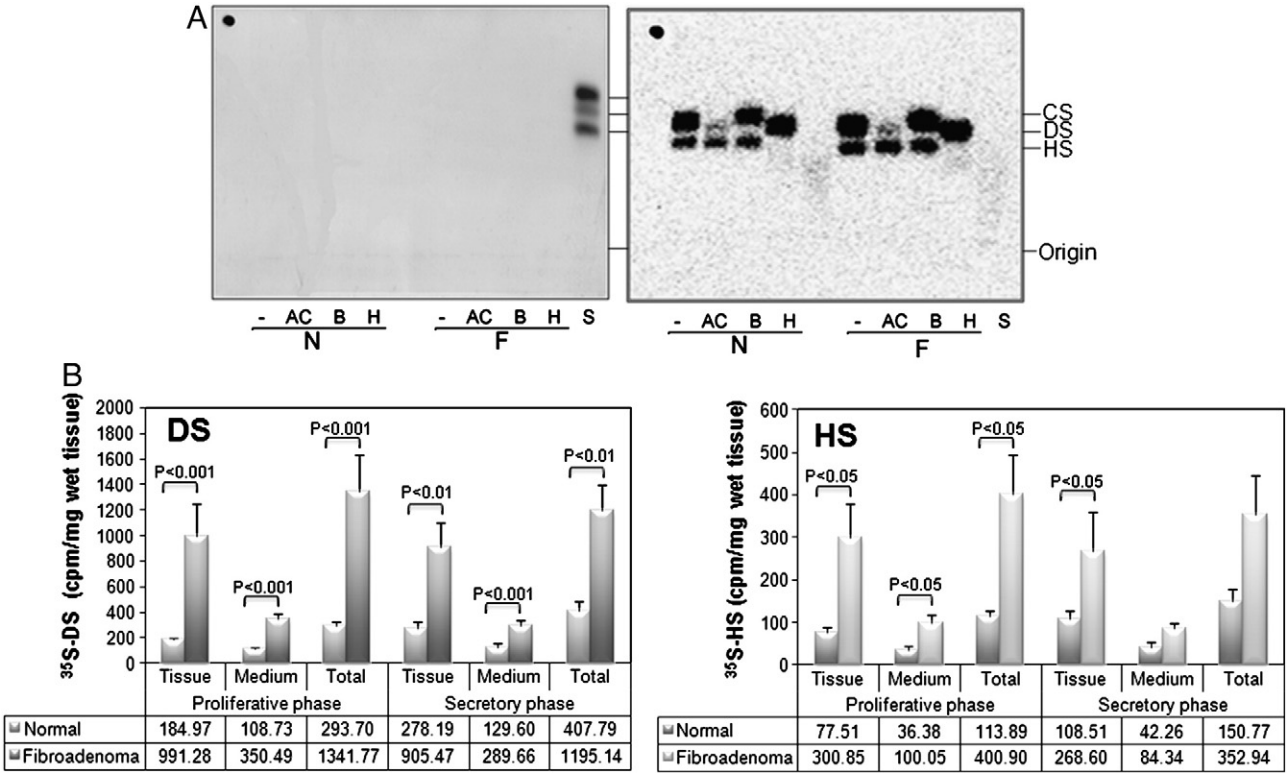


Fig. 3. Agarose gel electrophoresis of ^{35}S-PGs from normal breast (N) and fibroadenoma (F) tissue explants. Fibroadenoma and adjacent normal breast samples (200–500 mg, wet weight) were metabolically labeled with ^{35}S-sulfate. ^{35}S-PGs were isolated from the conditioned media (M) and the tissue explants (PG), and then tissue residues were submitted to total proteolysis and the ^{35}S-GAGs were also analyzed (GAG). These compounds were analyzed by agarose gel electrophoresis (PDA buffer), and localized in the gel by Toluidine Blue staining (A) and radioautography (B). The radioactivity of the bands containing ^{35}S-labeled compounds was quantified as described in Section 2.4. Individual paired results, and mean ± standard error are shown in (C) and (D), respectively. Brackets indicate statistically significant differences between normal and tumoral tissue explants. S, standard mixture of GAGs; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate.

[35] supporting the theory that fibroadenomas are the product of hyperplastic processes associated with aberration of the normal maturation of the breast, rather than true neoplasms [36]. The proliferation forms duct-like spaces surrounded by fibroblastic stroma.

The growth of normal breast epithelial cells is controlled by the ovarian steroid hormones estrogen and progesterone, as well as by cytokines and associated transcription factors, suggesting that multiple receptor signaling pathways could be involved in its growth and differentiation [37,38]. The synthesis of extracellular matrix macromolecules is also controlled by cytokines and hormones, and a reciprocal regulation of extracellular matrix proteins and ovarian steroid activity has been demonstrated in the mammary gland [39]. In fact, an antimetastatic role has been proposed for decorin, a member of the small leucine-rich proteoglycan gene family and the main PG of breast extracellular matrix [40]. It is possible that the production of extracellular matrix components in fibroadenomas is also under hormonal control. The epithelium is of normal appearance, but it was shown that the characteristic cyclic behavior that occurs in normal mammary epithelium with the menstrual cycle does not occur in fibroadenoma epithelium: there was no difference in the mitotic index and nuclear volume between proliferative and secretory phases [41], suggesting that endocrine hormones are not the main control mechanism in fibroadenoma growth. Our results on GAG and PG synthesis seem to corroborate this notion.

The present paper describes the fibroadenoma and the normal breast PGs, as well as the PGs synthesized under tissue culture



	Proliferative phase			Secretory phase		
DS	Tissue	Medium	Total	Tissue	Medium	Total
Normal	184.97	108.73	293.70	278.19	129.60	407.79
Fibroadenoma	991.28	350.49	1341.77	905.47	289.66	1195.14

	Proliferative phase			Secretory phase		
HS	Tissue	Medium	Total	Tissue	Medium	Total
Normal	77.51	36.38	113.89	108.51	42.26	150.77
Fibroadenoma	300.85	100.05	400.90	268.60	84.34	352.94

Fig. 4. ^{35}S-GAG chains released from PGs. The ^{35}S-GAG chains were released from ^{35}S-PGs by proteolysis, and identified by a combination of agarose gel electrophoresis and enzymatic degradation with specific GAG lyases. ^{35}S-GAGs from fibroadenoma (F) and from normal breast (N), both intact and after incubation with chondroitin AC lyase (AC), chondroitin B lyase (B), and heparitinase II (H), were analyzed by agarose gel electrophoresis and localized in the gel slabs by Toluidine Blue staining and radioautography (A). (B) Shows the quantitative data (mean ± standard error) for ^{35}S-HS and ^{35}S-DS released from tissue explants (Tissue) and conditioned culture medium (Medium).

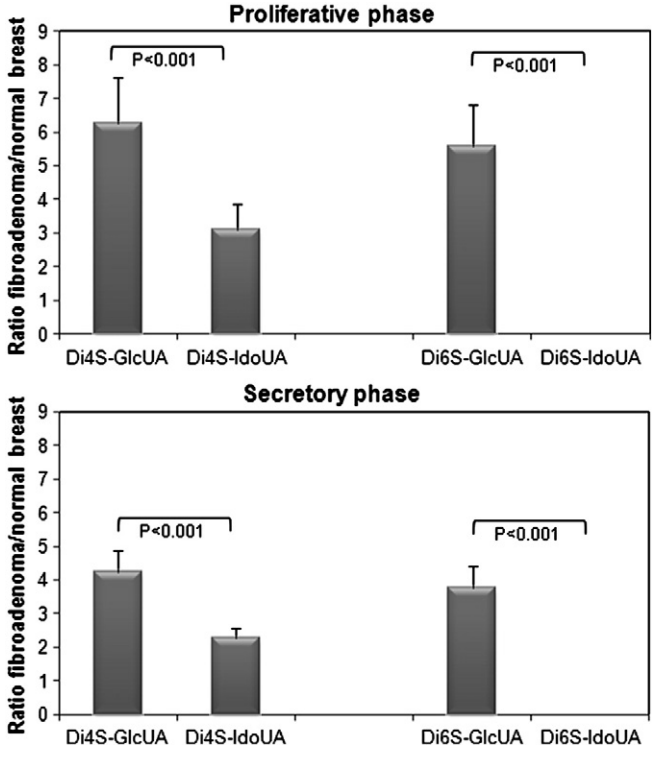


Fig. 5. Ratio fibroadenoma/normal breast of ^{35}S-labeled DS disaccharides in proliferative and secretory phases of the menstrual cycle. The amounts of 4-sulfated and 6-sulfated disaccharide units containing either β-D-glucuronic acid (Di4S-GlcUA and Di6S-GlcUA) or α-L-iduronic acid (Di4S-IdoUA and Di6S-IdoUA) in DS chains were measured after incubation with chondroitin AC or B lyases and analysis of their degradation products.

conditions, and the changes that occur during the menstrual cycle. The "normal breast" here studied is adjacent to diseased tissue, and all investigated tissues were derived from fibroadenoma patients. Only six of the 28 patients here studied were parous (22 nulliparous).

In normal breast, the PG concentration fluctuated with the menstrual cycle, achieving higher concentrations during the secretory phase (it is important to notice that, in the present paper, PGs were quantified by their GAG contents).

In fibroadenoma, the PG concentration also oscillated, but there was no difference between the mean values obtained during the proliferative and the secretory phases. Nevertheless, the PG concentration was always higher in fibroadenoma than in normal breast.

The main breast GAGs were HS (71%–74%) and DS (26%–29%). Both GAGs increased in fibroadenoma, but DS increased more, becoming 35%–37% of total.

Chondroitin sulfate (CS)/DS proteoglycans are ubiquitous compounds of extracellular matrices that have been implicated in several biological processes, such as cell signalling, proliferation and would healing (review in [42]). CS and DS chains consist of repeating disaccharide units of uronic acid (UA) and N-acetylgalactosamine (GalNAc). The only UA found in CS is β-D-glucuronic acid (GlcUA), while DS contains also its C5-epimer α-L-iduronic acid (IdoUA). The epimerization of GlcUA to IdoUA in DS is catalyzed by two DS-epimerases, recently cloned and named DS-epi1 and DS-epi2 [43]. The presence of IdoUA increases the chain conformational flexibility, which facilitates interactions, and is critical in mediating several DS biological functions, such as binding to growth factors [44] and heparin cofactor II [45].

IdoUA moieties in DS can be clustered together to form long blocks or interspersed among unmodified GlcUA moieties. It was recently shown that reduced expression of either DS-epimerase affects IdoUA block formation, and it is possible that the relative abundance of the two enzymes might be a regulatory mechanism for the IdoUA amounts and distribution [43]. The expression of DS-epimerase (SART2, [46])

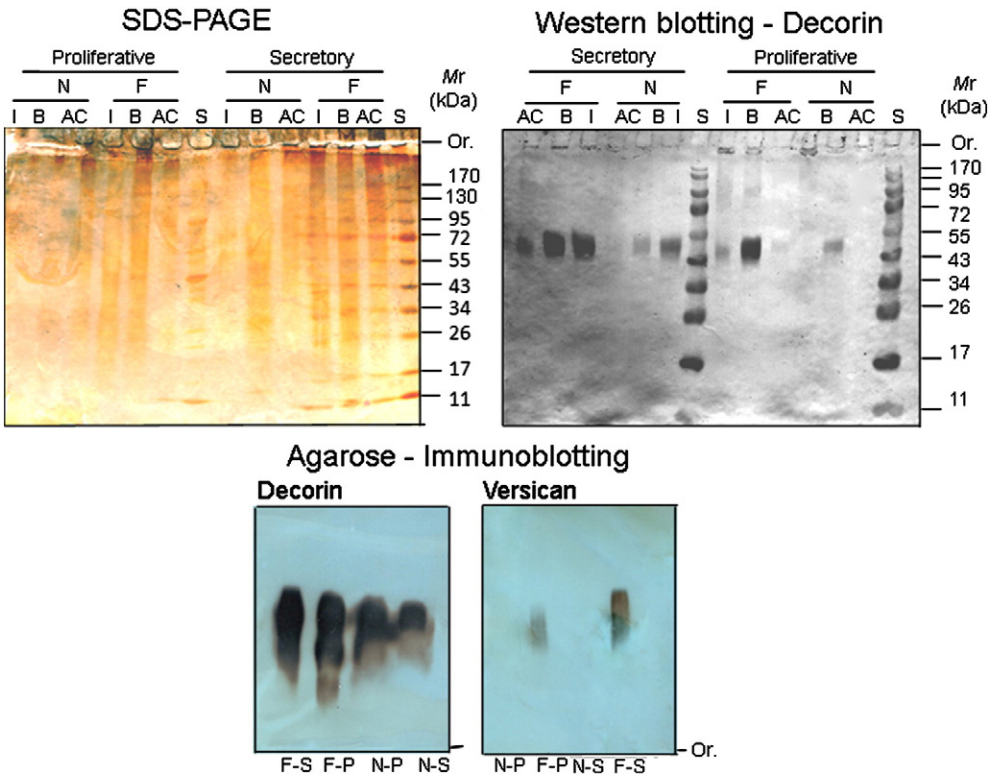


Fig. 6. SDS-PAGE (silver staining) and immunoblotting of PGs from normal breast tissue and fibroadenoma probed with either anti-decorin or anti-versican antibodies. PGs extracted from normal breast tissue (N) and fibroadenoma (F), obtained either during the proliferative or the secretory phase of the menstrual cycle, were incubated with chondroitin B lyase (B) or chondroitin AC lyase (AC). Intact PGs (I) were incubated with heat inactivated enzymes. Intact and chondroitin lyase-treated PGs were submitted to SDS-PAGE (3–20% gradient). The bands were visualized by silver staining (SDS-PAGE) and by Western blotting probed with anti-decorin antibody. The M_r of prestained protein standards is shown for SDS-PAGE gel. Intact PGs were also analyzed by agarose gel electrophoresis in PDA buffer, transferred to nitrocellulose membranes, and probed with either a polyclonal anti-decorin antibody (Decorin) or a monoclonal anti-versican antibody (Versican). Abbreviations used are: F-S, PGs from fibroadenoma, secretory phase; F-P, PGs from fibroadenoma, proliferative phase; N-P, PGs from normal breast, proliferative phase; N-S, PGs from normal breast, secretory phase; Or., origin.

was detected in different breast cell lines and tumor tissue using RT-PCR methods and *in situ* hybridization [47].

Furthermore, CS and DS chains can also be modified by sulfation of positions 4 and 6 of GalNAc and position 2 of UA by specific sulfotransferases, and sulfation patterns also affects the biological functions of these compounds [48]. The epimerization affects the sulfation. For instance, the long IdoUA-GalNAc stretches are never found with 6-sulfates on GalNAc. Our results are in agreement with these data.

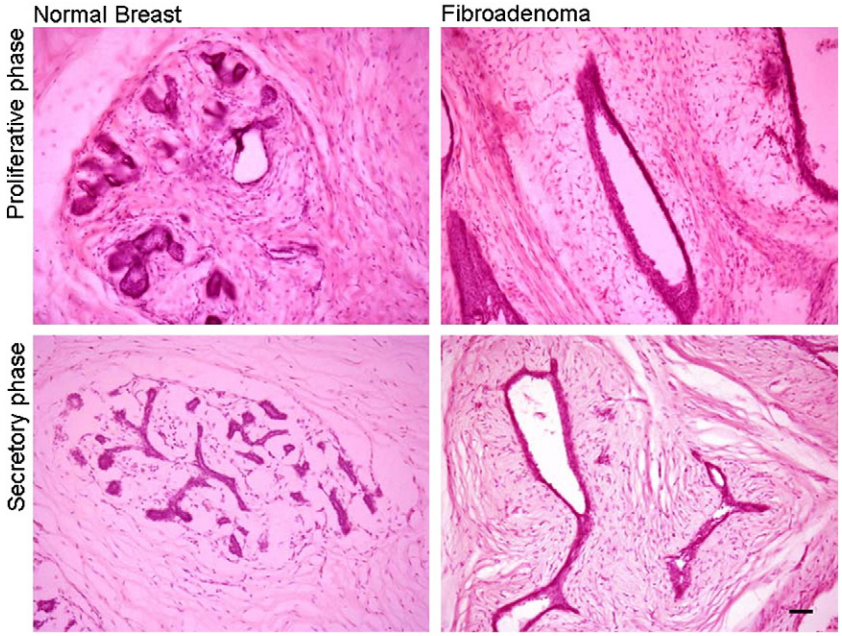


Fig. 7. Optical microscopy of fibroadenoma and adjacent normal breast during the proliferative and the secretory phases of the menstrual cycle. Tissue samples collected during surgery were fixed with 4% buffered formaldehyde, cut in 12 μm sections in cryostat, transferred to gelatin-coated slides, dehydrated, rehydrated, and stained with H&E. Bar = 50 μm.

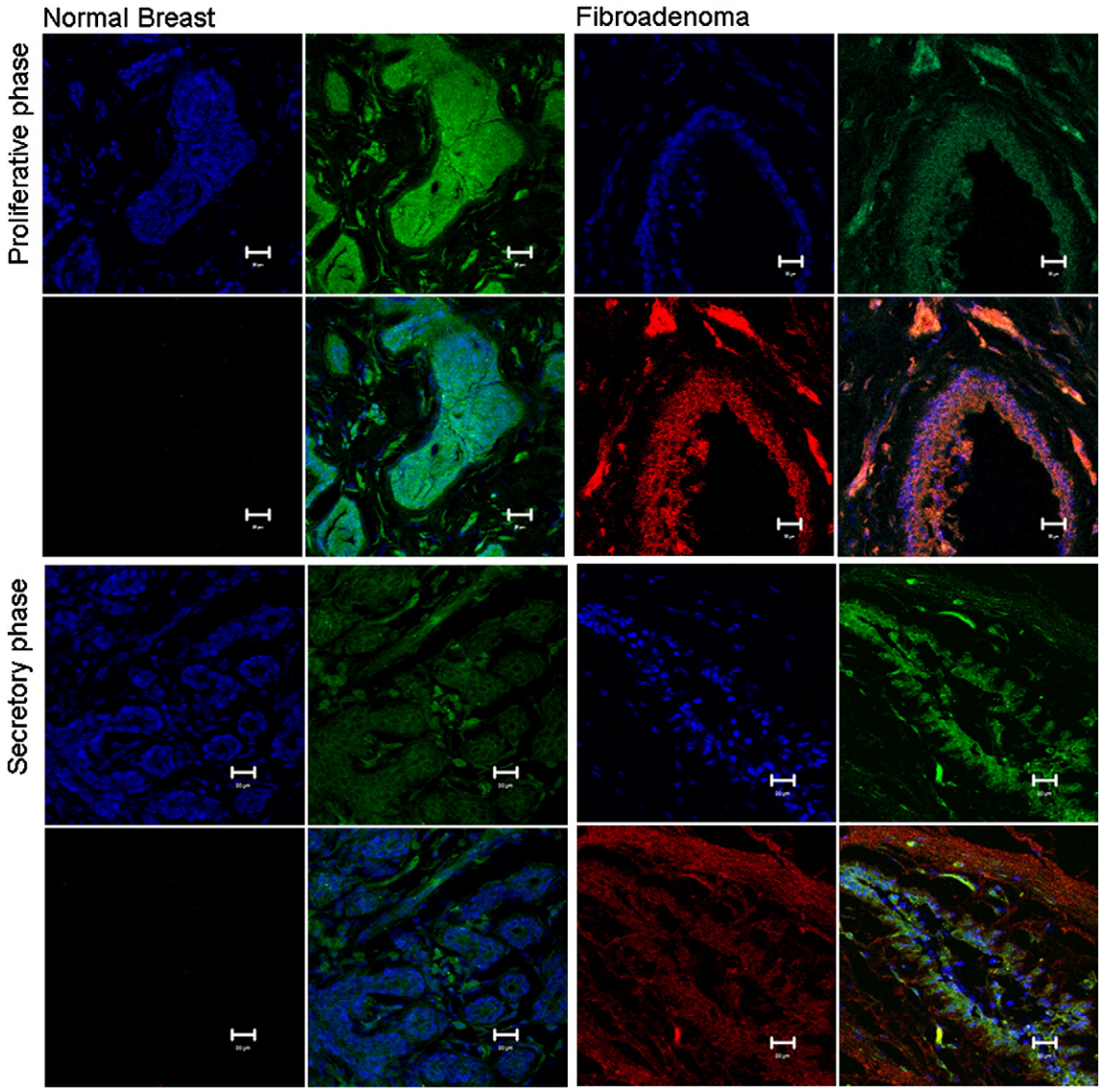


Fig. 8. Confocal microscopy for decorin (B, green) and versican (C, red) of human normal breast and fibroadenoma. Normal breast and fibroadenoma cryostat sections were double immunostained with polyclonal rabbit anti-decorin (B, green) and monoclonal mouse anti-versican antibodies (C, red). Nucleus was stained by DAPI (A, blue), and D shows overlaid images. The images were obtained by confocal microscopy. Note that versican was detected only in fibroadenoma. Bars = 20 μm.

Enzymatic analysis have shown that fibroadenoma DS is a hybrid polymer, containing three types of disaccharide units: (A) 2-acetamido-2-deoxy-3-O-(β-D-glucuronic acid)-4-O-sulfo-D-galactose; (B) 2-acetamido-2-deoxy-3-O-(α-L-iduronic acid)-4-O-sulfo-D-galactose; (C) 2-acetamido-2-deoxy-3-O-(β-D-glucuronic acid)-6-O-sulfo-D-galactose. Type (C) disaccharides (6-sulfated) were not detected in normal breast DS, and the increased amounts of types (A) and (C) in fibroadenoma indicate that the GlcUA/IdoUA ratio was increased in comparison to normal breast. It is possible that the polymers synthesized in fibroadenoma were not completely processed, maybe due to limiting activities of DS-epimerases. This possibility was also raised by others, who analyzed benign and malignant tumors of the uterus [20,49].

Concerning the protein core, the main extracellular matrix PG found in normal breast was decorin. Hallberg et al. [50] reported the gene expression (mRNA) decorin (and also syndecan-1 and -4) in normal breast, and found a decrease in decorin expression in the secretory phase, only for parous women. The glycosylation was not analyzed. In the present study, increased concentrations of decorin were found in fibroadenoma, which also contained versican, a high molecular weight PG of the hyalectan gene family. Versican was not found in normal breast. Decorin (and also versican in fibroadenoma) was concentrated in the periductal regions (lobular stroma) of normal and tumoral tissues.

Increased expression of versican has been detected in many malignant tumors, and is associated with the degree of tumor malignancy [51,52]. Versican was also found to be overexpressed in uterine leiomyoma [53], and its distribution and expression were found to be regulated by steroid hormones in mouse uterus [54]. To our knowledge, this is the first study to document the expression of versican in fibroadenoma.

The increased expression of decorin here reported could explain the self-limited growth of fibroadenoma. The decorin core protein modulates collagen fibrillogenesis and matrix assembly [55], and it has been shown that it negatively regulates the growth of a variety of tumor cells. It specifically binds and down-regulates the epidermal growth factor (EGF) receptor and blocks the transforming growth factor β (TGF-β) signaling pathway [56,57]. Decorin causes reduction of ErbB2 levels in tumor xenografts *in vivo*, and prevents metastatic spreading to the lungs [39]. Low levels of decorin in invasive breast

carcinomas are associated with poor outcome, in comparison to patients expressing high levels [58]. Furthermore, it was recently shown that decorin has antiangiogenic activities [59], while the tumor associated chondroitin sulfate seems to inhibit metastasis [60]. Decorin was proposed as a therapeutic agent against breast cancer due to its inhibition of tumor growth and metastatic spreading [39].

4.2. Conclusions

Taken together, these data highlight the relevance of PGs in the regulation of tumor biology. Our results demonstrate that the synthesis rate and the concentration of PGs is increased in fibroadenoma as compared to normal breast, but the menstrual cycle has less influence on fibroadenoma than on normal breast PGs. Decorin was the main extracellular matrix PG expressed in both normal and tumoral tissues, at higher concentrations in the lobular stroma, while versican was found only in fibroadenoma. These changes may be related to modifications in the tissue architecture that lead to changes in the control of cell proliferation, adhesion and migration.

Acknowledgements

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasília, DF, Brazil; Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília, SP, Brazil; Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), São Paulo, SP, Brazil; and Sociedade Paulista para o Desenvolvimento da Medicina (SPDM), São Paulo, SP, Brazil.

References

[1] K.M. Alle, J. Moss, R.J. Venegas, I. Khalkhali, S.R. Klein, Conservative management of fibroadenoma of the breast, Br. J. Surg. 83 (1997) 992–993.
[2] R. Greenberg, Y. Skornick, O. Kaplan, Management of breast fibroadenomas, J. Gen. Intern. Med. 13 (1998) 640–645.
[3] R.K. Hansen, M.J. Bissell, Tissue architecture and breast cancer: the role of extracellular matrix and steroid hormones, Endocr. Relat. Cancer 7 (2000) 95–113.
[4] C.K. Osborne, K. Hobbs, G.M. Clark, Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice, Cancer Res. 45 (1985) 584–590.
[5] R.M. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (1988) 889–895.
[6] R.A. Ignotz, J. Massague, Transforming growth factor-2 stimulates the expression of fibronectin and collagen and their incorporation to the extracellular matrix, J. Biol. Chem. 261 (1986) 4337–4345.
[7] Y. Berthois, X.F. Dong, P.M. Martin, Regulation of epidermal growth factor-receptor by estrogen and antiestrogen in the human breast cancer cell line MCF-7, Biochem. Biophys. Res. Commun. 159 (1989) 126–131.
[8] S.A. Chrysogelos, R.I. Yarden, A.H. Lauber, J.M. Murphy, Mechanisms of EGF receptor regulation in breast cancer cells, Breast Cancer Res. Treat. 31 (1994) 227–236.
[9] D.G. Fernig, J.T. Gallagher, Fibroblast growth factors: an information network controlling tissue growth, morphogenesis and repair, Prog. Growth Factor Res. 5 (1994) 353–377.
[10] S.F. Penc, B. Pomahac, T. Winkler, R.A. Dorschner, E. Eriksson, M. Herndon, R.L. Gallo, Dermatan sulfate released after injury in a potent promoter of fibroblast growth factor-2 function, J. Biol. Chem. 273 (1998) 28116–28121.
[11] M. Lyon, J.T. Gallagher, Bio-specific sequences and domains in heparan sulphate and the regulation of cell growth and adhesion, Matrix Biol. 17 (1998) 485–493.
[12] S.H. Kim, J. Turnbull, S. Guimond, Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor, J. Endocrinol. 209 (2011) 139–151.
[13] C.P. Dietrich, H.A. Armelin, Y.L. Nogueira, H.B. Nader, Y.M. Michelacci, Turnover, changes of composition with rate of cell growth and effect of phenylxyloside on synthesis and structure of cell surface sulfated glycosaminoglycans of normal and transformed cells, Biochim. Biophys. Acta 717 (1982) 387–397.
[14] T.N. Wight, D.K. Heinegård, V.C. Hascall, Proteoglycans: structure and function, in: E.D. Hay (Ed.), Cell Biology of Extracellular Matrix, 2nd edition, Plenum Press, 1991, pp. 45–78.
[15] J.C. Adams, F.M. Watt, Regulation of development and differentiation by the extracellular matrix, Development 117 (1993) 1183–1198.
[16] L.O. Sampaio, C.P. Dietrich, O. Gianotti Filho, Changes in sulfated mucopolysaccharide composition of mammalian tissues during growth and cancerization, Biochim. Biophys. Acta 448 (1977) 123–131.
[17] A.G.A. Berto, L.O. Sampaio, C.R.C. Franco, R.M. Cesar-JR, Y.M. Michelacci, A comparative analysis of structure and spatial distribution of decorin in human leiomyoma and normal myometrium, Biochim. Biophys. Acta 1619 (2003) 98–112.
[18] S.M.B. Jerônimo, A.O. Sales, M.Z. Fernandes, F.P. Melo, L.O. Sampaio, C.P. Dietrich, H.B. Nader, Glycosaminoglycan structure and content differ according to the origins of human tumors, Braz. J. Med. Biol. Res. 27 (1994) 2253–2258.
[19] C. Ricciardelli, K. Mayne, P.J. Sykes, W.A. Raymond, K. McCaul, V.R. Marchall, W.D. Tilley, J.M. Skinner, D.J. Horsfall, Elevated stromal chondroitin sulfate glycosaminoglycans predicts progression in early-stage prostate cancer, Clin. Cancer Res. 3 (1997) 983–992.
[20] A.G. Berto, Y.M. Michelacci, L.O. Sampaio, Galactosaminoglycans from normal myometrium and leiomyoma, Braz. J. Med. Biol. Res. 34 (2001) 633–637.
[21] G. Parry, E.Y. Lee, D. Farson, M. Koval, M.J. Bissell, Collagenous substrata regulate the nature and distribution of glycosaminoglycans produced by differentiated cultures of mouse mammary epithelial cells, Exp. Cell Res. 156 (1985) 487–499.
[22] M.J. Warburton, R.A. Kimbell, S.A. Ferns, A.R. Hayman, N. Perunsinghe, P. Monaghan, Characterization of chondroitin/dermatan sulfate proteoglycans synthesized by rat mammary mioepithelial and fibroblastic cell lines, Biochim. Biophys. Acta 1117 (1992) 291–300.
[23] J.E. Fergusson, A.M. Schor, A. Howell, M.W. Fergusson, Changes in the extracellular matrix of the normal human breast during the menstrual cycle, Cell Tissue Res. 268 (1992) 167–177.
[24] M.F. Rego, M.A.L.H. Navarrete, G. Facina, R. Falzoni, R. Silva, E.C. Baracat, A.C.P. Nazario, Analysis of human mammary fibroadenoma by Hi-67 index in the follicular and luteal phases of menstrual cycle, Cell Prolif. 42 (2009) 241–247.
[25] L.O. Sampaio, M.T. Bayliss, T.E. Hardingham, H. Muir, Dermatan sulfate proteoglycan from human articular cartilage. Variation in its contents with age and structural comparison with a small chondroitin sulfate proteoglycan from pig laryngeal cartilage, Biochem. J. 254 (1988) 757–764.
[26] E.S. Soriano, M.S. Campos, Y.M. Michelacci, Effect of epithelial debridement on glycosaminoglycan synthesis by human corneal explants, Clin. Chim. Acta 295 (2000) 41–62.
[27] C.P. Dietrich, S.M.C. Dietrich, Eletrophoretic behaviour of acidic mucopolysaccharides in diamine buffers, Anal. Biochem. 70 (1976) 645–647.
[28] J.A.K. Aguiar, C.R. Lima, A.G.A. Berto, Y.M. Michelacci, An improved methodology to produce *Flavobacterium heparinum* chondroitinases, important instruments for diagnosis of diseases, Biotechnol. Appl. Biochem. 37 (2003) 115–127.
[29] H.B. Nader, M.A. Porcionatto, I.L. Tersariol, M.A. Pinhal, F.W. Oliveira, C.T. Moraes, C.P. Dietrich, Purification and substrate specificity of heparitinase I and heparitinase II from *Flavobacterium heparinum*. Analyses of the heparin and heparan sulfate degradation products by 13C NMR spectroscopy, J. Biol. Chem. 265 (1990) 16807–16813.
[30] V.L. Petricevich, Y.M. Michelacci, Proteoglycans synthesized *in vitro* by nude and normal mouse peritoneal macrophages, Biochim. Biophys. Acta 1053 (1990) 135–143.
[31] Y.M. Michelacci, C.P. Dietrich, A comparative study between a chondroitinase B and a chondroitinase AC from *Flavobacterium heparinum*. Isolation of a chondroitinase AC-susceptible dodecasaccharide from chondroitin sulphate B, Biochem. J. 151 (1975) 121–129.
[32] C.R. Merril, D. Goldman, M.L. Van Keuren, Gel protein stains: silver stain, Methods Enzymol. 104 (1984) 441–447.
[33] J. Sambrook, E.F. Fritsch, T. Maniatis, second ed., Molecular Cloning. A Laboratory Manual, vol. 3, 1989, pp. 18.69–18.75.
[34] L.A. Brinton, M.P. Vessey, R. Flavel, D. Yeates, Risk factors for benign breast disease, Am. J. Epidemiol. 113 (1981) 203–214.
[35] S. Noguchi, K. Motomura, H. Inaji, S. Imaoka, H. Koyama, Clonal analysis of fibroadenoma and phyllodes tumor of the breast, Cancer Res. 53 (1993) 4071–4074.
[36] N. Sawhney, N. Garrahan, A.G. Douglas-Jones, E.D. Williams, Epithelial–stromal interactions in tumors. A morphologic study of fibroepithelial tumors of the breast, Cancer 70 (1992) 2115–2120.
[37] S. La Rosa, F. Sessa, L. Colombo, M.G. Tibiletti, D. Furlan, C. Capella, Expression of acidic fibroblast growth factor (aFGF) and fibroblast growth factor receptor 4 (FGFR4) in breast fibroadenomas, J. Clin. Pathol. 54 (2001) 37–41.
[38] C.J. Watson, C.H. Olivera, W.T. Khaled, Cytokine signalling in mammary gland development, J. Reprod. Immunol. 88 (2011) 124–129.
[39] S.Z. Haslam, T.L. Woodward, Reciprocal regulation of extracellular matrix proteins and ovarian steroid activity in the mammary gland, Breast Cancer Res. 3 (2001) 365–372.
[40] S. Goldoni, D.G. Seidler, J. Heath, M. Fassan, R. Baffa, M.L. Thakur, R.T. Owens, D.J. McQuillan, R.V. Iozzo, An antimetastatic role for decorin in breast cancer, Am. J. Pathol. 173 (2008) 844–855.
[41] M.M. Simomoto, A.C.P. Nazário, L.H. Gebrim, M.J. Simões, E.C. Baracat, G.R. de Lima, Morphometric analysis of the epithelium of mammary fibroadenomas during the proliferative and secretory phases of the menstrual cycle, Breast J. 5 (1999) 256–261.
[42] S. Yamada, K. Sugahara, S. Özbek, Evolution of glycosaminoglycans. Comparative biochemical study, Commun. Integr. Biol. 4 (2011) 150–158.
[43] B. Pacheco, A. Malmström, M. Maccarana, Two dermatan sulfate epimerases form iduronic acid domains in dermatan sulfate, J. Biol. Chem. 284 (2009) 9788–9795.
[44] C.D. Nandini, K. Sugahara, Role of the sulfation pattern of chondroitin sulfate in its biological activities and in the binding of growth factors, Adv. Pharmacol. 53 (2006) 253–279.

[45] A. Raghuraman, P.D. Mosier, U.R. Desai, Understanding dermatan sulfate-heparin cofactor II interaction through virtual library screening, ACS Med. Chem. Lett. 1 (2010) 281–285.
[46] M. Maccarana, B. Olander, J. Malmström, K. Tiedemann, R. Aebersold, U. Lindahl, J.-P. Li, A. Malmström, Biosynthesis of dermatan sulfate. Chondroitin-glucuronate C5-epimerase is identical to SART2, J. Biol. Chem. 281 (2006) 11560–11568.
[47] C.S. Schuetz, M. Bonin, S.E. Clare, K. Nieselt, K. Sotlar, M. Walter, T. Fehm, E. Solomayer, O. Riess, D. Wallwiener, R. Kurek, H.J. Neubauer, Progression-specific genes identified by expression profiling of matched ductal carcinomas in situ and invasive breast tumors, combining laser capture microdissection and oligonucleotide microarray analysis, Cancer Res. 66 (2006) 5278–5286.
[48] E.M. Koźma, G. Wisowski, D. Kusz, K. Olczyk, The role of decorin and biglycan dermatan sulfate chain(s) in fibrosis-affected fascia, Glycobiology 21 (2011) 1301–1316.
[49] M. Sobue, J. Takeuchi, K. Yoshida, S. Akao, T. Fukatsu, T. Nagasaka, N. Nakashimu, Isolation and characterization of proteoglycans from human nonepithelial tumors, Cancer Res. 47 (1987) 160–168.
[50] G. Hallberg, E. Andersson, T. Naessén, G.E. Ordeberg, The expression of syndecan-1, syndecan-4 and decorin in healthy human breast tissue during the menstrual cycle, Reprod. Biol. Endocrinol. 8 (2010) 35–41.
[51] M.E. Tsara, A.D. Theocharis, D.A. Theocharis, Compositional and structural alterations of proteoglycans in human rectum carcinoma with special reference to versican and decorin, Anticancer Res. 22 (2002) 2893–2898.
[52] C. Ricciardelli, J.H. Brooks, S. Suwiwat, A.J. Sakko, K. Mayne, W.A. Raymond, R. Seshandri, R.G. Lebaron, D.J. Horsfall, Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer, Clin. Cancer Res. 1054 (2002) 1054–1060.
[53] R.M. Salgado, L.P. Capelo, R.R. Favaro, J.D. Glazier, J.D. Aplin, T.M. Zorn, Hormone-regulated expression and distribution of versican in mouse uterine tissues, Reprod. Biol. Endocrinol. 7 (2009) 1–10.
[54] T.N. Seagroves, J.P. Lydon, R.C. Hovey, B.K. Vonderhaar, J.M. Rosen, C/EBPβ (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development, Mol. Endocrinol. 14 (2000) 359–368.
[55] C.C. Reed, R.V. Iozzo, The role of decorin in collagen fibrillogenesis and skin homeostasis, Glycoconj. J. 19 (2003) 249–255.
[56] J.X. Zhu, S. Goldoni, G. Bix, R.T. Owens, D.J. McQuillan, C.C. Reed, R.V. Iozzo, Decorin evokes protracted internalization and degradation of the epidermal growth factor receptor via caveolar endocytosis, J. Biol. Chem. 280 (2005) 32468–32479.
[57] Y. Yamaguchi, D.M. Mann, E. Ruoslahti, Negative regulation of transforming growth factor-beta by the proteoglycan decorin, Nature 346 (1990) 281–284.
[58] S. Troup, C. Njue, E.V. Kliewer, M. Parisien, C. Roskelley, S. Chakravarti, P.J. Roughley, L.C. Murphy, P.H. Watson, Reduced expression of the small leucine-rich proteoglycans, lumican, and decorin is associated with poor outcome in node-negative invasive breast cancer, Clin. Cancer Res. 9 (2003) 207–214.
[59] T. Neill, H. Painter, S. Buraschi, R.T. Owens, M.P. Lisanti, L. Schaefer, R.V. Iozzo, Decorin antagonizes the angiogenic network: concurrent inhibition of Met, HIF-1α and VEGFA and induction of thrombospondin-1 and TIMP3, J. Biol. Chem. 287 (2012) 5492–5506.
[60] R.D. Prinz, C.M. Willis, A. Viloria-Petit, M. Klüppel, Elimination of breast tumor-associated chondroitin sulfate promotes metastasis, Genet. Mol. Res. 10 (2011) [Epub ahead of print].