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# Small Leucine Rich Proteoglycans Are Differently Distributed in Normal and Pathological Endometrium

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**Abstract.** *Background: During the woman's fertile period, the non-pregnant uterus is subject to constant cyclic changes. The complex mechanisms that control the balance among proliferation, differentiation, cell death and the structural remodeling of the extracellular matrix can contribute to the benign or malignant endometrial pathological state. The small leucine-rich proteoglycans (SLRPs) are important components of cell surface and extracellular matrices. Materials and Methods: Using immunohistochemistry, we showed that the distribution patterns of SLRPs were completely modified in the pathological compared to normal endometrium. Results: The expression of SLRPs was low/absent in all endometrial pathologies examined compared to normal endometrium. We observed an increase of lumican from proliferative to secretory phase of the endometrium and a decrease of fibromodulin, biglycan and decorin. In menopause endometrial tissue, the level of expression of fibromodulin, biglycan, decorin and lumican dramatically decreased. Conclusion: The results revealed the prominence and importance of proteoglycans in the tissue architecture and extracellular matrix organization.*

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Proteoglycans consist of a large family of highly anionic glycoproteins ubiquitously expressed in connective tissues (1-5). Proteoglycans are sub-divided into two groups, large molecules, such as aggrecan, versican and perlecan, and the relatively small leucine-rich proteoglycan (SLRP) molecules consisting of five distinct members, such as fibromodulin (FMOD), biglycan, decorin, lumican and chondroadherin, containing leucine-rich repeat motifs in their protein cores (6-8).

The large proteoglycans help maintain tissue hydration and contribute to overall structural scaffolding in the extracellular matrix (ECM), whereas small molecules play important roles in binding to other matrix molecules, either aiding fibrillogenesis or acting as bridging molecules among various tissue elements (1).

Several studies provide insight concerning the participation of collagens and proteoglycans in the epithelial-mesenchymal interface (9, 10). Proteoglycans have been observed in the cytoplasm of several organs as lung, kidney, liver, colon, ovary, corneal stroma (11, 12), in the endothelial cells of small and large vessels, in the structure of the subcutaneous adipocytes (13) and in the composition of the articular disc (14, 15). Sometimes the interactions between growth factors and connective tissue may be causes of remodelling and pathological states (16, 17).

An emerging function of SLRPs is an intrinsic ability to affect cellular proliferation. For example, ectopic expression of decorin slows-down the growth of a wide variety of tumor cells. The decorin-induced growth arrest is associated with an induction of p21, a potent inhibitor of cyclin-dependent kinase activity (18-20).

The human endometrium, during every reproductive cycle, undergoes extensive tissue remodeling in response to cyclical hormonal changes. Estrogens stimulate

proliferation and re-establishment of the stromal and vascular components of the tissue. By contrast, glandular differentiation and stromal decidualization result from the influence of progesterone (21). Menstruation is finally initiated by the fall in estrogen and progesterone that results from the demise of the corpus luteum at the end of a non-fertile cycle (22, 23).

Deregulation of the balance occurring among cellular proliferation, differentiation and death predisposes endometrial cells to malignant transformation as in neoplastic and pre-neoplastic lesions. In recent years, increasing evidence has highlighted the proteoglycans' pathway in extracellular matrix remodeling. Changes in proteoglycan concentration and/or structure may correlate to changes in the overall architecture of the tissue, possibly affecting cell proliferation in different ways, such as cell adhesion, migration and cytokine availability.

On light of these observations, we investigated the distribution of the small leucine-rich proteoglycans (SLRPs) extracellular matrix components in normal endometrium, obtained from fertile patients and from patients in menopause. Then, we compared these data with the distribution of these proteins in different endometrial pathologies, such as hyperplasia and polyps through histochemical methods.

Table I. *Sampling of physiological endometrium.*

| | |
|---|---|
| Number of patients | 15 |
| Average age (years) | 49.8 |
| Endometrium | Proliferative phase 5 |
| | Secretory phase 5 |
| | Menopause 5 |
| Surgery | Laparotomic hysterectomy colpohysterectomy |
| | Operative hysteroscopy |
| Sampling | Novak |
| | Courette |
| | Bipolar loop |

Table II. *Sampling of pathological endometrium.*

| | |
|---|---|
| Number of patients | 10 |
| Average age (years) | 42.8 |
| Endometrial pathology | Polyps 5 |
| | Hyperplasia 5 |
| Surgery | Laparotomic hysterectomy colpohysterectomy |
| | Operative hysteroscopy |
| Sampling | Novak |
| | Courette |
| | Bipolar loop |

## Materials and Methods

*Samples.* Human endometrial samples were obtained under the approval of an appropriate ethics committee from patients undergoing surgery, such as laparotomic hysterectomy, colpohysterectomy and operative hysteroscopy. A total of 15 samples of physiological endometrium (5 in proliferative phase, 5 in secretory phase and 5 in menopause) (Table I) and 10 samples of pathological endometrium (5 of hyperplasia, 5 of post-menopausal polyps) (Table II) were used for the study. Removal of endometrial samples, both physiological and pathological, was carried-out by different surgical instruments (novak, courette and bipolar loop). The specimens, picked under sterile conditions with different instruments, were immediately fixed in formalin for immunohistochemistry.

*Immunohistochemistry.* Immunohistochemistry was carried-out essentially as described previously (24-26). Briefly, sections from each specimen were cut at 5 μm, mounted on glass and dried overnight at 37˚C. All sections were then deparaffinized in xylene, rehydrated through a graded series of alcohol and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide and blocked with PBS–6% non-fat dry milk (Bio-Rad Laboratories S.r.l. Milano, Italy) for 1 h at room temperature. Slides were then incubated at 4˚C overnight with antibodies raised against human fibromodulin, biglycan, decorin and lumican (Santa Cruz Biotechnology, Dallas, TX, USA), each at a 1:100 dilution. After several washes (3×5min) to remove excess antibody, the slides were incubated with diluted (1:200) anti-goat biotinylated antibodies (Vector Laboratories, Burlington, ON Canada) for 1 h. All the slides were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. 3, 3'-diaminobenzidine (DAB; Vector Laboratories) was used as a chromogen and hematoxylin was used as a nuclear counter stain. For each antibody, negative controls were prepared by substituting the primary antiserum with non-immune IgG and positive controls by using the antibody on a tissue section where the protein had already been demonstrated.

For each experiment, all the slides were stained in a single batch, thus ensuring equal staining for each. The staining pattern of fibromodulin, biglycan, decorin and lumican proteins were examined and scored for staining intensity signal: 0 (absent immunopositivity); 1 (low immunopositivity); 2 (moderate immunopositivity); 3 (intense immunopositivity) (27).

For each specimen, a HSCORE value was derived by summing the percentages of cells/areas that stained at each intensity and multiplying that by the weighted intensity of the staining. For example:

$$\text{HSCORE}=\Sigma Pi(i + 1)$$

where $i$ represents the intensity scores and $Pi$ the corresponding percentage of cells/areas. An average of 22 fields was observed for each tissue by three observers at different times and the average score was used. All values were expressed as mean±standard error of mean (S.E.M.) and differences were compared using the Student's $t$-test.

## Results

In the present study we describe the distribution of the SLRPs in the physiological and in the pathological endometrium.

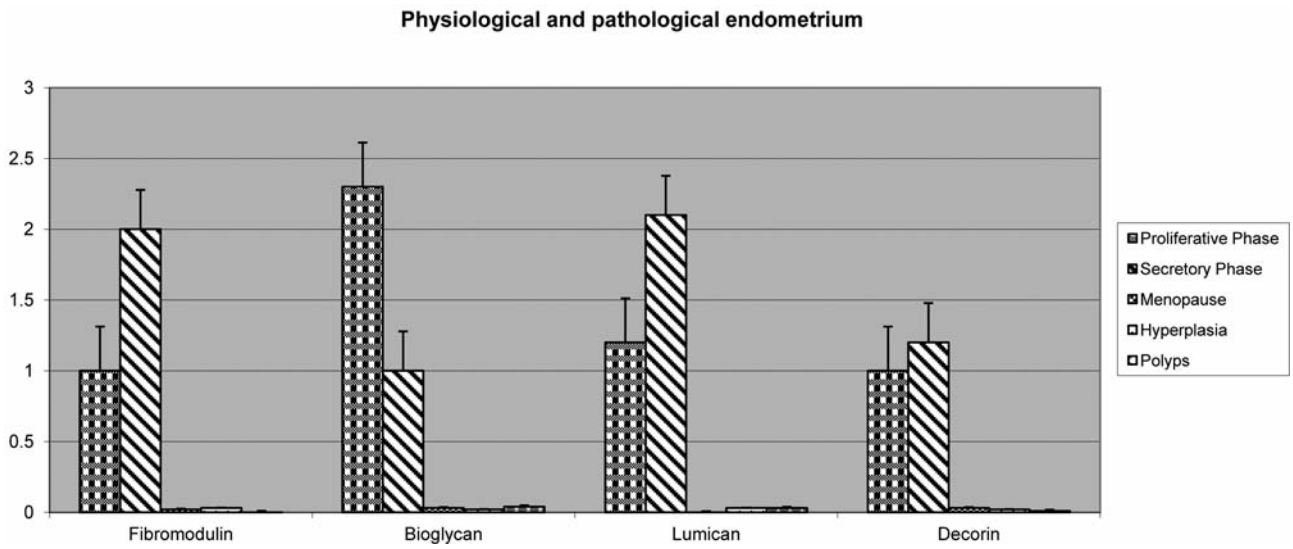


Figure 1. *Expression pattern of small leucine rich proteoglycan proteins in human endometrium during physiological reproductive cycle and in human pathological endometrium. Vertical lines show means±SEM.*

In Figure 1 the expression of the SRLPs (fibromodulin, biglycan, lumican and decorin) is depicted as detected by immunohistochemical staining intensity analysis. Student's *t*-test revealed significant score × immunopositivity differences ($p<0.05$) for fibromodulin, biglycan, lumican and decorin expression levels in the different physiological endometrial phases and pathological endometrium. In detail, we observed that the distribution patterns of SLRPs were completely modified in the pathological endometrium compared to normal endometrium.

Our results showed that the expression of biglycan and lumican was low in endometrial pathologies (polyps and hyperplasia), whereas the expression of fibromodulin and decorin was absent in the endometrial pathologies (polyps and hyperplasia) compared to normal endometrium.

Instead, in physiological endometrium, we observed a different modulation and localization of the SRLPs.

*Fibromodulin:* Fibromodulin showed a low level of expression in the endometrium stroma during the proliferative phase (Figure 2a). In the secretory phase, we observed an increase of fibromodulin immunopositivity at a low/moderate level of expression; it was localized especially in the apical portion of endometrial glands (Figure 2b).

*Biglycan:* During the proliferative phase, biglycan showed a moderate/intense immunopositivity in the basement membrane of the vessel (Figure 2c). In the secretory phase, we observed a low level of expression of biglycan in all the endometrial compartments (Figure 2d).

*Lumican:* In the samples examined, we observed that, in the proliferative phase, lumican was expressed at moderate levels in some epithelial cells and the vessel wall (Figure 2e). During the secretory phase, lumican expression increased at a moderate/intense level with a particular localization in the stroma and in the apical portion of endometrial glands (Figure 2f).

*Decorin:* In the samples examined, we observed that, both in the proliferative and secretive phases, the decorin expression level was very low (data not shown).

Expression of fibromodulin, biglycan, lumican and decorin was almost absent in all components of the endometrium in menopause (data not shown).

## Discussion

The switch from proliferation to differentiation is a process that requires growth factors and the cross-talk among different cellular pathways. The binding of growth factors to proteoglycans and the subsequent modulation of growth factor activities represent one of the major conceptual advances in the field; whether this binding is mediated by the protein core or the carbohydrate moiety, the final event is a perturbation (either negative or positive) of the growth factors' biological activity with significant consequences on the affected cell population. Moreover, this biological interaction provides a mechanistic explanation for the growth- and differentiation-promoting ability of the extracellular matrix.

Proteoglycans and glycosaminoglycans have been studied in uterine cells in culture and in normal uterus, especially during pregnancy (28-30), but there are few reports on proteoglycans in uterine pathology.

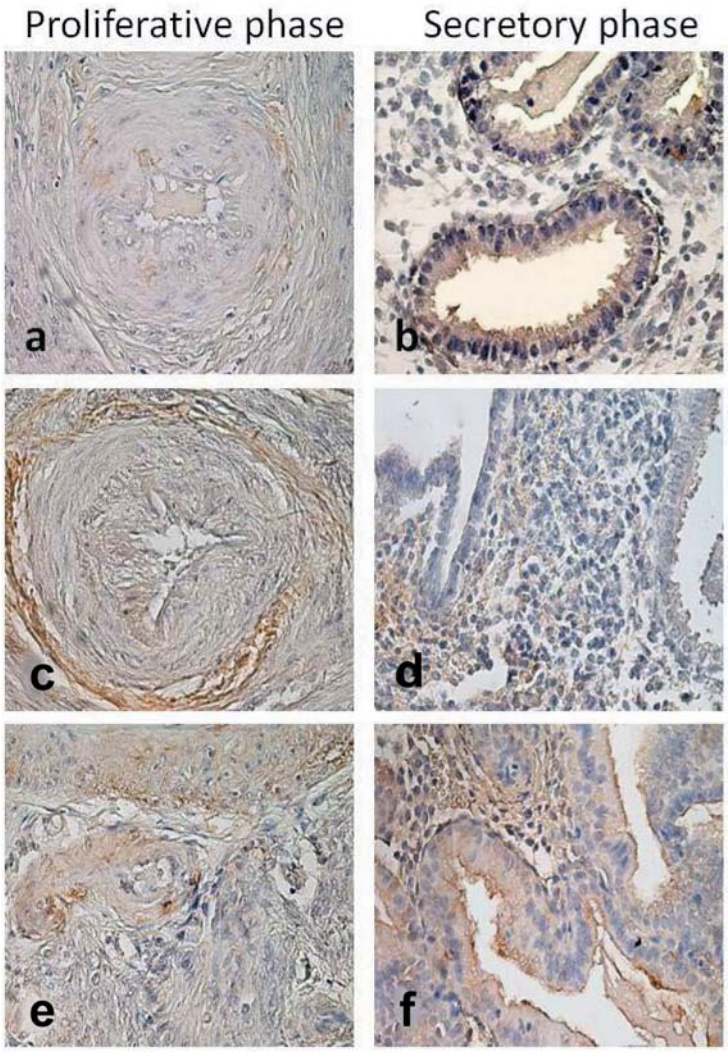


Figure 2. *Localization of small leucine rich proteoglycan proteins in human endometrium during the physiological reproductive cycle. (a) Fibromodulin low expression during the proliferative phase; ×300; (b) Fibromodulin low/moderate immunopositivity in the apical portion of endometrial glands during the secretory phase; ×300; (c) Biglycan moderate/intense immunopositivity in the basemant membrane of the vessel during the proliferative phase; ×300; (d) Biglycan low expression in the endometrial compartments during the secretory phase; ×640; (e) Lumican moderate immunopositivity in some epithelial cells and vessel wall of the proliferative phase; ×640; (f) Lumican moderate/intense immunoreactivity in the stroma and in the apical portion of endometrial glands in the secretory phase; ×300.*

In the present study we described the distribution of SLRPs in the physiological endometrium and in the pathological endometrium. To determine if the changes in proteoglycan expression correlate with modifications in the extracellular matrix organization, we compared the general architecture of physiological endometrium to the pathological endometrium.

Through immunohistochemistry, we showed that the distribution patterns of SLRPs were completely modified in the pathological endometrium compared to normal endometrium. Our results showed that the expression SLRPs was low/absent in all the endometrial pathologies examined compared to normal endometrium. We observed an increase of lumican from proliferative to secretory phase of the endometrium and a decrease of fibromodulin, biglycan and decorin. In menopause endometrial tissue, the level of expression of fibromodulin, biglycan, decorin and lumican dramatically decreased.

Our data demonstrated a change in the tissue architecture and extracellular matrix organization in the compartment examined. Our data are supported by a

previous report that demonstrated, in "knockout" mice, that absence of decorin results in lax, fragile skin in which collagen fibril morphology is irregular with fusion of adjacent fibrils appearing to have occurred (31). Absence of biglycan resulted in an osteoporosis-like phenotype, with animals having a reduced growth rate and a decreased bone mass (32). Absence of lumican produced both skin laxity and corneal opacity with an increased proportion of abnormally thick collagen fibrils and delayed corneal epithelial wound healing (33). Absence of fibromodulin produced no change in the appearance of te mice but it was possible to observe an abnormal collagen fibril organization in tendons (34). This work clearly shows that collagen fibril architecture is impaired in tissues in which SLRPs are deficient and that the abnormal phenotype is specific for each proteoglycan.

Instead, at the molecular level, increased transforming growth factor beta (TGF-b) production is the hallmark of a number of fibrotic diseases that are characterized by abundant accumulation of extracellular matrix components. At least four SLRP members (fibromodulin, biglycan, lumican and decorin,) interact with TGF-b (35). These *in vitro* binding studies correlate well with the observation that ectopic expression of decorin leads to marked growth retardation and change in morphology and adhesion properties of TGF-b-dependent cells (36). Moreover, several lines of evidence support a specific protein/protein interaction between decorin and epidermal growth factor receptor (EGFR) (37). Because decorin and other SLRP members are intimately associated with fibrillar collagen, a complex picture in which multimeric interactions take place in an integrin-independent manner should be considered. An enhancement in decorin content in the newly-formed tumor stroma could trigger functional interaction with EGFR, which would, in turn, start a signaling cascade that directly influences the cell-cycle machinery. In this light, it is noteworthy that a double knockout of decorin and *p53*, a well-established tumor suppressor gene, shows a cooperative action between these two genes and an acceleration of lymphoma tumorigenesis (38).

In summary, the results presented herein reveal the importance of proteoglycans in the tissue architecture and extracellular matrix organization. Changes in matrix composition may be related to modifications in the tissue architecture that lead to alterations in several biological phenomena concerning the control of cell proliferation, such as growth factor and cytokine availability, cell adhesion and migration, as well as immune response.

SLRPs are involved both in the regulation and the organization of the connective tissue and control of cell growth; their lack of expression could explain the disorganization of the support tissue and, in the long run, phenomena as the uncontrolled growth of tissue.

## Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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