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Recovery of the pubic symphysis on primiparous young and multiparous senescent mice at postpartum

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Summary. It has been observed that parturition has a significant effect on female skeletal architecture and that age alters musculoskeletal tissues and their functions. We therefore hypothesized that multiparity affects the recovery of the pubic symphysis in senescent mice at postpartum and the morphology of the interpubic tissues. The pubic symphysis of primiparous young, virgin senescent (VS) and multiparous senescent (MS) Swiss mice was examined by light microscopy, transmission electron microscopy, morphometric analysis and immunohistochemistry. The mouse pubic symphysis was remodeled during the first pregnancy: the cellular phenotype and morphology changed to ensure a structurally safe birth canal, followed by recovery of the interpubic articulation after birth. The morphology of the pubic symphysis in the VS group was maintained in a state similar to that observed in virgin young mice. In contrast, MS mice exhibited an interpubic ligament characterized by extended fibrocyte-like cells, an opened interpubic articulation gap, compacted and thin collagen fibrils and scarce galectin-3-positive cells. Thus, we found that the cellular and extracellular characteristics of the pubic symphysis were altered by multiparity in senescent mice. These particular tissue characteristics of the MS group might be associated with an impaired recovery process at postpartum. Thus, a better understanding of the alterations that occur in the birth canal, including the pubic symphysis, due to multiparity in reproductively aged mice may contribute to our comprehension of the biological mechanisms that modify the skeleton and pelvic ligaments and even play a role in the murine model of pelvic organ prolapse.

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Introduction

Musculoskeletal remodeling, which makes part of the birth canal, facilitates the preparation for vaginal birth and is noticeable in such organs and structures as the uterus (Starcher and Percival, 1985), uterine cervix (Read et al., 2007; Buhimschi et al., 2009), vagina (Word et al., 2009) and pubic symphysis (Gardner, 1936; Crelin, 1969). Impairments in these organs and structures, which comprise both the birth canal and the pelvic floor, are strongly associated with urinary incontinence and pelvic organ prolapse (Liu et al., 2004; Drewes et al., 2007; Lee et al., 2008). Little is known about the pathophysiological mechanisms responsible for these conditions, but histomorphological changes in the musculoskeletal system have been suggested to result from biological and lifestyle factors (Delancey et al., 2008). More specifically, it is thought that two major risk factors for pelvic organ prolapse are vaginal birth and aging (Delancey et al., 2008; Abramowitch et al., 2009).

The mouse pubic symphysis is a sexually dimorphic structure that surrounds the pelvic cavity like a belt. It stabilizes articulation of the pubic bones and forms part of the musculoskeletal system that supports the gastrointestinal tract, bladder and reproductive organs (Iguchi et al., 1989; Ortega et al., 2003; Schimpf and Tulikangas, 2005). In some mammals, such as guinea pigs (Ortega et al., 2003), bats (Crelin and Newton, 1969) and mice (Crelin, 1969), the symphysis undergoes remodeling during pregnancy. The modifications of the pubic symphysis that occur in young animals during the first pregnancy have previously been defined as a process of metamorphosis (Gardner, 1936). They are

characterized by (a) the separation of the pubic bones, which results in a gradual expansion of the fibrocartilage into an interpubic ligament (IpL), and (b) IpL relaxation during late pregnancy (Talmage, 1947; Sherwood, 1994), due to hormonal factors (mainly estrogen, progesterone and relaxin) that help to regulate the processes involved in remodeling of the extracellular matrix such promoting interpubic ligament growth, which increases the size of the birth canal (Sherwood, 1994).

Because the connective tissues have a predominantly mechanical function and are hormonal-regulated, it is important to understand how their components provide specific mechanical attributes. Connective tissue mechanical behavior is primarily determined by the composition and organization of collagen (Provenzano and Vanderbay, 2006). Non-axial fibrils likely influence tissue viscoelasticity in the fibrocartilaginous tissues of virgin mice, whereas axial fibrils with characteristic crimp morphology indicate a more organized collagenous matrix. The collagen fibers demonstrated a very efficient design, which aided in maintaining the integrity, strength and elasticity of the interpubic ligament in primiparous mice throughout late pregnancy (Pinheiro et al., 2004). The crimp morphology functions to protect the collagen fibers from tensional forces (Franchi et al., 2010) and during late pregnancy (Pinheiro et al., 2004).

During late pregnancy in wild-type mice, the cells of the interpubic ligament possess a myofibroblast-like phenotype and are distributed along collagen fibers. These are one of the cell types responsible for the tightening of the pubic articulation in primiparous mice after parturition (Moraes et al., 2004). The postpartum recovery process is particularly important for the reproductive tract homeostasis (Buhimschi et al., 2009), as evidenced by the finding that abnormal connective tissues may be a key factor in the development of pelvic support disorders (Liu et al., 2004). Thus, a mouse pubic symphysis model can be used to study cellular phenotypes and the rapid synthesis and degradation of the extracellular matrix in the tissues of young female mice. However, the morphology of the pubic symphysis depends upon the species, age, sex and physiological reproductive stage being studied (Ortega et al., 2003). Despite this, since Gardner (1936), little attention has been given to pubic symphysis in older mice after successive pregnancies.

The molecular mechanisms by which pregnancy, parturition, parity and aging lead to reduced pelvic organ support are not well understood. Nevertheless, physiological stress associated with breeding may accelerate the development of age-related phenotypes (Konigsberg et al., 2007; Conde-Perezprina et al., 2008), and the aging process is accompanied by changes in the extracellular matrix, including modifications in macromolecules and/or their interactions with other matrix components (Sell and Monnier, 1995). Aging specifically modifies the extracellular matrix by promoting changes in the metabolism of chondrocytes, including an increase in oxidative stress (Pelletier et al., 2000; Loeser et al., 2002).

Among the members of the galectin family of proteins, which possess diverse biological functions related to cellular homeostasis (Hsu and Liu, 2004; Dumic et al., 2006), galectin-3 has been described to play a protective role in chondrocyte survival (Colnot et al., 2001; Boileau et al., 2008). Moreover, galectin-3 can regulate myofibroblast activation in connective tissues (Henderson et al., 2006). Since myofibroblasts are important for contraction during wound closure after tissue injury (Desmouliere et al., 2005) and in drawing the pelvic bones together after the first parturition (Moraes et al., 2004), galectin-3 may play a critical role in the interpubic tissues by participating in tissue recovery at the cellular level.

Joint connective tissue remodeling directly affects the mechanical integrity of the pelvic girdle and its supportive tissues, and this integrity is reduced in the case of pelvic organ prolapse. The effects of multiparity and aging on matrix composition and the structure of the pubic symphysis are not well understood and should be considered to better understand the factors that lead to organ prolapse. To understand the cellular and extracellular effects of multiparity on the pubic symphysis in senescent mice better, we compared virgin, pregnant and postpartum young female mice (primiparous) as well as virgin senescent (VS) and multiparous senescent (MS) mice. Specifically, we sought to characterize recovery on the pubic symphysis in primiparous young and MS mice at postpartum by investigating the phenotypes of the associated chondroblasts and fibroblasts, the organization of the extracellular matrix and the distribution of galectin-3, which is relevant to chondrocyte survival and myofibroblast activation.

Materials and methods

Animals

Virgin young and old female Unib:SW mice (3-4 months and 12 months old, respectively) and reproductively aged, retired female breeders (12 months old) from the reproductive matrix after having given birth six to eight times and demonstrated a decline in fertility (Yeh and Kim, 2007) were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB) at Unicamp. Mating was encouraged by placing the young females in the same cage as breeding males overnight. Vaginal plug formation was considered an indicator of the first day of pregnancy (D1). The VS mice were maintained for 12 months without mating to control for physiological modifications that could occur due to aging alone. The reproductively aged mice that are referred to as the MS group in this work were maintained for a period of 40 days without reproduction to ensure that no remodeling

occurred as a result of parturition.

Pubic symphyses or interpubic ligaments were obtained from the following groups: virgins young in estrus (virgin, n=12) (Shorr, 1941), day 18 of pregnancy (D18, n=12), one day postpartum (1dpp, n=12), 40 days postpartum (40dpp, n=12), VS (n=12) and MS (n=12). Between 11:00 a.m. and 12:00 p.m., the animals were anesthetized using a mixture of 100-200 mg/kg ketamine and 5-16 mg/kg xylazine chloride, which was administered intraperitoneally (Agribrands do Brasil, Jacarei, Brazil). Following laparotomy, the distance between the pubic bones, which is known as the interpubic articulation gap, was measured using a caliper with a precision of one hundredth of a millimeter. The medial portions of the pubic bones (symphyses or ligaments) were then removed and processed after the mice were sacrificed by cervical dislocation. Seventy-two animals were evaluated for the purpose of this study, as detailed below. The animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, issued by the National Institutes of Health (NIH; Bethesda, MD, USA). All of the experimental protocols were approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/Unicamp, Protocol 1221-1).

Light microscopy

The interpubic tissue (symphyses or ligaments) was dissected and fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 24 h at 4°C and then decalcified in 5% ethylenedinitrilo tetraacetic acid (EDTA, Mallinckrodt Baker, Phillipsburg, NJ, USA) and 2% paraformaldehyde in 0.1 M PBS (pH 7.4) for five days at 4°C. The tissues of three animals per experimental group were dehydrated in graded concentrations of alcohol, embedded in historesin (Leica Microsystems Heidelberg, Germany) and sectioned transversely at a width of 2 μm. The resulting serial sections were mounted on slides and stained with Giemsa. The interpubic tissues of an additional three animals per group were decalcified and dehydrated in graded concentrations of alcohol, embedded in paraffin and sectioned transversely at a width of 5 μm. Serial sections were mounted on slides and stained with Sirius red-F3B (Montes, 1996) and Sirius red using a modified protocol (Wehrend et al., 2004). The sections were then examined and imaged using a Nikon Eclipse E800 light microscope.

Transmission electron microscopy

Small samples of pubic symphyses or interpubic ligaments from three animals per group were fixed with 2% glutaraldehyde (Electron Microscope Science, Hatfield, PA, USA) in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.3% tannic acid for 3 h at 4°C, followed by a post-fixation in 1% osmium tetroxide for 1 h at 4°C. The samples were dehydrated in a graded ethanol series and embedded in epoxy resin Epon 812 (Electron Microscope Science). Ultrathin sections were then collected on copper grids, stained with uranyl acetate and lead citrate and examined using a LEO 906 electron microscope.

For the quantitative collagen fibril analyses, three random electron micrographs obtained at a magnification of 21,560x were selected for each group. The diameters of the collagen fibrils that were randomly selected from each micrograph were measured, and a histogram was obtained for each group, as described by Muellner et al. (2001). The mean fibril diameter and the standard error (SE) were also calculated. The collagen fibril analyses were specifically performed using Image Pro-Plus 4.1.0.1 software (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemistry

The symphyses and interpubic ligaments from three animals per group were fixed as previously described without EDTA treatment, embedded in paraffin and sectioned transversely. Briefly, after paraffin removal, the slides were heated in a Panasonic microwave oven (model NN 7809-BH; 1380W; Manaus, AM, Brazil) for 60 s. The endogenous peroxidase activity was inhibited using 3% hydrogen peroxide, and non-specific binding was blocked with a 1% bovine serum albumin solution. The sections were incubated overnight with a rat monoclonal antibody against galectin-3 at a final concentration of 0.10 mg/mL (TIB 166, M3/38), which was kindly provided by Dr. Roger Chammas (Faculty of Medicine, University of Sao Paulo, SP, Brazil), followed by goat anti-rat IgG antibody at a final concentration of 0.02 mg/mL (Dako, CA, USA) for 1 h. A streptavidin-peroxidase conjugate (Dako) was then applied to the slides, followed by a substrate mixture of 0.5 mg/mL 3.3-diaminobenzidine (Sigma, St Louis, MO, USA) and 0.3% hydrogen peroxide. The sections were then counterstained with Harris hematoxylin. The immunohistochemical experiment included a negative control, replacing the primary antibody with normal serum of rat (as primary antibody). The sections were observed and imaged using a light microscope.

The cells that were positive for galectin-3 were identified based on their immunoreactivity. An index was defined based on the ratio of galectin-3-positive cells to total cells, which was determined by counting a minimum of 100 cells per group within different regions of the pubic symphysis at a magnification of 400x, as described by Veridiano et al. (2007). Three random images were analyzed from each animal for each day of the pregnancy.

Statistical analysis

The data are presented as the mean values ± Standard Error (SE). The interpubic articulation gap, mean collagen fibril diameter and the ratio of galectin-3-

positive cells to total cells were compared among the groups using Kruskal-Wallis test followed by Mann-Whitney test. Statistical significance was defined as $P<0.05$.

Results

Cellular phenotype and interpubic tissue morphology in primiparous, VS and MS mice

Because the cellular phenotype and the interpubic tissue are important during mouse pregnancy for both the preparation of the birth canal for parturition and birth canal closure, we analyzed representative histological images of the interpubic tissues of primiparous, VS and MS mice and measured the interpubic articulation gap.

Examination of Giemsa-stained transversal tissue sections showed that the articular surfaces of the pubic symphysis are covered by hyaline cartilage on either side and are the margins of a central fibrocartilaginous disk in virgin mouse (Fig. 1A). In contrast, the IpL is formed by dense connective tissue organized along the opening axis of the articulation in MS (Fig. 1B). Morphological analysis on high magnification indicated that the first

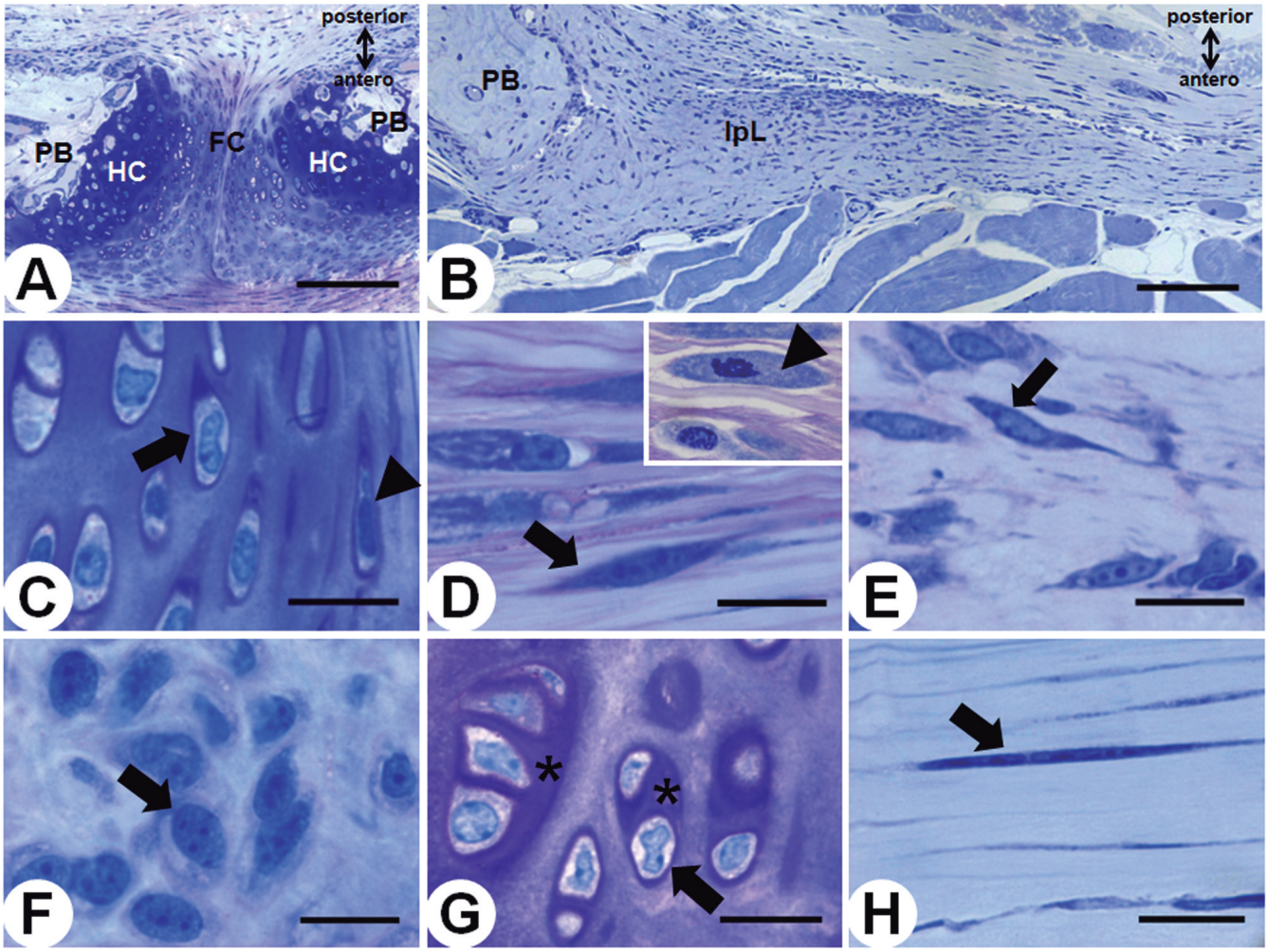

Fig. 1. Light microscopy of pubic symphysis in virgin **(A)**, IpL in MS **(B)** and cellular phenotypes in the pubic symphyses in virgin **(C)**, 40dpp **(F)** and VS **(G)** mice, and in the interpubic ligaments in D18 **(D)**, 1dpp **(E)** and MS **(H)** mice. The medial ends of the pubic bones demonstrate a horizontal orientation in all of the photomicrographs. **A.** Virgin mouse pubic symphysis histoarchitecture with the fibrocartilaginous disk (FC) placed between thin layers of hyaline cartilage (HC) and pubic bones (PB) at both sides. **B.** IpL formed by a dense connective tissue placed between the PB in the MS group. **C.** The arrow shows a typical chondrocyte, and the arrowhead indicates a typical fibrochondrocyte; both are surrounded by a characteristic extracellular matrix. **D and E.** The arrows show a fibroblast-like cell, and the arrowhead in the inset in **D** indicates a fibroblast-like cell undergoing mitosis. **F.** The arrow indicates a polygonal chondrocyte-like cell surrounded by a characteristic extracellular matrix. **G.** The arrow shows cell surrounded by noticeable extracellular matrix (asterisks). **H.** The arrow highlights a fibrocyte with its axis parallel to the opening axis of the symphysis, compacted chromatin and flattened and extended morphology. All of the samples shown were stained with Giemsa. Scale bars: A, B, 150 μm; C-H, 15 μm

pregnancy induces a change in the cellular phenotype from fibrochondrocytes of the typical fibrocartilage (Fig. 1C) to fibroblast-like cells of the newly formed interpubic ligament (Fig. 1D-E). The cellular alignment also changed from a perpendicular (Fig. 1C) to a parallel (Fig. 1D-E) orientation relative to the opening axis of the articulation. After parturition, the cells in the 40dpp mice showed an evident reorganization into chondroblast-like cells (Fig. 1F), whereas the VS group showed cells in their lacunae with a noticeable territorial extracellular matrix (Fig. 1G), and the cells in the MS group demonstrated a fibrocyte-like phenotype with a parallel alignment relative to the ligament along the opening axis (Fig. 1H). We often observed cell division at the interpubic ligament in the D18 mice (Fig. 1D), but we were unable to observe this phenomenon in the VS and MS mice.

To investigate further whether a typical

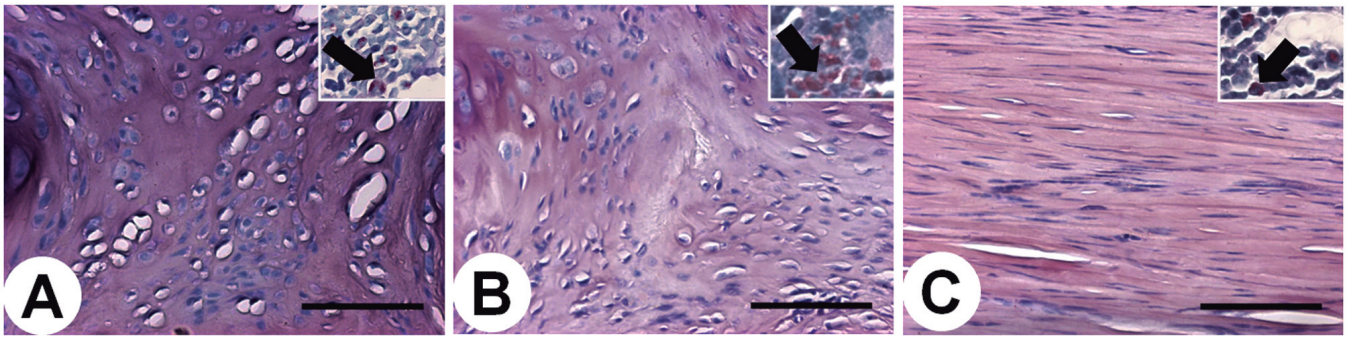

Fig. 2. Light microscopy of the pubic symphyses in 40dpp **(A)** and VS **(B)** mice, and the interpubic ligament in MS **(C)** mouse, which demonstrated no evidence of granulocyte influx, such as eosinophil and neutrophil. The arrows in the insets indicate granulocytes in the pubic bone marrow on the same slides as the positive control. Alkaline Sirius red counterstaining with hematoxylin. Scale bars: 30 μm

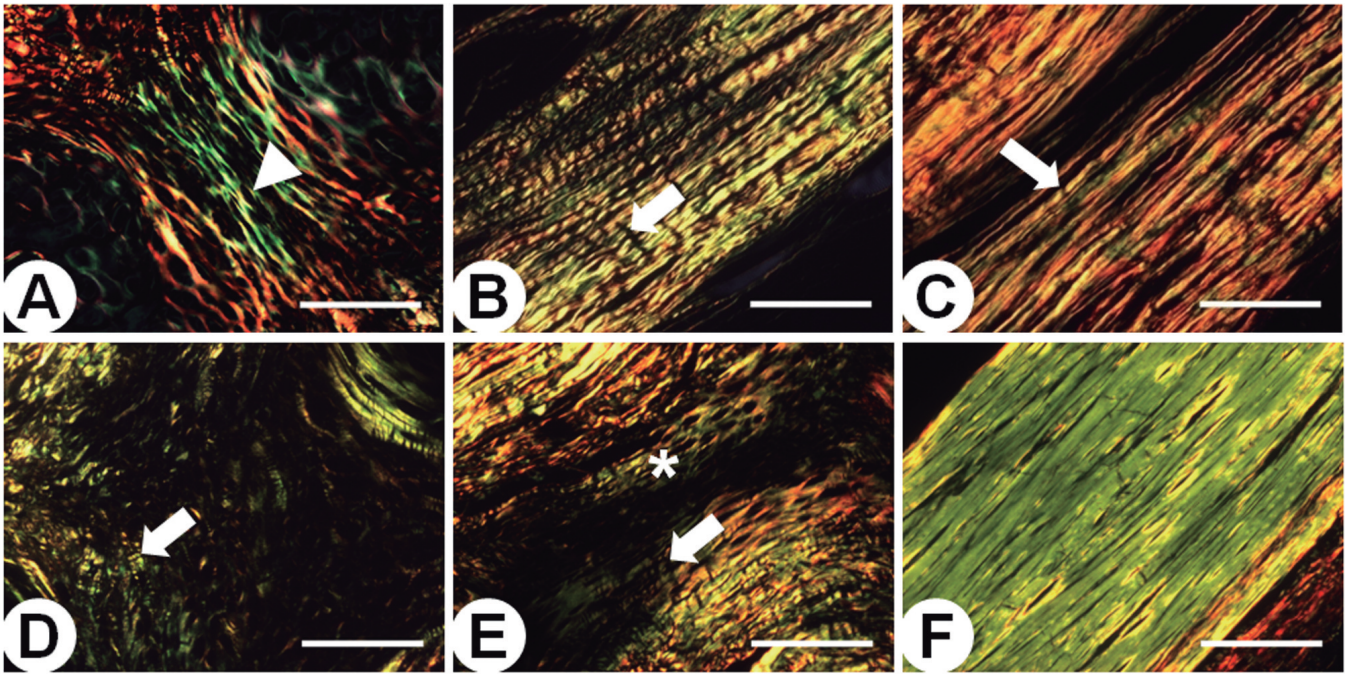

Fig. 3. Sirius red-stained photomicrographs under polarizing illumination of the pubic symphyses in virgin **(A)**, 40dpp **(D)** and VS **(E)** mice and the interpubic ligaments in the D18 **(B)**, 1dpp **(C)** and MS **(F)** groups. The medial ends of the pubic bones have the same orientation in all of the photomicrographs (lower left and upper right). **A.** The arrowhead indicates a thin birefringent collagen arrangement around the fibrochondrocytes. **B, C, D and E.** The arrows indicate the crimp. **E.** The fibrochondrocytes are found in the region indicated by an asterisk. **F.** Observe the aligned and compacted collagen that lacks a crimp structure. Scale bars: 30 μm

inflammatory process occurs in the interpubic articulation during recovery process after parturition, we examined alkaline Sirius red-stained tissue sections for the presence of granulocytic influx, which is indicative of an inflammatory reaction. We did not observe any such influx in the 40dpp, VS or MS groups (Fig. 2A-C).

The morphometrical analysis of the interpubic articulation gap (the distance between pubic bones) yielded the following values for each group: virgin, 1.59±0.08 mm; D18, 5.50±0.30 mm; 1dpp, 3.29±0.07 mm; 40dpp, 1.60±0.13 mm; VS, 1.64±0.18 mm; and MS, 5.62±0.18 mm. The differences between the virgin and D18, D18 and 1dpp, 1dpp and 40dpp, 40dpp and MS, VS and MS groups were significant (P<0.001) according to Kruskal-Wallis and Mann-Whitney tests.

Extracellular matrix in primiparous, VS and MS mice

The extracellular matrix constitutes a mechanical buffer that protects the tissue against tensional forces. However, the mechanical properties and morphology of the extracellular matrix may be affected by pregnancy and aging. To investigate this possibility, we analyzed the pubic symphysis in primiparous, VS and MS mice using polarized light and transmission electron microscopy.

An examination of Sirius red-stained sections under polarized light revealed that random and non-axial birefringent collagen fibrils were arranged around the fibrochondrocytes in the symphysis in the virgin group (Fig. 3A). However, in the D18 mice the collagen was aligned with the opening axis of the articulation, and crimp structures had formed (Fig. 3B). In contrast, fewer helicoidal structures were observed in this tissue in the 1dpp mice (Fig. 3C). The collagen network of the 40dpp group was arranged similarly to that observed in the virgin mice, although helicoidal structures were noted (Fig. 3D). Qualitatively, crimp structure could also be observed in VS group and birefringent collagen was greater than in the virgin group but did not seem to be aligned and compacted in the central region of the pubic symphysis (Fig. 3E). By comparison, the collagen network showed a very compacted and aligned distribution in the MS mice, without evident crimp (Fig. 3F).

A tissue analysis using transmission electron microscopy allowed us to observe morphological differences between the thin and thick collagen fibrils in the pubic symphysis (Fig. 4A,B). The frequency of the collagen fibril diameters showed a peak value of 30 nm in the virgin, 40dpp, VS and MS groups, which were characterized predominantly by thin fibrils (Fig. 4A,D-F). In comparison, no maximum frequency of fibril

Table 1. Mean collagen fibril diameter (ηm) of the virgin, D18, 1dpp, 40dpp, VS and MS groups ± SE.

Group	Mean collagen fibril diameter ± SE	P value
Virgin	32.5±0.2	-
D18	86.3±1.1	P<0.001
1dpp	56.8±0.9	P<0.001
40dpp	36.0±0.7	P<0.001
VS	52.6±0.7	P<0.001
MS	41.8±0.7	P<0.001

Mean: arithmetic mean of collagen fibril diameter per group. P value refers to the statistical analysis to the group above.

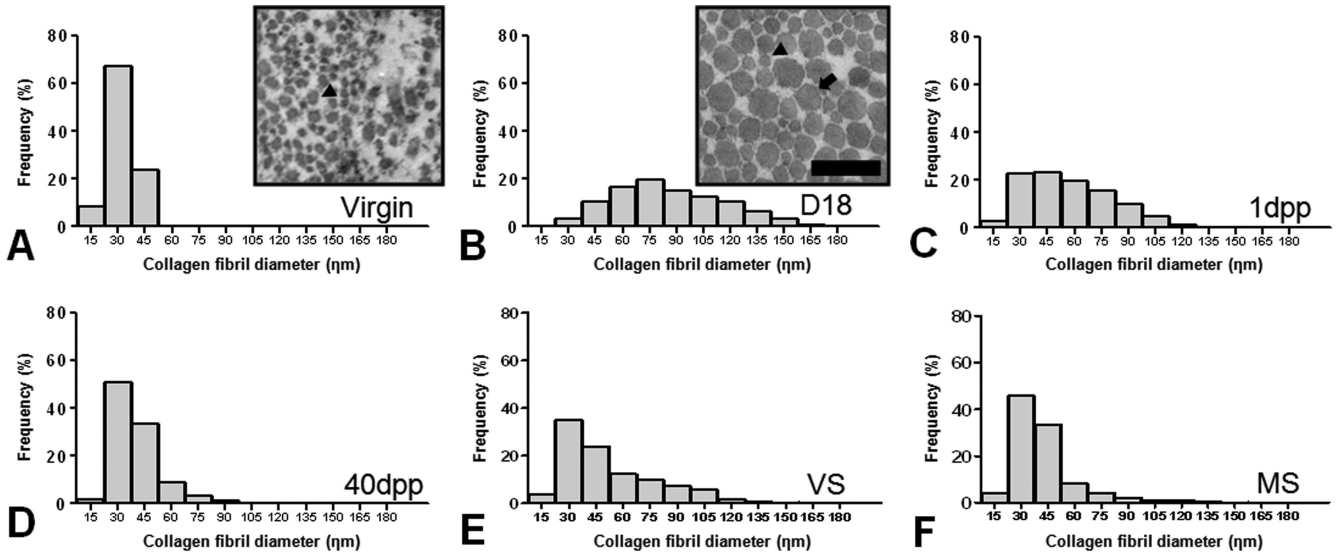

Fig. 4. Histograms showing the differential distribution of the collagen fibril diameter in virgin **(A)**, D18 **(B)**, 1dpp **(C)**, 40dpp **(D)**, VS **(E)** and MS **(G)** mice. In the insets, representative transmission electron micrographs showing the typical thin (arrowhead) and thick (arrow) collagen fibrils observed in the fibrocartilaginous pubic symphysis in virgin mice **(A)** and the interpubic ligament in D18 mice **(B)**. Scale bars: A, B, 300 nm

diameter was apparent in the D18 and 1dpp groups. Rather, a range from 15 nm to 185 nm, comprising both thin and thick fibrils at the pubic symphysis, was observed (Fig. 4B, C). A better insight of distribution of collagen fibril diameter was shown in a box plot with minimum, lower quartile, median, upper quartile and maximum, besides outliers (Fig. 5). Additionally, the mean collagen fibril diameter of the D18 was significantly higher than the virgin group (Table 1); at postpartum, the mean collagen fibril diameter of 1dpp and 40dpp was lower than D18, but the mean collagen fibril diameter of VS group was higher than virgin and 40dpp (Table 1). Finally, in the MS mice, the mean collagen fibril diameter was significantly greater than that observed in the 40dpp group, but was significantly lower than in the VS group (Table 1).

Galectin-3 in interpubic tissues in primiparous, VS and MS mice

The cells of the pubic symphysis are capable of changing their phenotype during the first pregnancy, and because galectin-3 plays an important role in chondrocyte differentiation and myofibroblast activation we hypothesized that this protein is expressed at the interpubic tissues in primiparous and MS mice.

An immunohistochemical analysis of galectin-3 expression revealed positive staining (Fig. 6A-C) in the chondrocytes of the hyaline cartilage (HC), the fibrochondrocytes of the fibrocartilage (FC) and the fibroblast-like cells of the interpubic ligament (IpL). Semi-quantitative data (Fig. 6D) indicated a significantly higher number of positive cells from the virgin (HC=0.30±0.02; FC=0.06±0.01) to the D18 (HC=0.40±0.03; IpL=0.41±0.02) mice. In comparison, a significantly lower number of immunopositive cells were detected in the 1dpp (HC=0.34±0.02; IpL= 0.25±0.01), 40dpp (HC=0.28±0.01; FC=0.22±0.01), VS (HC=0.25±0.02; FC=0.16±0.01) and MS (HC= 0.06±0.005; IpL=0.01±0.001) groups. We further observed that both chondrocytes and fibrocytes that were positive for galectin-3 were rarely found in the MS mice.

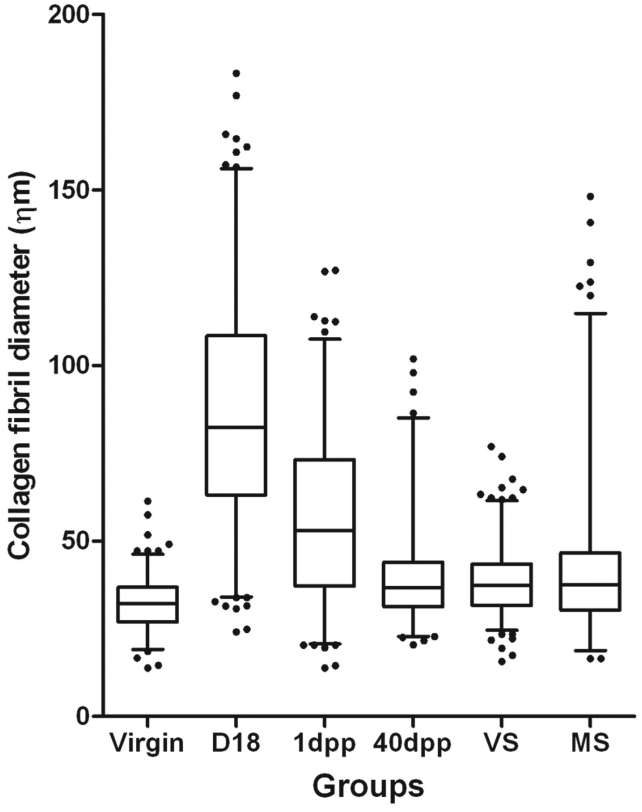

Fig. 5. Box plot showing the minimum, lower quartile, median, upper quartile, maximum and outliers of the collagen fibril diameters in virgin, D18, 1dpp, 40dpp, VS and MS mouse groups.

Discussion

The present study is intended to bring readers up to date with accumulating evidence that the pubic symphysis undergoes remarkable modifications in response to multiparity in senescent mice, even though parity considerations are rarely related in female mice or humans (Becker et al., 2010). Thus, an understanding of the alterations that occur in the birth canal, including the pubic symphysis, due to multiparity in reproductively aged mice, may contribute to our understanding of the biological mechanisms that modify the skeleton and pelvic ligaments and may even play a role in the murine model of prolapse. Thus, the present study focused on the comparisons of the interpubic tissues of primiparous, VS and MS mice, demonstrating the contrasting morphological and morphometrical characteristics and differences in galectin-3 immunostaining that led us to postulate that the persistence of the IpL of the MS group might be associated with an impaired recovery process at postpartum.

The pubic symphysis has a central fibrocartilaginous disk that primarily functions to dissipate impaction forces (Cunningham et al., 2007). However, as seen, the cells of the pubic symphysis are not terminally differentiated, but rather display a state of phenotypic modulation, because first pregnancy induced an increase in the interpubic articulation gap and a change in the overall cellular phenotype during interpubic ligament formation. After parturition, the tissue began to undergo restoration, and the interpubic articulation gap rapidly closed, approaching the virgin dimensions of 40dpp. The modification of the cellular phenotype from an oval to flattened shape suggests that the interpubic tissues in virgin and 40dpp mice are subjected to compressive stress, whereas the flattened morphology during the development of the interpubic ligament in the D18 group indicates that the interpubic tissues are under tension.

These modifications associated with pregnancy may be due to previously described hormonal adaptations to

parturition in mouse (Gardner, 1936; Hall, 1947; Storey, 1957; Crelin, 1969; Zhao et al., 1999, 2000). Besides uterus (Starcher and Percival, 1985), uterine cervix (Read et al., 2007; Buhimschi et al., 2009), vagina (Word et al., 2009), physiological remodeling of the pubic symphysis is seen for the timely onset of labor, and rapid and coordinated remodeling is one of the key processes for uncomplicated delivery, since relaxin knockout mice exhibit abnormal cervical and vaginal morphology, poor mammary apparatus development, and no elongation of the pubic symphysis (Zhao et al., 1999).

In the 12-month-old virgin mice, the interpubic articulation gap and anatomical structure (data not shown) of the pubic symphysis remained very similar to that of the virgin young mice (at 3 to 4 months old). Adding to this, the appearance of oval fibrochondrocytes and lacunae stained differently from the rest of the extracellular matrix. This morphology suggests that aging may produce modified proteoglycan core proteins, in addition to the changes induced by degradation, which regulate the age-dependent proportions and content of proteoglycans (Heinegard et al., 1985; Glant et al., 1986). It has been reported that proteoglycan and type II collagen-rich matrix transmit the compressive loads (Wang et al., 2006). These findings suggest that proteoglycans in the VS group may be involved in tissue remodeling and a dynamic stress profile. This is further supported by the analysis one day postpartum on mouse pubic symphysis at 380 days old that indicated a separation of the pubic bones ranging from 2 to 2.5 mm in length (Gardner, 1936), which indicates that the aged mouse pubic symphysis can be recovered after the first pregnancy.

To our knowledge, only Gardner (1936) and Becker et al. (2010) have examined the microscopic anatomy of the pubic symphysis in multiparous mice. We therefore sought to characterize the effects of multiparity on the cells and extracellular matrix during the interpubic tissue recovery of senescent mice. The interpubic ligament in MS mice displayed cells resembling fibrocytes, based on their compacted chromatin and flattened and extended morphology. This fibrocyte-like phenotype is supported by the lack of evidence of cell division in the multiparous mice, unlike the findings in primiparous animals, as documented in this and another study

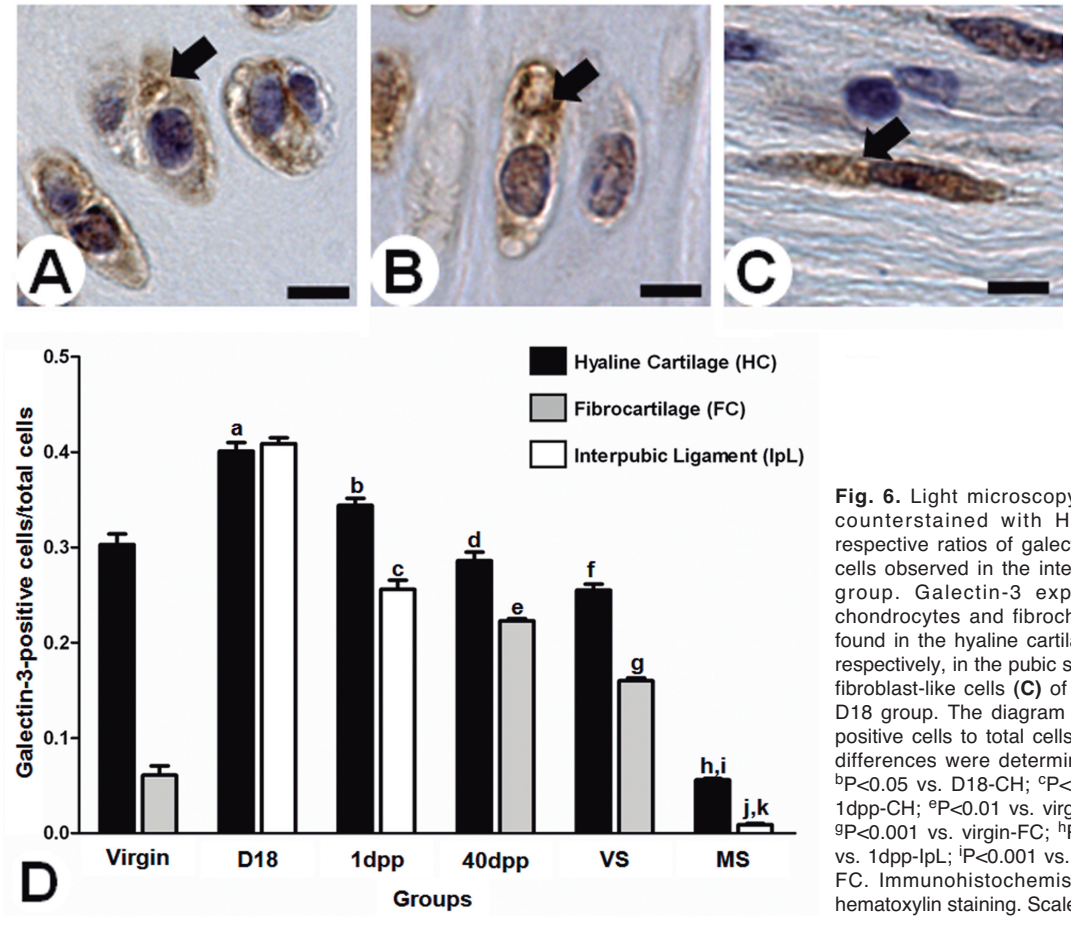

Fig. 6. Light microscopy of galectin-3-positive cells counterstained with Harris hematoxylin and the respective ratios of galectin-3-positive cells to the total cells observed in the interpubic tissues in each mouse group. Galectin-3 expression is present in the chondrocytes and fibrochondrocytes that are typically found in the hyaline cartilage **(A)** and fibrocartilage **(B)**, respectively, in the pubic symphysis in virgin mice, and in fibroblast-like cells **(C)** of the interpubic ligament in the D18 group. The diagram shows the ratio of galectin-3-positive cells to total cells **(D)** for all groups. Significant differences were determined at $^{a}P<0.01$ vs. virgin-CH; $^{b}P<0.05$ vs. D18-CH; $^{c}P<0.01$ vs. D18-IpL; $^{d}P<0.05$ vs. 1dpp-CH; $^{e}P<0.01$ vs. virgin-FC; $^{f}P<0.001$ vs. virgin-CH; $^{g}P<0.001$ vs. virgin-FC; $^{h}P<0.01$ vs. 40dpp-CH; $^{j}P<0.01$ vs. 1dpp-IpL; $^{i}P<0.001$ vs. VS-CH; and $^{k}P<0.001$ vs. VS-FC. Immunohistochemistry for galectin-3 and Harris hematoxylin staining. Scale bars: 1 μm

(Veridiano et al., 2007). In agreement with this evidence, it has been reported that fibroblasts from older mice have been shown to be less motile and proliferative (Arnesen and Lawson, 2006). Thus the reduced cellular proliferative potential of the MS mice may be related to "physiological wear out of breeding" to study physiological deterioration associated with breeding in female mice as a model for senescence and aging (Konigsberg et al., 2007).

Taken together, the evidence suggests that the cellular phenotype and morphology of the interpubic tissues change to allow a safe birth and then are restored following parturition. Such cells preferentially orient along extracellular matrix fibres as the fibroblasts actively sense and align parallel to the axis of stretch of pre-strained elastic substrates (Haston et al., 1983). With increasing age, there are few morphological modifications with regards to the extracellular matrix on the pubic symphysis. However, in the case of multiparity, the cells display a fibrocyte-like phenotype, and the interpubic articulation gap remains open in MS mice. The absence of granulocytes in all the groups confirms the suggestion that recovery does not occur as a result of granulocyte migration or maintenance in the interpubic tissues of primiparous mice, consistent with the observations of Rosa et al. (2008), or VS and MS mice in this study. These findings indicate that cells of the interpubic articulation may play an important role in tissue relaxation during remodeling of the pubic symphysis in pregnant mice (Rosa et al., 2008) and during tissue recovery after parturition following the first pregnancy.

Some studies have shown that parturition has a significant effect on skeletal architecture, and on female pelvic morphology in particular (Bowman and Miller, 1999; Schutz et al., 2009). Aging also alters the musculoskeletal tissues, causing them to become less well-adapted to their functions by reducing ligament elasticity (Barros et al., 2002; Sargon et al., 2004; Freemont and Hoyland, 2007). Nevertheless, evidence for a role of mechanical stimulation in the recovery of the cartilaginous interpubic tissues in primiparous mice is still lacking, despite being a well-known process in the enthesis organ (Benjamin et al., 2006). We believe that the lack of crimps in the pubic symphysis in MS mice may impair the stability of the ligament by preventing recoil after parturition-related stretching. This hypothesis is supported by the finding that the pubic symphysis in multiparous women has greater anteroposterior sagital movements than in nulliparous women (Walheim et al., 1984).

The tensile strength of the pubic articulation increases with the collagen fibril diameter to remodel the birth canal as the first pregnancy progresses. This increased fibril diameter is also observed in other tissues during development and maturation (Parry et al., 1978). In contrast, the diameter of the collagen fibrils decreased in the 40dpp mice in the present study, indicating a compression at the site of articulation to close the birth canal. These modifications during and after a first pregnancy occur in addition to changes in the cellular phenotype, thus restoring the fibrocartilaginous articulation site. Therefore, the differential distribution of collagen fibrils in the mouse pubic symphysis during a first pregnancy may be directly related to the mechanical properties of the tissue. The VS mice showed a slender deposition of organized collagen fibrils, continuous with the periosteum, surrounding the pubic symphysis, but the fibrocartilage remained with non-axial fibrils that surrounded the fibrochondrocytes.

Our data suggests an impaired recovery process at postpartum in MS mice. These animals exhibited thin collagen fibrils and the cellular phenotype remained fibrocyte-like, even under an apparent compression of the interpubic articulation after parturition. Further evidence was seen in the extracellular matrix morphology of the interpubic ligament, indicating aligned, thin and compacted collagen fibrils at the pubic symphysis articulation. This finding is supported by the changes with age in the density and structure of cross-links and in the fibril morphology of the collagenous tissue (Shadwick, 1990). In addition, the collagen fibrils become more compacted and oriented (de Carvalho and Vidal Bde, 1994), and the synthesis of collagenolytic enzymes decreases with age (O'Brien, 1997). One factor in impaired recovery in adults is the progressive decrease in the percent of synthesized proteoglycans (Plaas and Sandy, 1984), which can be related to tensile stress in tissue (Wang et al., 2006). Thus, the compacted morphology of the interpubic ligament in MS mice is suggestive of an age-dependent reduction in the amount of proteoglycans, indicating an adaptation in matrix organization and composition at the mouse pubic symphysis.

Regulation of cellular homeostasis by galectin-3 (Hsu and Liu, 2004) not only aids in regulating chondrocyte differentiation (Kasper et al., 2009) and metabolism, which are deemed necessary to maintain functional cartilage (Colnot et al., 2001; Boileau et al., 2008), but also supports liver myofibroblast activation (Henderson et al., 2006), prevents mitochondrial damage and generation of reactive oxygen species (Matarrese et al., 2000; Yu et al., 2002). Galectin-3 has been identified at embryonic stem cell-derived chondrogenic cells (Fecek et al., 2008) and has been suggested as a promising therapeutic target for rheumatoid arthritis (Ohshima et al., 2003; Neidhart et al., 2005) due to its anti-apoptotic functions (Hsu and Liu, 2004).

In the present study, the higher number of galectin-3-positive cells on the pubic symphysis during a first pregnancy and in the VS group supports the hypothesis that chondrocytes in the pubic symphysis and myofibroblasts (Moraes et al., 2004) in the interpubic ligament require galectin-3 for survival and activation, respectively. Thus, the scarce number of galectin-3-positive cells observed in the MS mice may indicate impaired chondrocyte survival and/or myofibroblast activation, which would be required to tighten the belt-

like pubic symphysis. Supported by Veridiano et al. (2007), it is tempting to speculate that galectin-3 may induce a transient resistance to cellular apoptosis in the D18, 1dpp, 40dpp and VS mice, whereas this resistance may be diminished in MS mice. These findings are consistent with an *in vivo* modification of the pubic symphysis at the cellular level due to multiparity in senescent mice. However, the molecular pathways involved remain unclear.

After multiple pregnancies, the cellular and extracellular features of the interpubic tissues may be related to an impaired recovery process at postpartum due to multiparity in senescent mice. In contrast, recovery is preserved in the interpubic ligament in primiparous mice (Moraes et al., 2004) that have not been subjected to the physiological stress of breeding and the related age-associated deterioration at the cellular level (Konigsberg et al., 2007). The observation that the morphology of the pubic symphysis in the VS group is maintained in a similar state to that observed in virgin young mice indicates that multiparity plays an important role in the morphology of MS mice. We cannot confirm whether the aged extracellular matrix encourages a fibrocyte-like cell phenotype or whether aged fibrocyte-like cells cause changes in the extracellular matrix, but this is certainly highly relevant to the reproductive and gerontological fields. The scarce number of galectin-3-positive cells in the MS mice may support the second possibility, which postulates that reduced levels of galectin-3 may impair the capacity of interpubic cells to remodel and restore the pubic articulation after pregnancy.

Because the female pelvis provides support for pelvic organs (Schimpf and Tulikangas, 2005) and the pelvic skeleton forms the margins of the birth canal (Weaver and Hublin, 2009), the importance of elucidating female age and reproductive history is clear, since abdominal muscle attachments may be shifted by the development of the interpubic ligament to enlarge the birth canal which explains, in part, the hernias in mouse (Gardner, 1936). Animal models, such as mice with null mutations in genes that encode proteins related to elastic fiber synthesis and assembly, including lysyl oxidase-like 1 (Liu et al., 2004), fibulin-5 (Drewes et al., 2007) and fibulin-3 (Rahn et al., 2009), have facilitated the study of pelvic organ prolapse. Despite the differences in posture and fetal size between mice and humans, mouse models improve our understanding of the pathogenesis of prolapse because, as observed in humans, mice can develop this condition both after pregnancy (Liu et al., 2004; Drewes et al., 2007) and during aging (Rahn et al., 2009). However, although no specific data is available regarding pubic symphysis, because it comprises a part of the margins of the birth canal (Weaver and Hublin, 2009) and is not solely a semi-genital target organ (Rundgren, 1974), this articulation site may play a role in the pathophysiology of pelvic organ prolapse. More specifically, adaption of pelvic bones and ligaments that were previously used in multiple pregnancies may be limited due to impaired recovery at postpartum.

The modifications in pubic symphysis length and cervical dilatability in pregnant mice are part of an adaptive physiological process that provides mechanical protection of the birth canal (Steinetz et al., 1957; O'Brien, 1997; Read et al., 2007). Prior studies have demonstrated that the length of the pubic symphysis increases rapidly between days 16-17 of pregnancy, whereas the cervical dilatability increases rapidly between days 18-19 (Sherwood, 1994; Read et al., 2007). Therefore, when Rahn et al. (2008) performed a vaginal distention using a balloon to simulate parturition at day 14 of pregnancy, the birth canal was not physiologically prepared, as evidenced by a significant increase in subsequent vaginal wall protease activity.

In conclusion, we believe to have shown that multiparity in senescent mice alters the pubic symphysis at both the intracellular and extracellular level, and we consider the pubic symphysis to be important for pelvic tissue support. However, the major limitation for this study was to determine a specific time point to characterize a model to study senescence. Mice age gradually, but there is no a priori reason to expect them to age for the same causes and mechanisms. Thus, in order to validate the results obtained in these animals, we paid attention to signs of reproductive senescence that can be seen in retired female breeders (12 months old) from the reproductive matrix after having given birth six to eight times and demonstrated a decline in fertility (Yeh and Kim, 2007). Therefore, the mouse pubic symphysis model may contribute to future research investigating the physiopathological and molecular pathways that underlie the changes in the reproductive tract and aged connective tissue of MS or genetically modified mice.

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