cord. Because the spike wave reflects action potentials, it shows propagation along the axonal pathway toward the cranial region. The amplitude of the spike wave is less influenced by the frequency of the stimulation, whereas the gentle waves do not propagate and decrease their amplitude with high frequency stimulation because of synaptic fatigue (Ertekin, 1976; Cracco, 1973; Saiki, 1979; Shimoji et al., 1972). In our experiment, E1 showed polyphasic configurations probably reflecting plural axonal currents. This could possibly be due to differences in nerve root length or the conductivity of each neural fiber in the spinal cord.

In this study we observed two different types of magnetic signals: propagating sharp waves in the short latency time and stationary gentle waves in the late latency time.

Comparing the results of the recorded SCEPs and SCEFs within the same subject, the latency of E1 in SCEPs and M1 in SCEFs at the center of the quadrupolar magnetic fields was nearly the same at the same vertebral level, and similarly, the latency and duration of E2 in SCEPs almost corresponded to that of M2 in SCEFs (Fig. 5). In addition, the conduction velocity of E1 and M1 was close at around 60–100 m/s, which is reasonable for the physiological value of neural conduction velocity (Akaike, 1973; Fukuoka et al., 2002). In the isomagnetic field maps, unlike the propagating quadrupolar fields, the dipolar fields sustained their position and, similar to E2 in SCEPs, they became unclear when high frequent stimuli were applied. Though the complicated volume currents by vertebral structures and gaps of them have a possibility to affect segmental magnetic events, removing dorsal bony interruption by lumbar laminectomy would decrease the effects of these volume currents. Thus, the quadrupolar field was considered to be constructed from M1 and was generated from the primary afferent nerves and the white matter, while the dipolar fields were thought to be constructed from M2 and originated from synaptic activities.

Two different types of estimated current sources, the conductive current flow and stationary currents, would support this conclusion. The forward and backward current flow, corresponding to the quadrupolar magnetic field in the latency, would account for the intra-axonal current flow derived from the action potential propagating from the sciatic nerve to the spinal cord. The following stationary and sustained currents, which emerged and faded away spontaneously, corresponded to the location of the dipolar fields, respectively. The minimum-norm estimation is a popular method for estimating the current distribution in the human brain from MEG data. We adopted this method to estimate current sources. Visualized current sources helped us to better understand simultaneous and adjacent phenomenon more easily.

In most of the subjects, the isomagnetic fields and the estimated currents showed that apparently plural static current sources emerged at different levels on the spinal cord.

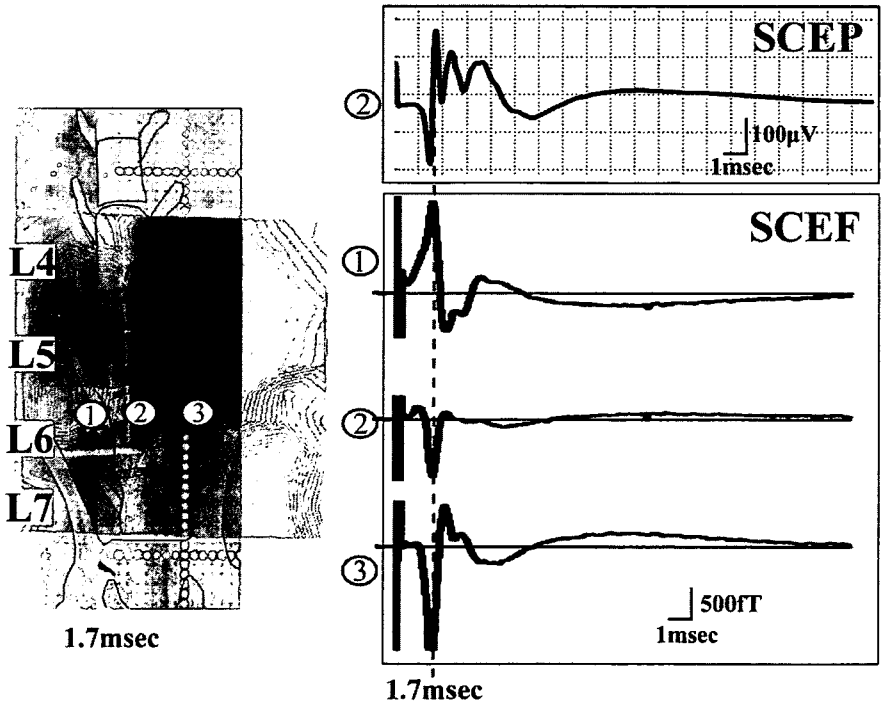

Fig. 5. Isomagnetic contour maps of the quadrupolar pattern and stationary dipolar fields were projected on the X-ray image. When comparing the results of the measurements of SCEP and SCEF at about the L6 vertebral level, the latency of the spike wave in SCEP (1.7 ms) corresponded to that of the center of the quadrupolar magnetic field. This indicates that the quadrupolar magnetic field was generated from the primary afferent. The latency and duration of the following gentle waves in both SCEP and SCEF were also the same, indicating that stationary and sustained dipolar fields were derived from synaptic activities.

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Neuromagnetic recordings were thought to be able to visualize axonal activities in the sciatic nerve which flowed into the spinal cord from L6 to S2 nerve roots, climbing up the spinal tracts and activating synaptic transmissions at L6, L5 and L4, in order. The difference in the nerve root length which diverged from the sciatic nerve would reflect the order of appearance of each segmental synaptic activity. The last sustained dipolar field, fired at about the L6 level, indicates that the main segment of sciatic nerve of a rabbit may exist at around the L6 level.

The direction of the synaptic currents did not show uniformity. Although our system is not suitable to measure vertical elements of currents because pick-up coils are placed horizontal to the X–Y plane and designed to measure only orthogonal elements of evoked magnetic fields, further analysis to examine current directions on the Y–Z or X–Z plane would be required for rational explanation of the incoherent synaptic current directions.

In the case of clinical use, stimulation of the peripheral nerve has a great advantage compared with stimulation of the spinal cord using an epidural catheter electrode because peripheral nerves are easily stimulated non-invasively from the skin. The ultimate aim of neuromagnetic recordings is to evaluate the function or the lesion level of the spinal cord without using invasive techniques. The measurement of segmental-SCEFs could become a helpful method to evaluate both the function of multi-segmental synaptic activities and conductive axonal activities in the spinal cord at one time.

Acknowledgements

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厚生労働科学研究費補助金（長寿科学総合研究事業）
高齢者の腰痛症に係る効果的な診断、治療、リハビリテーション等の確立
分担研究報告書

高齢者腰痛症の一因を成す椎間板変性に関する分子生物学的解析

分担研究者：千葉一裕　慶應義塾大学医学部整形外科　准教授

研究要旨：閉経後骨粗鬆症は脊椎圧迫骨折を引き起こし腰痛の原因となる。また椎間板変性も腰痛症の一因と考えられており、閉経によるエストロゲン欠乏が、椎間板変性にどのような影響を与えるのか、ラットを用いて骨粗鬆症モデルを作成し椎間板変性における分子生物学的な解析を行った。in vivo、in vitro の実験を経て、エストロゲン欠乏は椎体軟骨終板の変性を引き起こし、椎間板細胞における２型コラーゲンの mRNA 転写を抑制することで、椎間板変性を加速させる結果となった。以上より、閉経に伴うエストロゲン欠乏は、椎間板変性のリスクファクターに成り得る可能性が考えられた。

A. 研究目的

閉経後のエストロゲン欠乏による骨粗鬆症性脊椎圧迫骨折や椎間板変性は、中高齢者の慢性的な腰痛症の原因となる。エストロゲン欠乏によって引き起こされる骨粗鬆症と椎間板に関する報告は画像解析を中心として散見されるが、エストロゲンの椎間板に対する直接作用に関する報告はない。われわれは、卵巣摘出術後ラットを用いて骨粗鬆症モデルを作成し、エストロゲン欠乏が椎間板の恒常性にどのような影響を与えるかを分子生物学的に検討した。

B. 研究方法

8 週齢ラットを用いて卵巣摘出モデルを作成し（OVX 群）、術後１週から７ヶ月の間で、経時的に尾椎から髄核と線維輪を摘出し、Real-time PCR でⅡ型コラーゲンの mRNA 発現量を Sham 群と比較した。同時に組織学的変化の解析も行った。また、椎間板におけるエストロゲン受容体（ERα, ERβ）の発現を免疫染色で確認した。椎間板細胞および軟骨細胞に対するエストロゲンの直接作用を検討するため、8 週齢ラット尾椎から摘出した椎間板線維輪細胞と新生仔マウス由来肋軟骨細胞を用いて、アルジネートビーズまたは単層培養で培養し、17β-estradiol（E2）, TGF-β1（10ng/ml）刺激および SB431542（ALK-4/5/7 阻害剤）を添加した状態でのⅡ型コラーゲンの発現量を解析した。また、ER を介したⅡ型コラーゲン遺伝子（Col2a1）の転写活性を検討するために、ERα・ERβ・Dominant Negetive-ERα または活性型 Smad3 発現ベクターとともに Col2a1 promoter-Luciferase ベクターを用いて、Cos7 細胞でレポーターアッセイを行った。

C. 研究結果

組織学的所見として、術後 3 ヶ月、7 ヶ月の OVX 群では、髄核細胞、線維輪細胞数が有意に減少し、2 次性の骨化像からなる終板の変性が認められた。Ⅱ型コラーゲンの mRNA 発現量は髄核と線維輪において OVX

群で術後 2 週以降有意に減少した。ERαとERβは髄核、線維輪、椎体終板、成長板ともに発現しており、その局在は核内であった。椎間板細胞、肋軟骨細胞ともに、E2 とTGF-β1 刺激によってⅡ型コラーゲンのmRNA 発現量は相加的に増加し、SB431542 の添加により抑制された。E2 刺激によるCol2a1 転写活性の上昇は、ERβではなくERαに依存しており、転写開始点から 1kb 以内に存在する putative なエストロゲン応答配列（ERE）を削ることによって、その活性が低下した。またERα依存性のCol2a1 転写活性は、活性型 Smad3 により相乗的に増加した。

D. 考察

エストロゲン欠乏によるラット椎間板変性の機序として、リガンドと結合した活性型エストロゲン受容体の Col2a1 に対するgenomic な直接的作用と、軟骨終板の変性を介した拡散障害による間接的作用が考えられた。E2 存在下の Col2a1 の転写制御にはERαと Smad3 の相互作用が関与することが判明した。エストロゲンはERαを介して、TGF-βシグナルによって調節される椎間板細胞および軟骨細胞の生理的な代謝に影響を与え、直接的および間接的に椎間板組織の恒常性維持に寄与している可能性が示唆された。

E. 結論

エストロゲンは椎間板細胞の恒常性維持に寄与していることが確認されたことから、将来的な椎間板変性抑制への 1 つの治療法として臨床の場へ還元される可能性が示唆された。

F. 発表業績

1. 論文発表

2. 学会発表

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2) M. Kato, H. Takaishi, K. Chiba. Degeneration of Intervertebral Disc in Ovariectomized Rats. 29th The American Society for Bone and Mineral Research. September 2007

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4) 加藤雅敬、高石官成、千葉一裕: ラット卵巣摘出モデルにおいて椎間板変性は経時的に進行する．第 22 回日本整形外科学会基礎学術集会　2007 年 10 月

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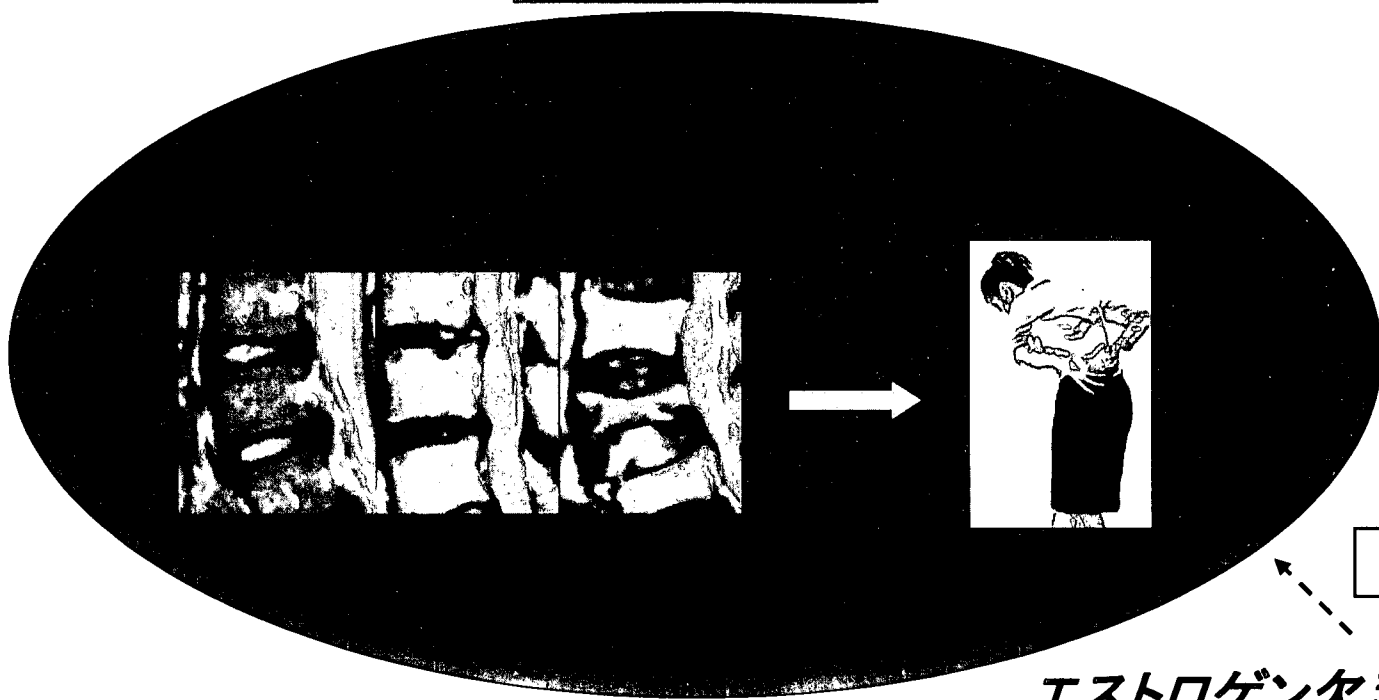
G. 知的財産権

予定していない。

目的 / 方法



♀ Wistar rat 8週齢

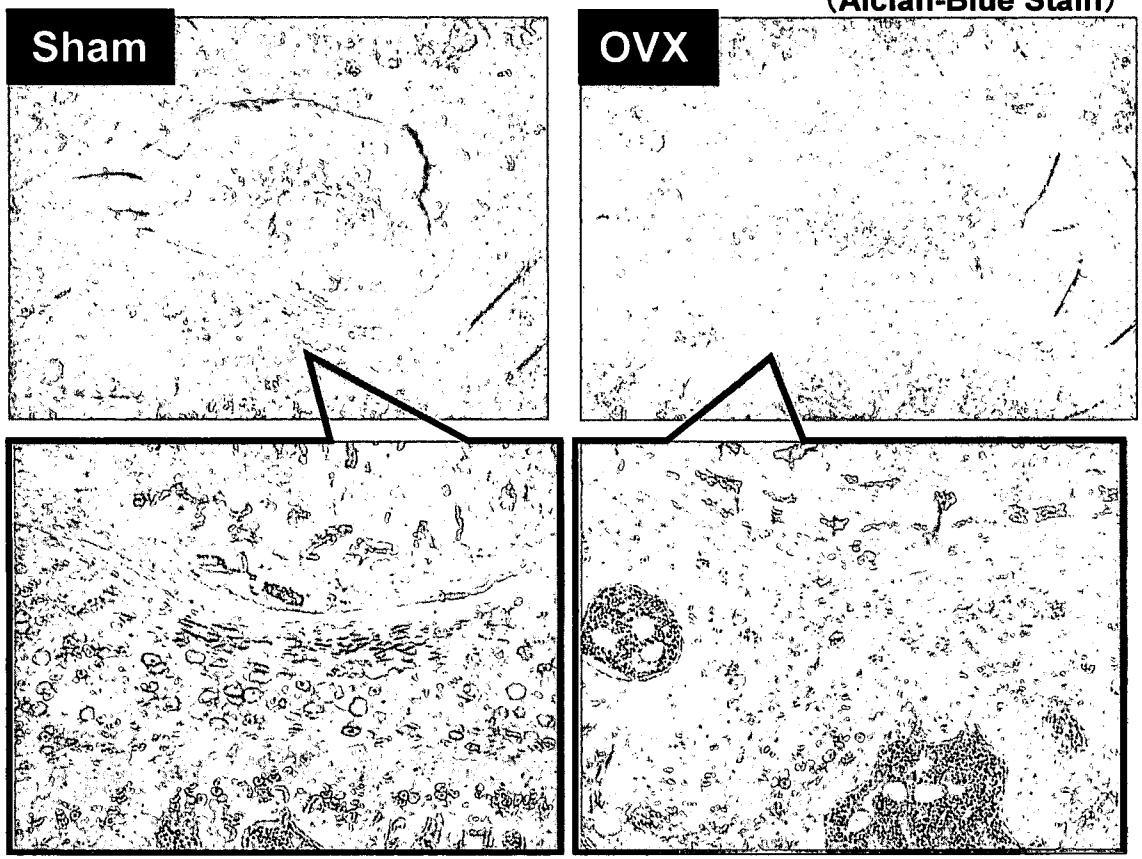
- ☆ OVX椎間板の組織学的検討
- ☆ OVX後の髄核(NP)・線維輪(AF)のII型コラーゲンmRNA発現変化
- ☆ 髄核・線維輪におけるエストロゲン受容体(ER)の発現
- ☆ 椎間板培養細胞に対するE2刺激によるII型コラーゲンmRNA発現
- ☆ II型コラーゲンプロモーターを用いたレポーターアッセイ

結果

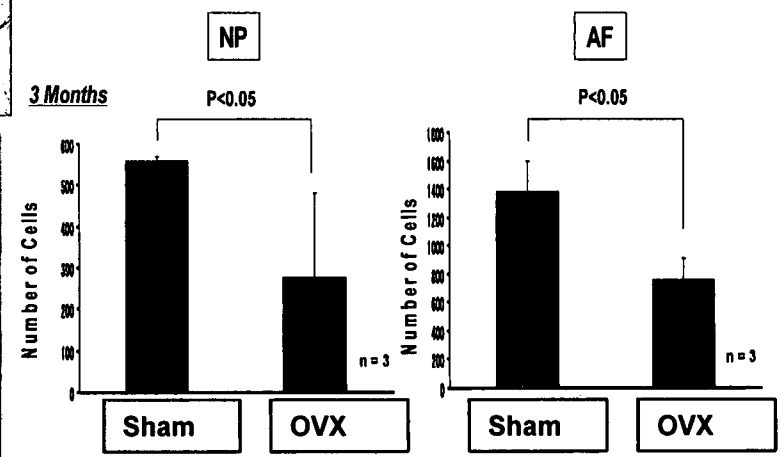
卵巣摘出術後3ヶ月のラット椎間板 / 軟骨終板では変性が進行する

術後3ヶ月

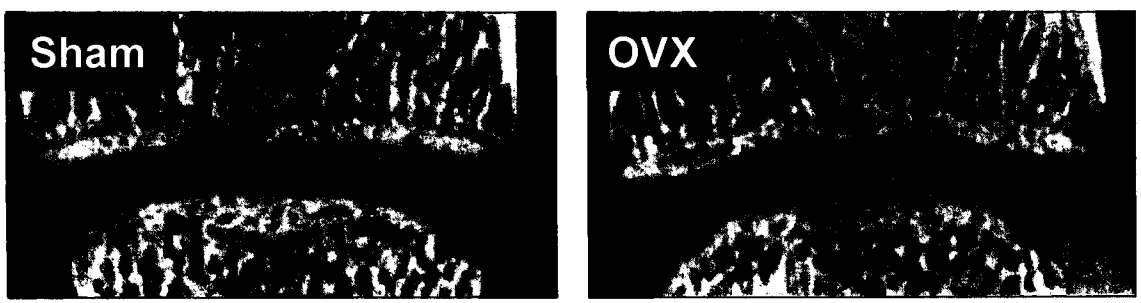
(Alcian-Blue Stain)



【 髄核・線維輪の細胞数減少 】



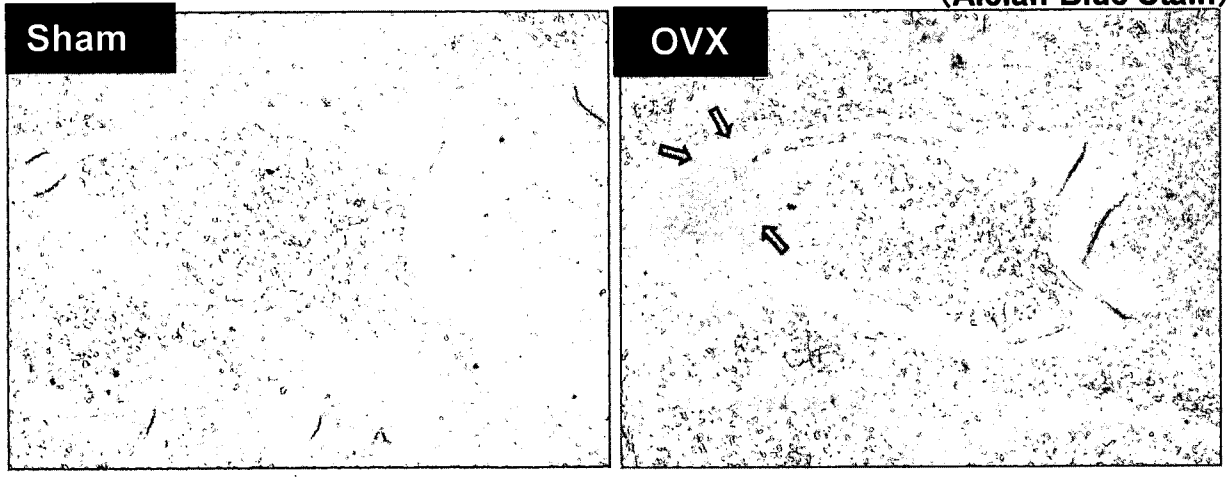
【 マイクロCT: 終板の変形 】



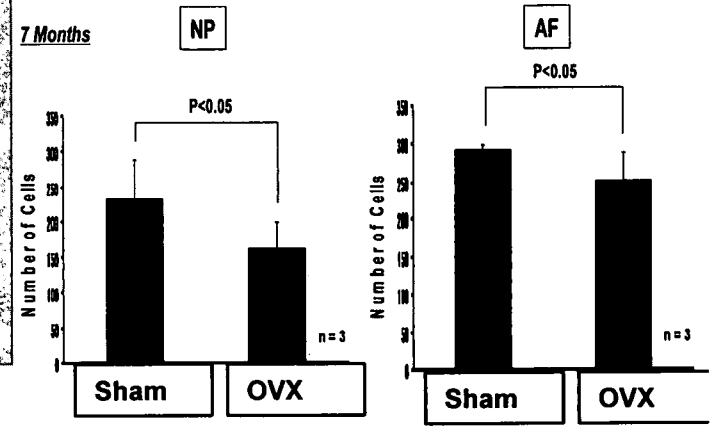
卵巣摘出術後7ヶ月・1年のラット椎間板/軟骨終板では変性が進行する

術後7ヶ月

(Alcian-Blue Stain)

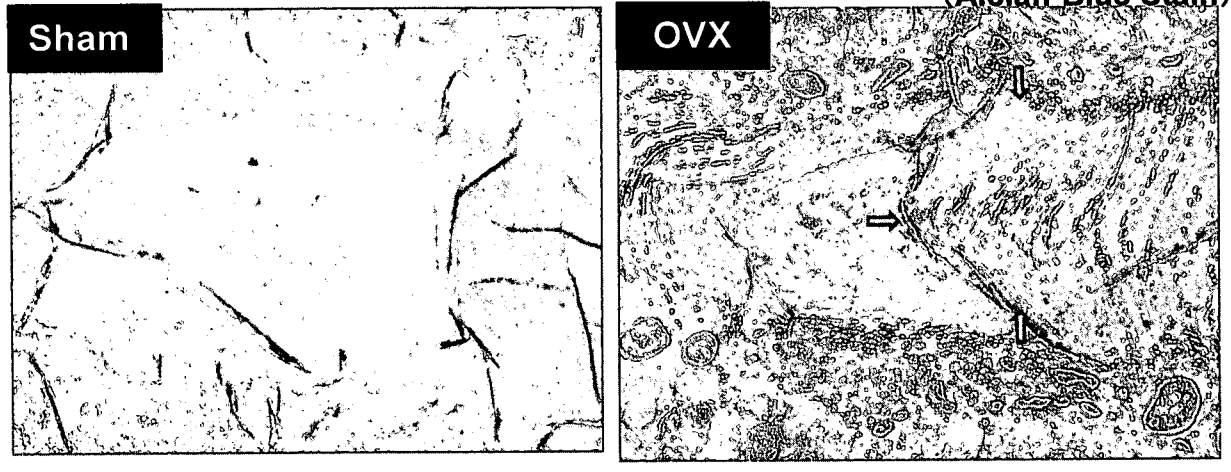


【 髄核・線維輪の細胞数減少 】



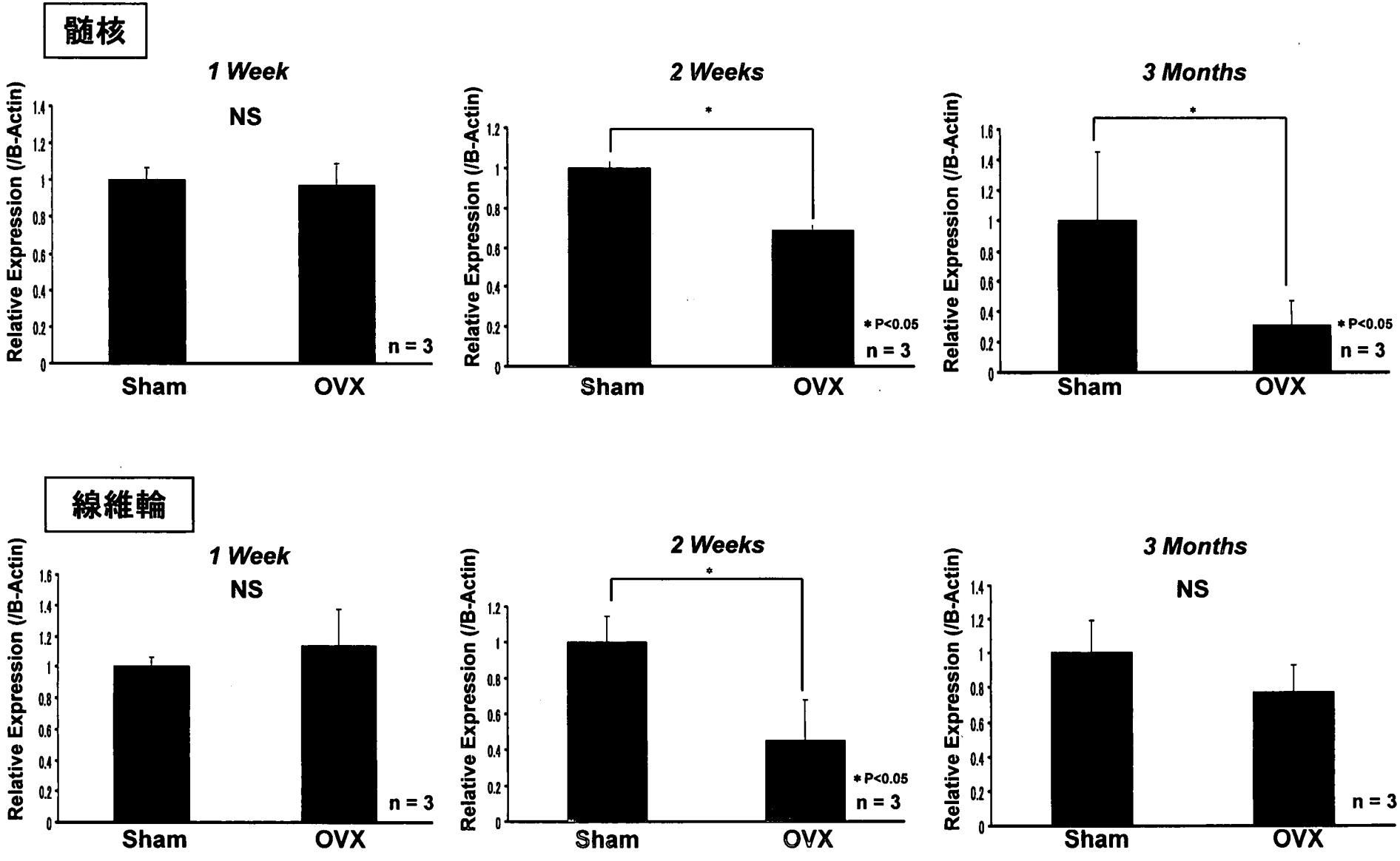
術後1年

(Alcian-Blue Stain)

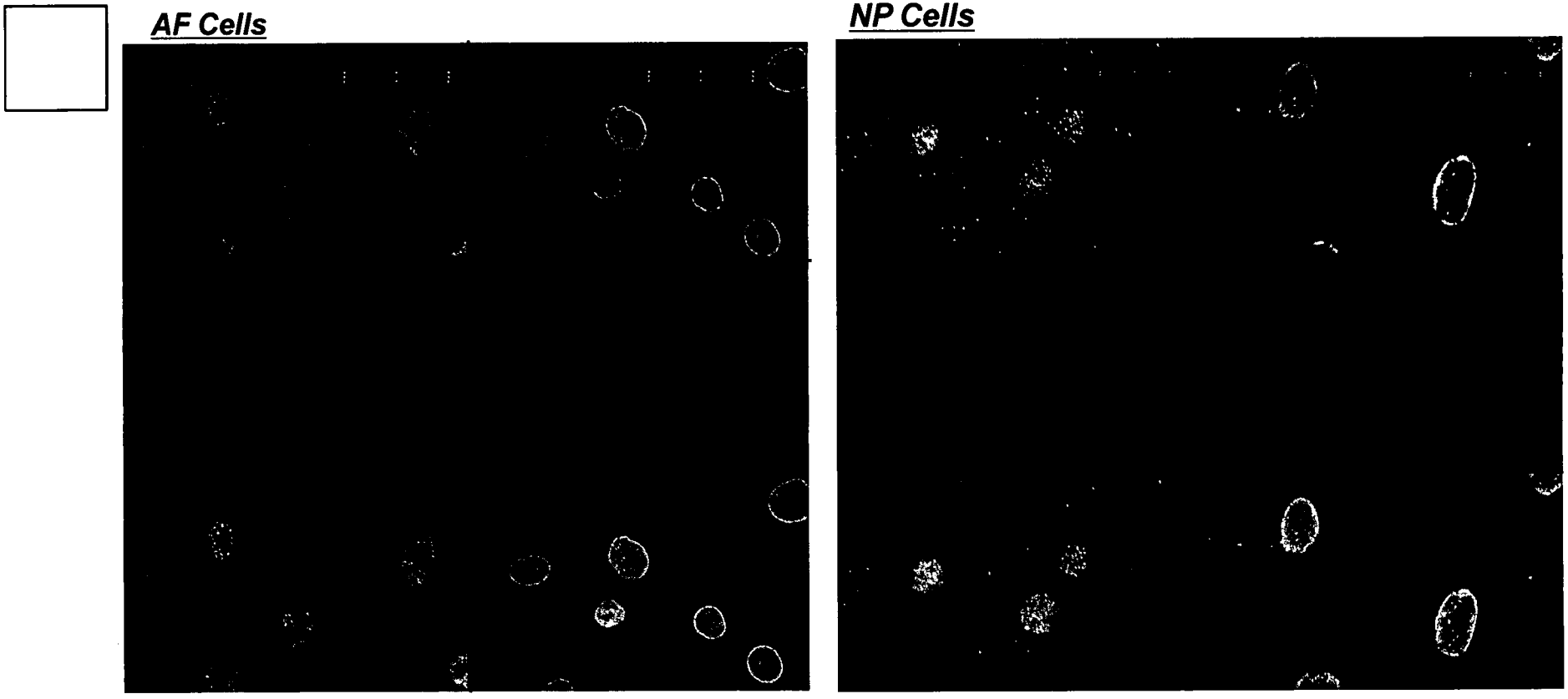


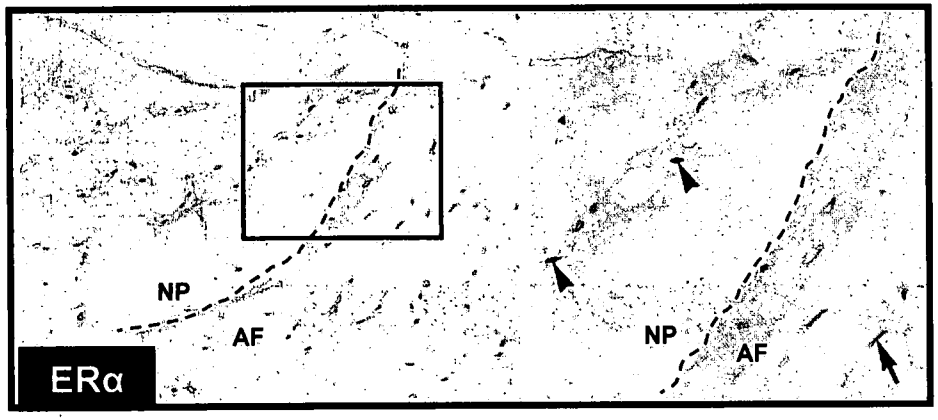
【 MRIにおける椎間板輝度の低下 】

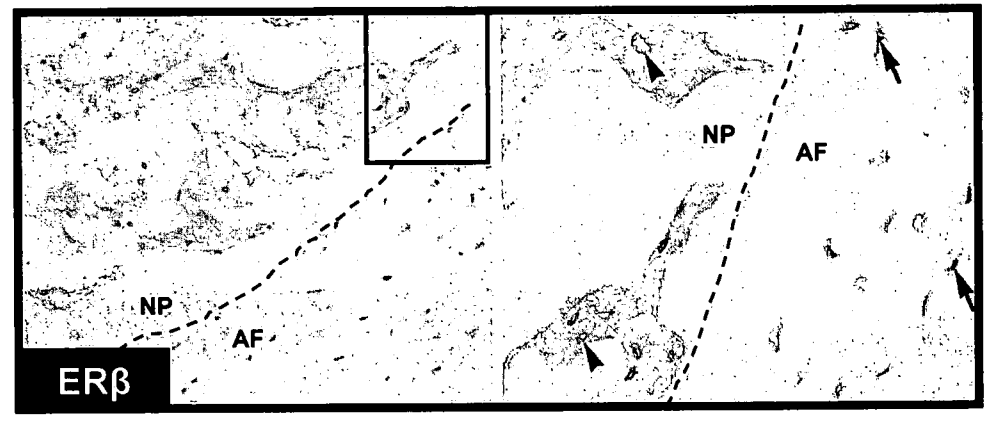
髄核・線維輪のII型コラーゲンmRNAはOVXで低下する



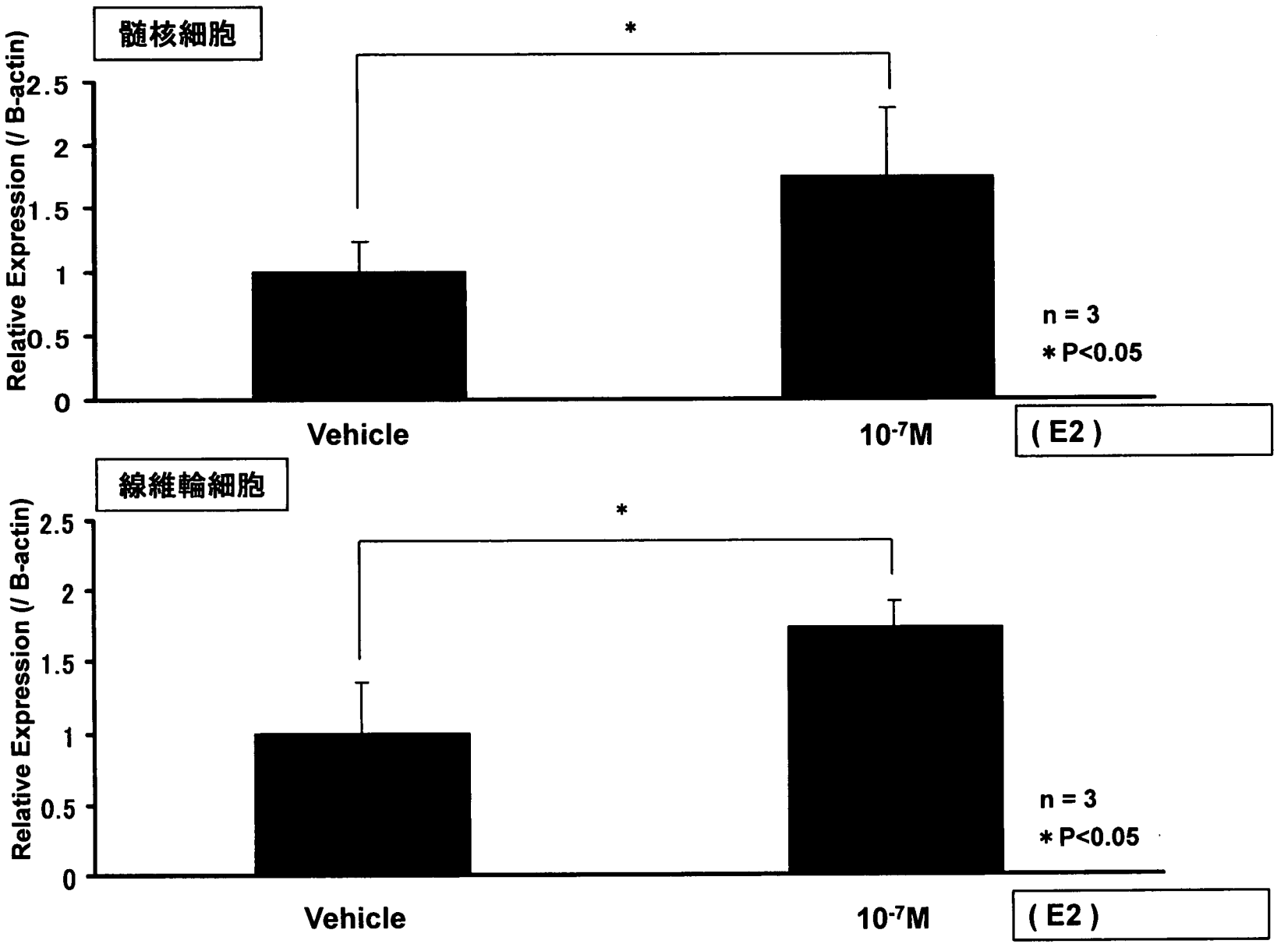
椎間板におけるエストロゲン受容体の発現



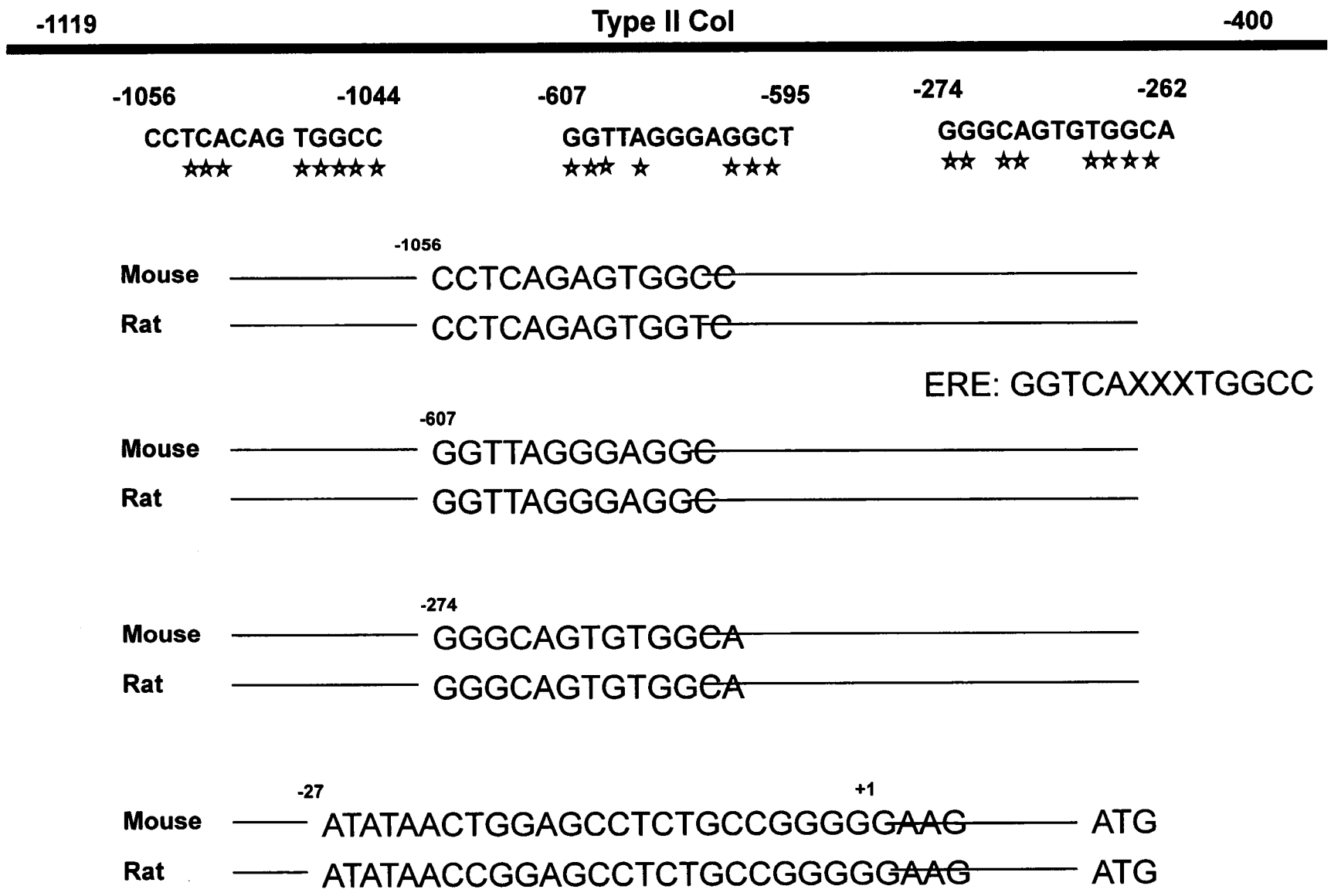




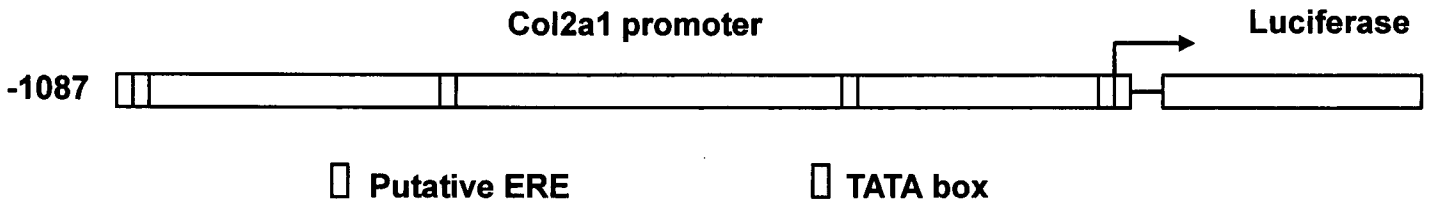
髄核・線維輪細胞のII型コラーゲンmRNAに対する17βエストラジオール(E2)の効果

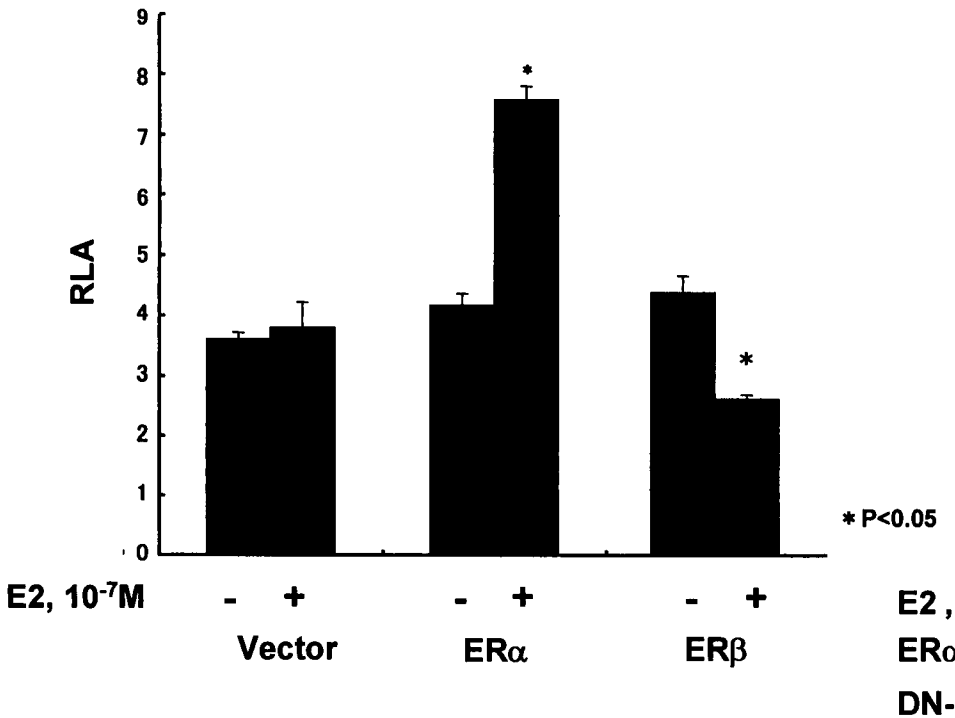


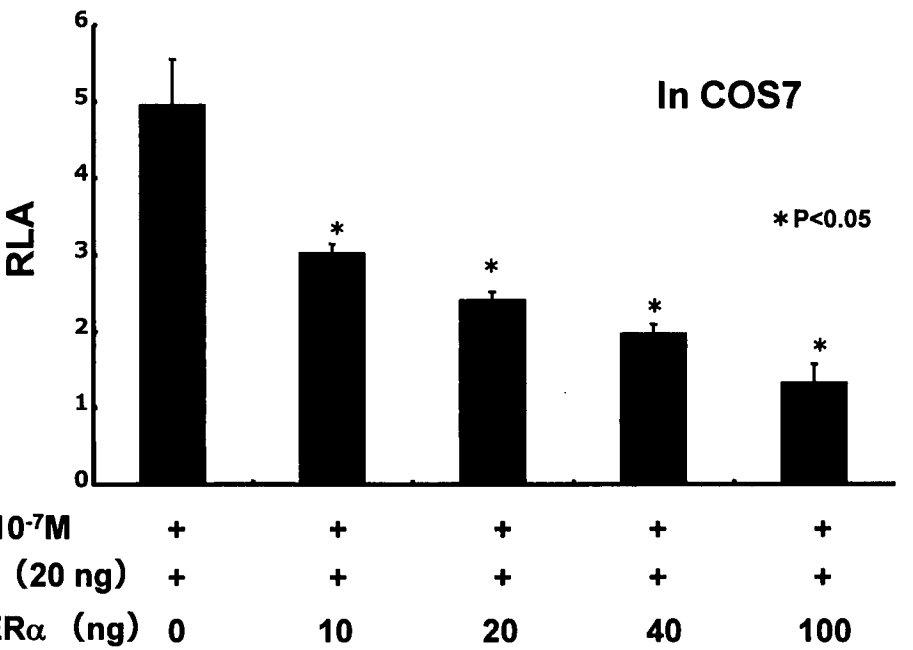
Putative ERE in the Type II Collagen Promoter



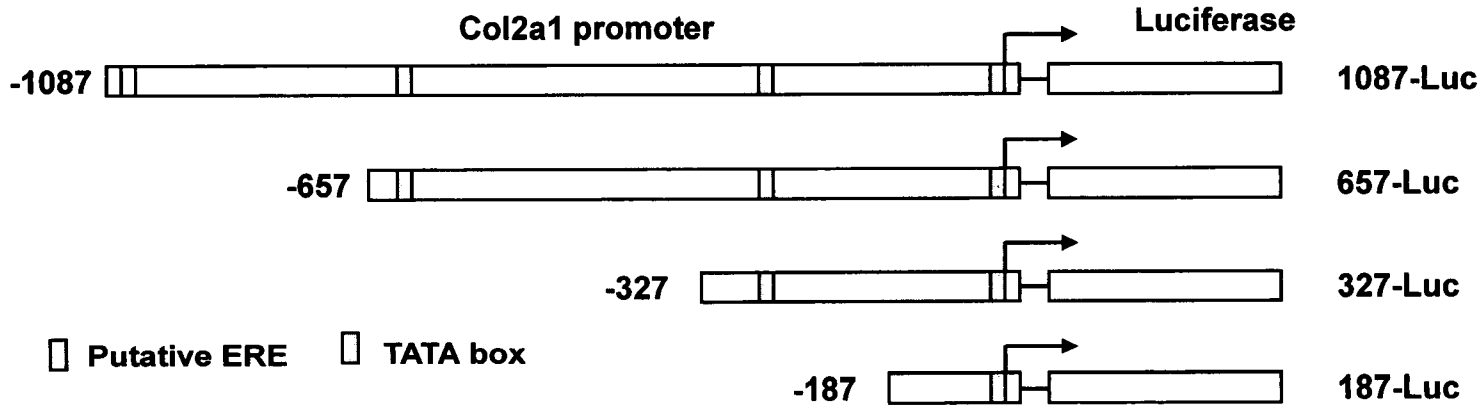
*E2はERαを介してI*型コラーゲン転写活性を促進する

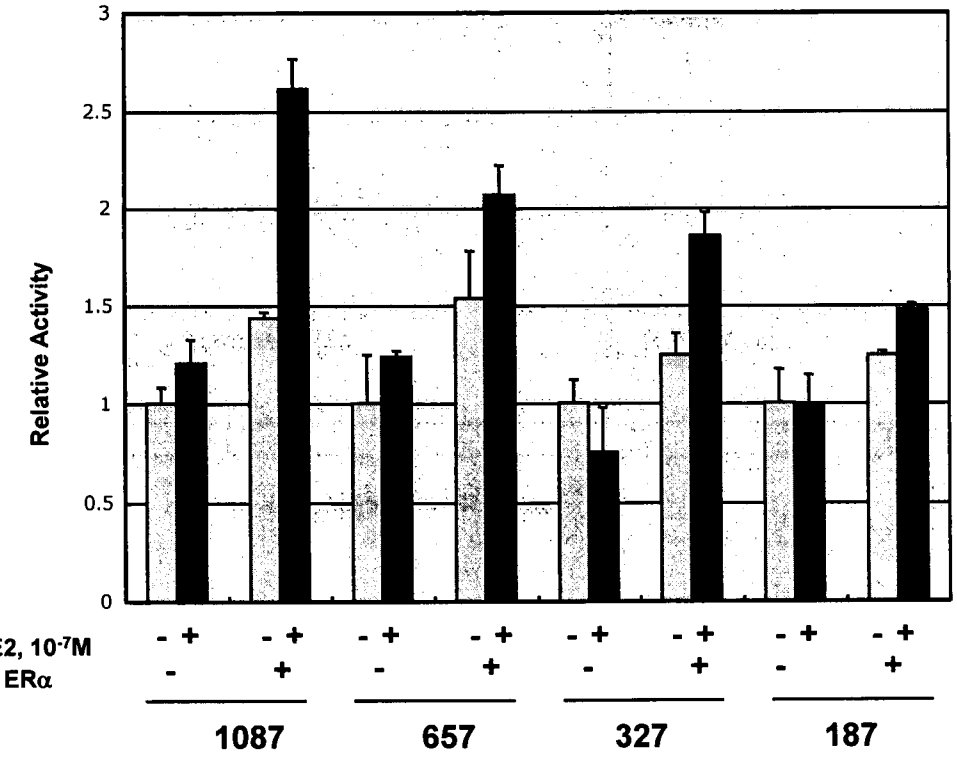




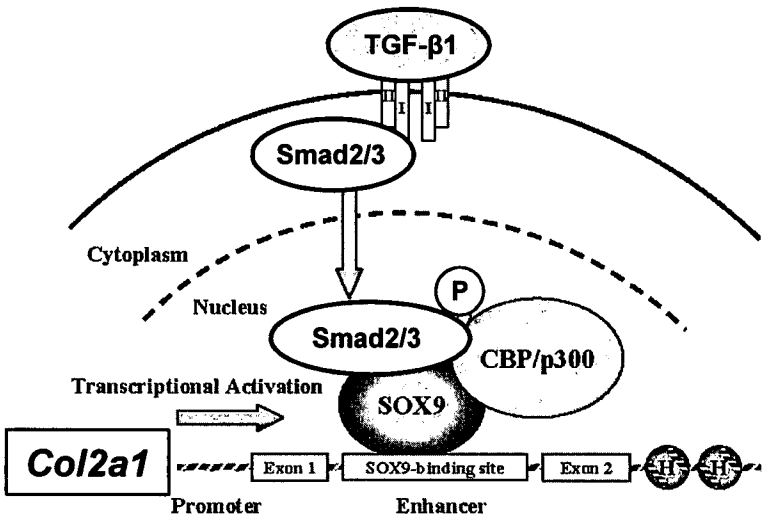


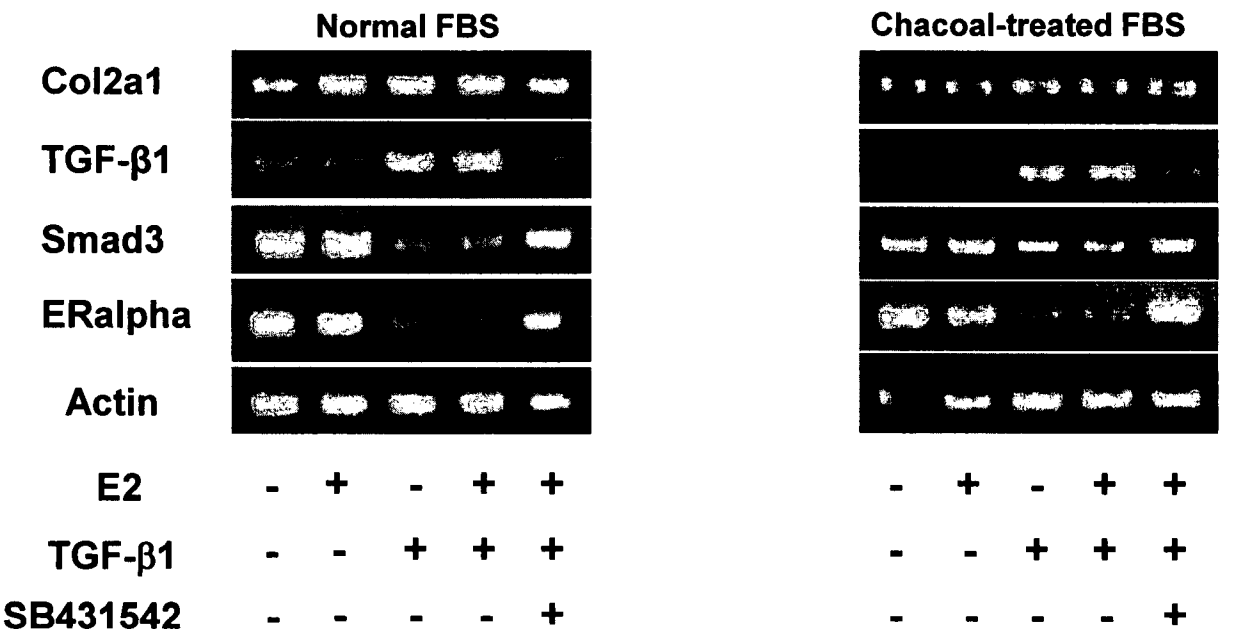
*E2*依存性の*II*型コラーゲン転写活性の促進は*ERα-ERE*を介する



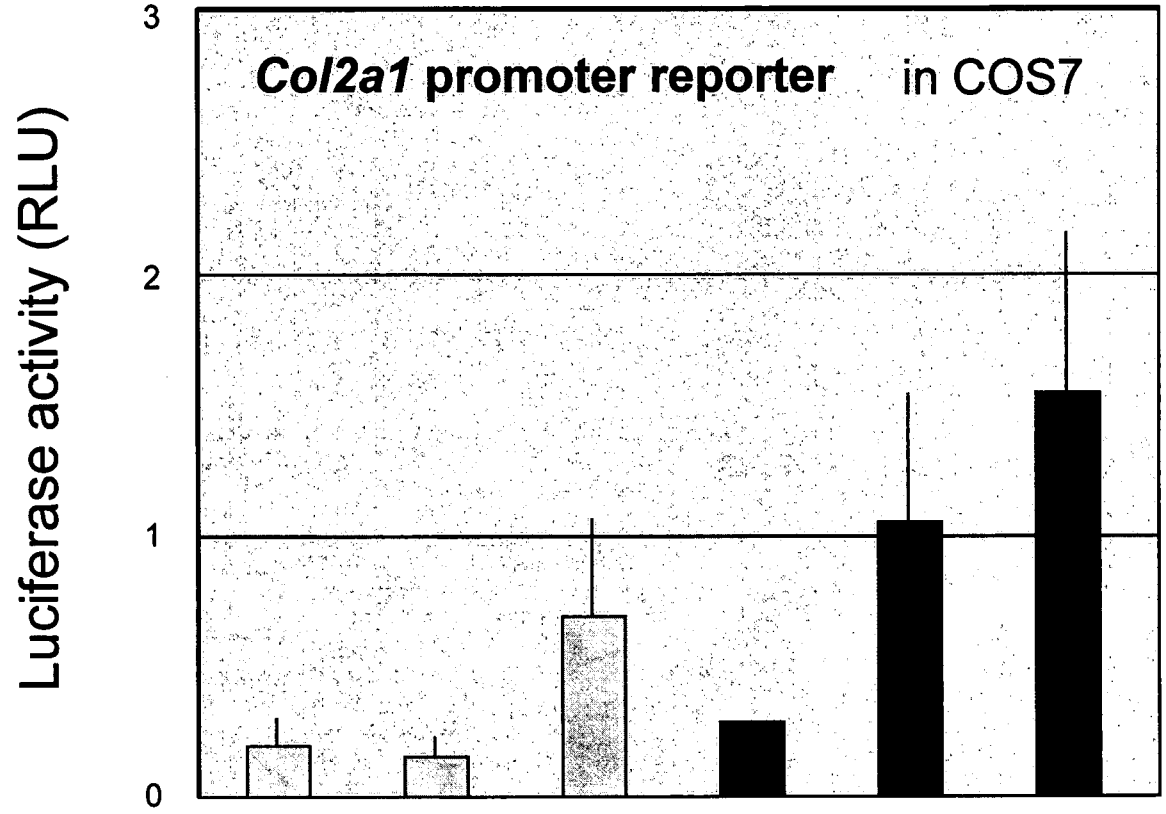


軟骨細胞のII型コラーゲンmRNA発現に対するTGF-β1とE2の影響





*Smad3*を介する*I*型コラーゲン転写活性は*E2*濃度依存性である



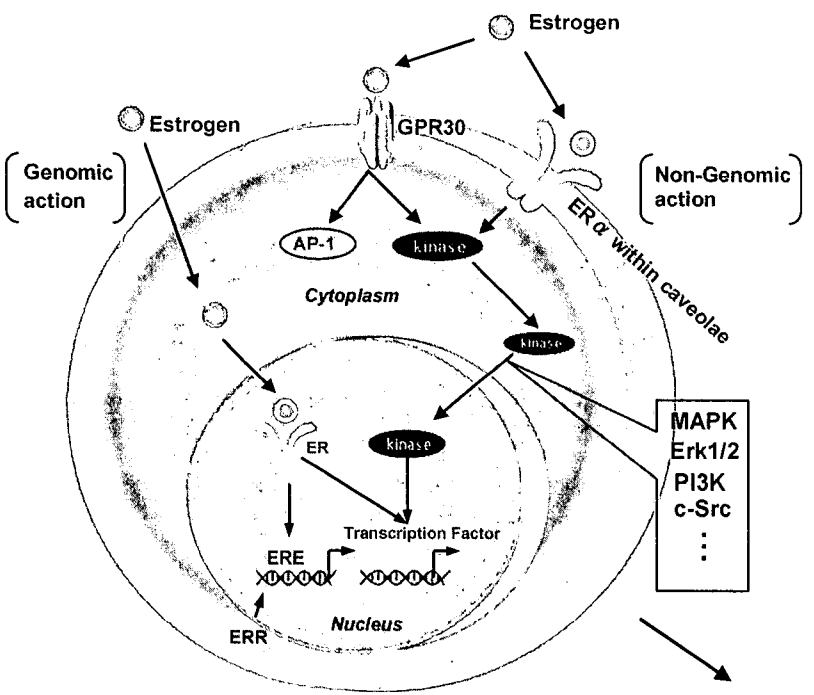
ERα (ng/well)	5	5	5	5	5	5
Sox9 (ng/well)	5	5	5	5	5	5
Smad3D (ng/well)	–	–	–	5	5	5
E2 (M)	10^{-9}	10^{-8}	10^{-7}	–	10^{-9}	10^{-8}

Discussion

エストロゲン欠乏

椎間板に対する直接作用

〔 II型コラーゲン発現抑制
アポトーシス促進？



軟骨終板に対する作用

↓

軟骨終板の変性・血管侵入・
二次骨化・終板の骨性閉鎖

↓

椎間板への拡散障害

↓

椎間板変性

結語

- OVXラットの椎間板組織では、II型コラーゲンの発現が抑制されており、経時的に椎間板変性が進行した.
- エストロゲン受容体(ERα, ERβ) は、ラット椎間板髄核・線維輪において核局在していた.
- ラットII型コラーゲンのプロモーター領域には推定上のエストロゲン反応領域(ERE)が存在し、リガンド刺激による反応性があった.
- ラットII型コラーゲン遺伝子の転写活性促進、mRNA発現誘導にはTGF-βシグナルとエストロゲン受容体シグナルの相互作用が関与している可能性が示唆された.
- エストロゲンは椎間板の恒常性維持に関与しており、その欠乏は椎間板変性の危険因子のひとつになると考えられた.

A Functional Polymorphism in *COL11A1*, Which Encodes the α1 Chain of Type XI Collagen, Is Associated with Susceptibility to Lumbar Disc Herniation

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Lumbar disc herniation (LDH), degeneration and herniation of the nucleus pulposus of the intervertebral disc (IVD) of the lumbar spine, is one of the most common musculoskeletal diseases. Its etiology and pathogenesis, however, remain unclear. Type XI collagen is important for cartilage collagen formation and for organization of the extracellular matrix. We identified an association between one of the type XI collagen genes, *COL11A1*, and LDH in Japanese populations. *COL11A1*, which encodes the α1 chain of type XI collagen, was highly expressed in IVD, but its expression was decreased in the IVD of patients with LDH. The expression level was inversely correlated with the severity of disc degeneration. A single-nucleotide polymorphism (c.4603C→T [*rs1676486*]) had the most significant association with LDH ($P = 3.3 \times 10^{-6}$), and the transcript containing the disease-associated allele was decreased because of its decreased stability. These observations indicate that type XI collagen is critical for IVD metabolism and that its decrease is related to LDH.

Lumbar disc herniation (LDH), degeneration and herniation of the nucleus pulposus of intervertebral disc (IVD) of the lumbar spine, is one of the most common musculoskeletal diseases.[1–3] Its etiology and pathogenesis, however, remain unclear. Genetic factors have been implicated in the etiology of lumbar disc degeneration.[4,5] Genetic abnormalities of the extracellular matrix (ECM) are implicated in disc degeneration and LDH. Phenotypes of transgenic mice and human mutations underscore the candidacy of ECM genes as susceptibility genes for LDH.[6,7] Several researchers have reported the association of ECM protein genes, including genes for type IX collagen[8,9] and aggrecan,[10] with lumbar disc disease (LDD). We reported elsewhere that cartilage intermediate layer protein and asporin—ECM proteins highly expressed in IVD, as well as articular cartilage—are implicated in LDD.[11,12]

Type XI collagen is a cartilage-specific ECM protein important for cartilage collagen fibril formation and for ECM organization.[13–16] Type XI collagen is composed of three α-chains, α1(XI), α2(XI), and α3(II), which are encoded by *COL11A1*, *COL11A2*, and *COL2A1*, respectively. The three chains fold into triple-helical heterotrimers to form procollagen, which is secreted into the ECM, where it participates in fibril formation with other cartilage-specific collagens, type II and IX collagens.[13] Type XI collagen regulates the diameter of cartilage collagen fibrils. Its N-terminal noncollagenous region limits the appositional lateral growth of the fibril by blocking further accretion of type II collagen.[14,15] Chondrodysplasia in mouse (*cho*) is an autosomal recessive disorder due to a frame-shift mutation of *COL11A1*.[16] The collagen fibrils of *cho* mice are much thicker than normal.[16,17] Thus, type XI collagen has a critical role in the organization of the supramolecular architecture of cartilage collagen.

Type XI collagen is present in IVD, both in the annulus fibrosus and nucleus pulposus,[18] but its significance in LDH is not known. Type XI collagen is a quantitatively minor component of cartilage collagen fibrils, but it is essential for the interaction between proteoglycan (PG) aggregates and collagens. It binds with high affinity to PG, which is important in vivo for anchoring cartilage PG to the collagen fibrillar network.[19] Mutations in type XI collagen cause various types of chondrodysplasias in human, including Stickler syndrome type II (MIM #604841), Marshall syndrome (MIM #154780), and oto-spondylo-mega-epiphyseal dysplasia (MIM #215150). These disorders are collectively termed "type XI collagenopathies,"[20] and all are complicated by abnormalities of the spine, including narrowing of the IVD. In particular, patients with Stickler syndrome have spondylar abnormalities and Schmorl's node (disc herniation into the vertebral body).[21] These human mutations are in vivo evidence that type XI collagen is critical for IVD integrity; thus, the type XI collagen genes are good candidates for the gene that causes LDH.

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Table 1. Clinical Characteristics of Subjects

Screening and Group	No. of Subjects	Age (years) Mean ± SD	Range	Male (%)	BMI[a]
1st:					
Case:					
LDD[b]	188	26.5 ± 10.4	13–74	40.0	21.0
LDH only	130	25.5 ± 6.9	13–66	54.0	21.1
Control	179	58.7 ± 11.7	23–81	6.0	23.0
2nd[c]:					
Case	359	41.4 ± 14.6	15–77	62.4	23.1
Control	286	69.6 ± 9.2	38–87	58.4	24.3
3rd[c]:					
Case	334	41.8 ± 15.1	11–83	61.3	23.4
Control	376	53.9 ± 9.7	13–86	47.6	22.2

[a] BMI calculated as body weight in kilograms divided by the square of height in meters.
[b] Includes disc degeneration only and LDH.
[c] Case group in the 2nd and 3rd screenings has LDH only.

Here, we present evidence that *COL11A1*, one of the type XI collagen genes, contributes to the genetic risk of LDH in Japanese. We have observed significant association between LDH and a functional SNP in *COL11A1* in independent Japanese populations. *COL11A1* was highly expressed in IVD, but its expression was decreased in the IVD of patients with LDH. *COL11A1* expression level was inversely correlated with the severity of disc degeneration in patients with LDH, and the transcript containing the disease-associated allele of the SNP was decreased.

Material and Methods

Study Population

All subjects were Japanese who were living in the middle part of the Honshyu island in Japan (table 1). They visited the participating hospitals and received medical examinations. For the initial screening, we recruited 188 case patients with LDD and 179 control subjects. The mean ages of the case and control groups were 26.5 and 58.7 years, respectively. The case group included 58 patients who had no herniation (disc degeneration only) and 130 patients with LDH. The mean age of the LDH case patients was 25.5 years. For the second screening (replication study), we recruited 359 patients with LDH and 286 control subjects. The mean ages of the case and control groups were 41.4 and 69.6 years, respectively. For the third screening, we recruited 334 patients with LDH and 376 control subjects. The mean ages of the case and control groups were 41.8 and 53.9 years, respectively. Subjects for the initial, second, and third screenings were recruited at the participating hospitals in the Toyama, Tokyo, and Kyoto areas, respectively. All LDH case patients had unilateral pain radiating from the back along the femoral or sciatic nerve to the corresponding dermatome of the nerve root with duration of >3 mo. Radiographic examination, including functional four-direction images and magnetic resonance imaging (MRI) (sagittal and axial images obtained with a 1.5-T imaging system), revealed positive findings indicating disc herniation. The degree of disc degeneration was evaluated by MRI and was scored according to Schneiderman's classification.[22] Of the affected individuals, 787 case patients underwent surgical treatment, and the other individuals with LDH were treated conservatively. All were followed up for >1 year. We excluded from the study individuals with spinal canal stenosis, spondylolisthesis, spondylosis, synovial cysts, spinal tumor, and trauma. We also excluded those who had occupational and/or habitual risk factors, such as heavy manual laborers, occupational drivers, and heavy smokers. We obtained informed consent from each subject, as approved by the ethical committees at the SNP Research Center of RIKEN and the participating hospitals.

Genotyping

We selected sequence variations of the type XI collagen genes (*COL11A1*, *COL11A2*, and *COL2A1*) for the first screening from the International HapMap Project database and JSNP Database. The SNPs covered >90% of the alleles with an r^2 value ≥ 0.8. We identified additional sequence variations in *COL11A1* by direct sequencing of a 230-kb region of DNA from 24 case patients. We extracted genomic DNA for genotyping from peripheral blood leukocytes of the subjects and genotyped SNPs as described elsewhere.[11,12]

Haplotype Structure and Statistical Analyses

We estimated haplotype frequencies, using the expectation-maximization algorithm and pairwise linkage-disequilibrium index (D' and Δ in 465 control individuals, as described elsewhere).[23] χ^2 tests were used to compare cases with controls for allelic and genotypic frequencies; the odds ratio (OR) and its 95% CI were calculated. We used a permutation test to adjust significance in the analysis of association between the *COL11A1* SNPs and LDH.[24] We performed 10^7 permutations of the cases and the controls. Bonferroni correction was applied when significance was adjusted for the number of SNPs genotyped. MRI data, real-time PCR data, and mRNA stability data were tested using Student's t test.

Analysis of COL11A1 *Expression*

We extracted and purified total RNAs and synthesized randomly primed cDNAs, using Multiscribe reverse transcriptase (PE Ap-

Table 2. Association between LDH and c.4603C→T (*rs1676486*) in *COL11A1*

Screening and Case Group	No. of Cases with Genotype CC	CT	TT	Total No. of Cases	No. of Controls with Genotype CC	CT	TT	Total No. of Controls	T Allele Frequency Case	Control	*P*	OR (95% CI)
1st:												
LDD[a]	85	86	17	188	99	67	13	179	.31	.26	.076	1.34 (.97–1.84)
LDH only	55	60	15	130	99	67	13	179	.34	.26	.020	1.51 (1.07–2.14)
2nd:												
LDH only	149	163	47	359	154	108	21	283	.35	.26	.00038	1.55 (1.21–1.97)

[a] Includes disc degeneration only and LDH.