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Expression and localization of estrogen receptor-β in annulus cells of the human intervertebral disc and the mitogenic effect of 17-β-estradiol in vitro

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Abstract

Background: Recent evidence suggests that estrogens exert effects in different tissues throughout the body, and that the estrogen receptor β (ERβ) may be important for the action of estrogen (17-β-estradiol) on the skeleton. The cellular localization of ERβ in the human intervertebral disc, however, has not yet been explored.

Methods: Human disc tissue and cultured human disc cells were used for immunocytochemical localization of ERβ. mRNA was isolated from cultured human disc cells, and RT-PCR amplification of ERβ was employed to document molecular expression of this receptor. Cultured human disc cells were tested to determine if 17-β-estradiol stimulated cell proliferation.

Results: In this report data are presented which provide evidence for ERβ gene expression in human intervertebral disc cells in vivo and in vitro. Culture of annulus cells in the presence of 10^{-7} M 17-β-estradiol significantly increased cell proliferation.

Conclusions: These data provide new insight into the biology of cells in the annulus of the intervertebral disc.

Background

Estrogen receptors belong to a single receptor superfamily which also includes receptors for androgens, progesterone, glucocorticoids, mineralocorticoids, thyroid hormone, retinoic acid and vitamin D [1–4]. Estrogen receptor-alpha is expressed in cells which are classic estrogen targets; estrogen receptor-β is a newly described receptor which has now been found in cells of tissues which in the past might not have been traditionally viewed as estrogen targets, including cells in prostate, ovary, vascular cells, astrocytes, cells of the osteoblast lineage, osteoclasts, and articular chondrocytes [5–9]. Annulus cells share a

mesenchymal origin with osteoblasts and chrondrocytes. Since no previous work has addressed the question of whether intervertebral disc cells have estrogen receptor-β domains, the present work adds to this list cells found in the annulus of the human intervertebral disc and also demonstrates a proliferative effect of 17-β-estradiol in vitro.

Materials and Methods

Disc Procurement and Cell Culture

Donor disc specimens were obtained from disc surgeries and the NCI Cooperative Human Tissue Network (CHTN). Studies were approved by the human subjects Institutional Review Board. Disc tissue studied here was from the annulus. Patient specimens were obtained from surgical procedures performed on individuals with herniated discs. For immunolocalization studies, annulus specimens were examined from 5 subjects, aged 32 to 46 years (4 females and 1 male). Cells from the annulus were cultured as previously described in monolayer culture [17,18]. Discs used for cell culture studies reported here for RT-PCR were from lumbar discs of three surgical patients (30 year old male, 28 year old female, 37 year old female), and a thoracic disc from a 35 year old male donor. Cells used for in vitro ERβ immunolocalization were from a lumbar site from a 54 year old female donor. Disc tissue in our collection of morphology specimens has been previously described [19]. Cells tested for the proliferative effect of 17-β-estradiol were derived from annulus tissue from surgical specimens obtained from a 30 year old male (lumbar), a 32 year old female (cervical), a 53 year old male (lumbar) and a donor thoracic disc from a 33 year old female.

Immunocytochemistry

Localization was performed on paraffin-embedded disc tissue or on cultured disc cells [7] using rabbit anti-estrogen receptor beta primary antibody (PA1-311, Affinity Bioreagents, #PA1-311) and streptavidin localization. PA1-311 was produced by Affinity Bioreagents by immunizing New Zealand white rabbits with a synthetic peptide corresponding to the N-terminal amino acid residues 55–70 of rat and mouse ER beta conjugated to KLH; this sequence is completely conserved in human ER beta protein. For cultured cell studies, cells were grown on chambered slides, fixed in 10% neutral buffered formalin, rinsed twice in PBS (5 minutes/rinse), incubated in 0.5% H_2O_2 in PBS for 10 minutes, rinsed twice in PBS (5 minutes/rinse), and incubated in 5% normal goat serum (Sigma) in 0.25% Triton X-100 (Sigma) in PBS for 2 hours. The solution was drained off and sections incubated overnight at room temperature in primary antibody at a concentration of 2 μg/ml in 2% normal goat serum in 0.25% Triton X-100 in PBS. Sections were rinsed twice in PBS (5 minutes/rinse), then incubated in biotinylated goat anti-rabbit (Sigma) solution, 1:50 dilution in 2% normal goat serum in 0.25% Triton X-100 in PBS for 2 hours at room temperature. Sections were rinsed twice in PBS (5 minutes each rinse), incubated in peroxidase-conjugated streptavidin (Dako) for 20 minutes and room temperature, rinsed twice in PBS (5 minutes/rinse), and incubated in DAB (Sigma) for 10 minutes. Cells were then rinsed with water, chamber gaskets removed, counterstained with light green, dehydrated, cleared and mounted. Negative controls were included with omission of primary antibody; a specimen of benign hyperplasia of the prostate was included as a positive control.

For tissue sections, antigen retrieval was carried out for 20 minutes in a steamer using a solution consisting of 1 mM EDTA (Sigma, St. Louis, MO) with pH adjusted to 8.0 with 10 M NaOH (Sigma). Sections were cooled for 20 minutes at room temperature, rinsed twice in PBS (5 minutes/rinse), incubated in 0.5% H_2O_2 in PBS for 10 minutes and processed as described above with the exception that slides were incubated overnight at room temperature in primary antibody at a concentration of 4 μg/ml in 2% normal goat serum in 0.25% Triton X-100 in PBS. Negative controls were included with omission of primary antibody.

Molecular studies

Disc cells were grown to confluence in flasks with 75 cm^2 growing area. Flasks were cooled on ice, and culture medium removed and cell layers washed with phosphate buffered saline. Cells were scraped from flasks and disrupted in 4 M guanidinium thiocyanate in 25 mM sodium citrate buffer, pH = 7.0, containing 0.5% sarcosyl and 0.1 M β-mercaptoethanol. DNA and proteins were removed by extraction with phenol and chloroform/isoamyl alcohol (49:1) in 200 mM sodium acetate buffer, pH = 4.0. RNA was precipitated using isopropanol.

One μg of total RNA was dissolved in 10 μl of annealing buffer containing 20 mM Tris-HCl, pH 8.3, and 0.4 M KCl and mixed 1 μl of 20 μM oligo dT. The annealing reaction was incubated at 65°C for 10 min and then allowed to cool slowly to room temperature. Oligonucleotide primers were prepared according to published sequences (ERβ, forward: 5'TGT GTT GTG GCC AAC ACC TG 3'; reverse: 5'AGC CAC ACT TCA CCA TTC CC 3') [8]. Primer extension (40 μl) was begun by adding 4 μl of 10 × RT buffer (500 mM Tris-HCl, pH 8.3, 60 mM MgCl, 25 mM DTT), 4 μl of dNTP mix (2.5 mM each), 1 unit of RNasin, and 20 units of Superscript II (Life Technologies, Inc.), followed by incubation for 1 h at 42°C. The RT product (10%) was then amplified by PCR. Amplification was performed under the following cycle conditions: the first cycle at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes, and the last

cycle at 72°C for 10 minutes. PCR reactions were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

In vitro Proliferation Assays Testing the Mitogenic Activity of 17-β-estradiol

Cells were fed 0.5 ml per well Minimum Essential Medium with Earle's salts prepared as described previously except that fetal bovine serum (FBS) was supplemented at 1% (MEM1) for control conditions. This minimal level of FBS is needed for cell survival, and lowered FBS levels are employed to minimize amounts of growth factors and cytokines which are normally present in FBS. This design has been used in our previous studies of the effects of IGF-1 and PDGF on disc cells [16]. Cells were tested for their proliferative response following exposure to 10^{-7} M 17-β-estradiol (water-soluble, Sigma) for 10 days. Cells without the addition of 17-β-estradiol (i.e., in MEM1 only) or cells under routine conditions of 20% FBS served as controls. Cells were seeded at a monolayer density of 40,000 cells/well in replicates in 48 well tissue culture plates (Corning, Inc., Corning, N.Y.). Cells were fed thrice a week and the experiment was terminated after 10 days of exposure. Twenty-four hours prior to termination, cultures were pulse-labeled with 2 μCi/ml [^{3}H] thymidine (Amersham). At termination, cells in monolayer were trypsinized, centrifuged at room temperature for 5 minutes at 500 rpm and resuspended in 500 μl lysis buffer (58.44 mg/ml NaCl) (Sigma); 0.1 mg/ml trypsin inhibitor (Sigma); 0.1% (v/v) triton X-100 (Sigma). Aliquots were taken and total DNA content was analyzed using a fluorometric procedure. Thymidine incorporation was determined by liquid scintillation counting from an average of 4 replicate assays for each cell population tested and is expressed as mean cpm [^{3}H]-thymidine per μg DNA. Cells from four discs (described above) were tested for the proliferative effect of 17-β-estradiol. Analysis of data used routine methods and data were analyzed using t-tests; a p value of 0.05 was considered statistically significant.

Results

Morphologic identification of ERβ in vivo and in vitro

Localization of anti-ERβ antibodies in formalin-fixed, paraffin embedded disc specimens required antigen retrieval techniques; both cells and embedded tissues utilized an overnight incubation in primary antibody (Figure 1A and 2A). Localization was seen in the cytoplasm in all disc cells and was also cytoplasmic in a positive control tissue specimen of benign hyperplasia of the prostate (Figure 3A). Figure 3B and 3C show, respectively, negative controls in the absence of primary antibody (Fig. 3B) and a specimen run in the presence of antibody and immunizing peptide (Fig. 3C).

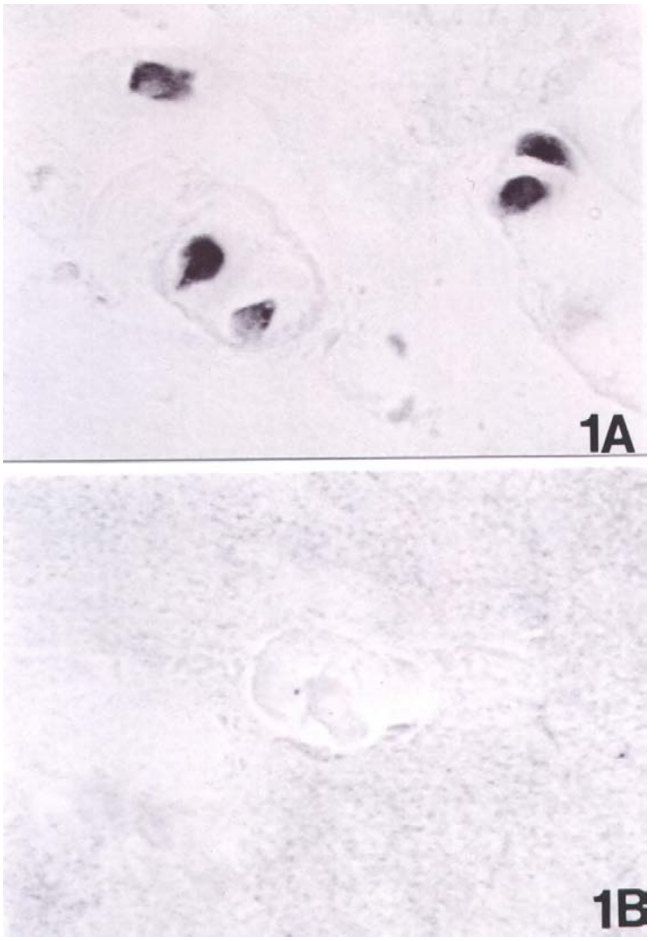

Figure 1

Fig. 1A Photomicrograph of disc tissue showing immunolocalization of ERβ in disc cells in the annulus of a cervical disc specimen from a 49 year old female patient. Localization is cytoplasmic. Fig. 1B, negative control. (original magnification × 640).

Expression of ERβ

Figure 4 is a photograph of the ethidium bromide-stained agarose gel electrophoresis showing the expression of ERβ and HPRT as single bands of expected sizes (323 bp for ERβ) from RT-PCR amplification of mRNA from the cultured specimens.

Proliferative Response to 17-β-Estradiol

Cell cultures from the annulus of four individuals were evaluated in a test of the proliferative effect of 17-β-estradiol on disc cells in monolayer culture. Compared to control cells grown in 1% serum (conditions needed for cell maintenance), disc cells exposed to 17-β-estradiol showed significantly increased proliferation (140.1% + 16.8 compared to 99.2% + 32.7 (seen in cells grown under our standard conditions of 20% FBS) (mean + S.D.) ($p = 0.034$).

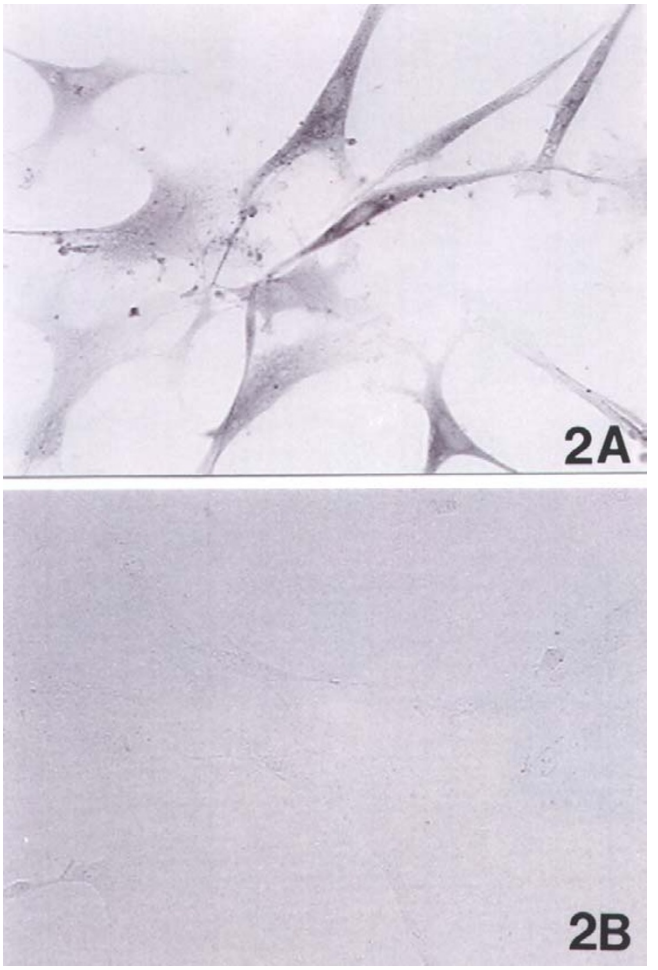

Figure 2
Fig. 2A: Photomicrograph of immunolocalization of ERβ in cultured disc cells in monolayer culture. Localization is cytoplasmic. Fig. 2B, negative control. (original magnification × 295).

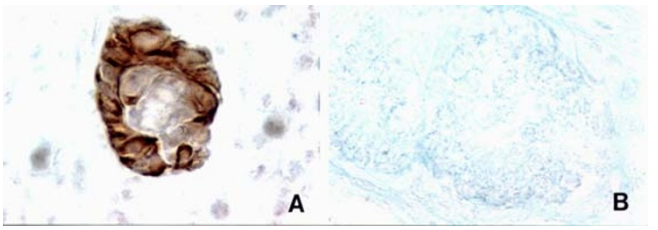

Figure 3
Fig. 3A presents a positive control photomicrograph of a specimen of benign hyperplasia of the prostate; localization is cytoplasmic. Fig. 3B, negative control performed with omission of primary antibody.

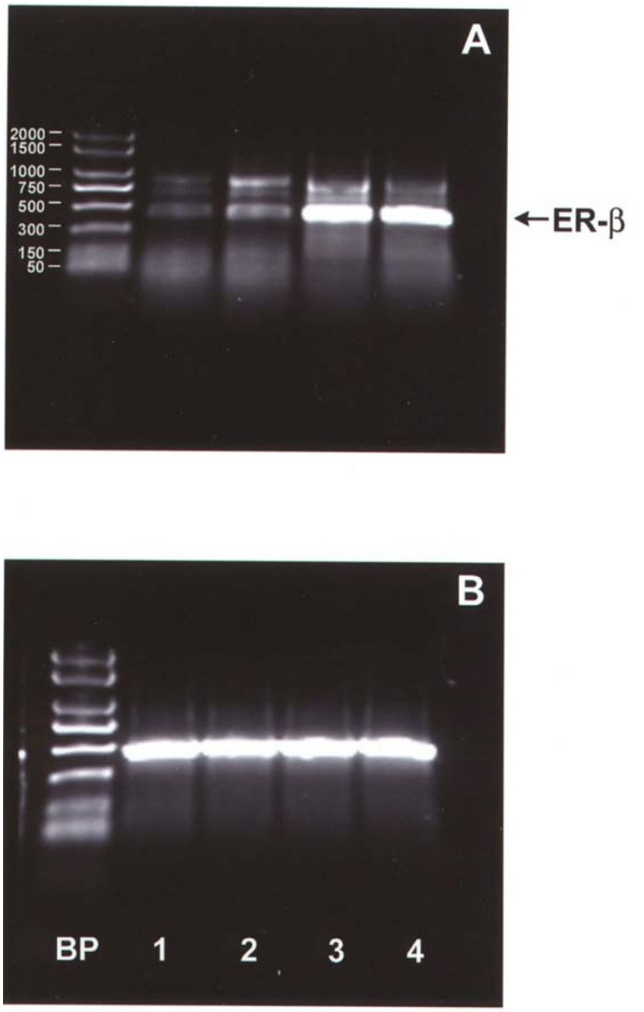

Figure 4
RT-PCR amplification of ERβ mRNA in cultured human disc cells from the annulus. Panel A is a photograph of an ethidium bromide-stained agarose gel electrophoresis showing gene expression of ERβ as a single band at the predicted 323 bp size. First lane, base pair controls; mRNA from lumbar disc cells from a 30 year old male (lane 1), mRNA from lumbar disc cells from a 28 year old female (lane 2), mRNA from lumbar disc cells from a 37 year old female (lane 3), mRNA from cells from a thoracic disc from a 35 year old male donor (lane 4). Panel B shows expression of the control HPRT gene in the lanes as described for Panel A (size markers as indicated in Panel A).

Discussion

This report provides novel information on the presence and expression of ERβ in cells in the annulus of the human intervertebral disc. Annulus cells exposed to 10^{-7} M 17-β-estradiol were also seen to show significantly greater proliferation compared to low serum controls than that which was elicited by growth in 20% FBS. Recent work by Manole et al showed that 17-β-estradiol is a strong mi-

togen for benign and malignant thyroid tumor cells acting by activation of the mitogen-activated protein kinase pathway [11].

The effect of estrogen on chondrocyte metabolism has recently been reviewed by Ushiyama et al [8]. Higher expression levels of both ERβ and ERalpha were found in articular chondrocytes derived in men compared to those from women. Corvol et al have reported that estrogen stimulated proteoglycan synthesis in rabbit chondrocytes in vitro [12]. Estrogen stimulated cell proliferation in rat chondrocytes from the growth plate [13], but other studies of rabbit chondrocytes failed to show this [14]. The latter study suggested that estrogen had a priming effect on the biological action of IGF-1. IGF-1 and/or IGF binding protein-5 have been suggested to function in estrogen-induced modulation of PTH action on osteoblast proliferation and cell function in recent work by Nasu et al [15]. Such findings may be relevant to the action of estrogen on disc cells in light of our previous report of the anti-apoptotic effect of IGF-1 on human disc cells [16].

The results presented here with a rabbit anti-estrogen receptor beta primary antibody (Affinity Bioreagents) showed cytoplasmic localization. Previous studies using this antibody have reported cytoplasmic localization in osteoclasts [5] and in Purkinje neurons of rat cerebellum and cells of the murine pituitary gland [20,21]. A previous study with an antibody produced in a specialized laboratory, however, showed nuclear localization in osteoblasts [9]. Such differences are likely due to specificity and characteristics of the particular antibodies, and point to the need for investigators to become familiar with the specific antibody they are employing since there is no external agency providing antibody certifications (in contrast to the color index system available for histologic stains).

In summary, this report has shown gene expression of ERβ by human cells from the annulus, localization of ERβ in both cultured cells and in disc tissue and the mitotic activity of 10^{-7} M 17-β-estradiol on cultured disc cells. These findings are important because they provide novel information on the fundamental cell biology of the disc cell and point to the need to explore the effects of estrogen on disc cell numbers and extracellular matrix production.

Conclusions

These findings in cells from the annulus are important because these data provide new information on the fundamental cell biology of the disc cell.

Competing Interests

None declared.

Acknowledgements

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References

1. Pelletier G: **Localization of androgen and estrogen receptors in rat and primate tissues.** *Histol. Histopathol.* 2000, **15**:1261-1270
2. Carson-Jurica MA, Schrader WT, O'Malley BW: **Steroid receptor family: structure and functions.** *Endocr. Rev.* 1990, **11**:201-234
3. Evans RM: **The steroid and thyroid hormone receptor superfamily.** *Science* 1988, **240**:889
4. Tsai MJ, O'Malley BW: **Molecular mechanisms of action of steroid/thyroid receptor superfamily members.** *Ann. Rev. Biochem.* 1994, **63**:451-486
5. Vidal O, Kindblom LG, Ohlsson C: **Expression and localization of estrogen receptor-β in murine and human bone.** *J. Bone Mineral Res.* 1999, **14**:923-929
6. Windahl SH, Norgård M, Kuiper GGJM, Gustafsson JÅ, Andersson G: **Cellular distribution of estrogen receptor β in neonatal rat bone.** *Bone* 2000, **26**:117-121
7. Toesca A, Pagnotta A, Specchia N: **Evidence of type II estrogen receptor in human osteoblast-like cells.** *Cell Biol. Int.* 2000, **24**:303-309
8. Ushiyama T, Ueyama H, Inoue K, Ohkubo I, Hukuda S: **Expression of genes for estrogen receptors α and β in human articular chondrocytes.** *Osteoarthritis Cartilage* 1999, **7**:560-566
9. Braidman IP, Hainey L, Batra G, Selby PL, Saunders PTK, Hoyland JA: **Localization of estrogen receptor β protein expression in adult human bone.** *J. Bone Miner. Res.* 2001, **16**:214-220
10. Wade CB, Robinson S, Shapiro RA, Dorsa DM: **Estrogen receptor (ER)α and ERβ exhibit unique pharmacologic properties when coupled to activation of the mitogen-activated protein kinase pathway.** *Endocrinology* 2001, **142**:2336-2342
11. Manole D, Schildknecht B, Gosnell B, Adams E, Derwahl M: **Estrogen promotes growth of human thyroid tumor cells by different molecular mechanisms.** *J. Clin. Endocrinol. Metab.* 2001, **86**:1072-1077
12. Corvol M-T, Carrascosa A, Tsagris L, Blanchard O, Rappaport R: **Evidence of a direct in vitro action of sex steroids on rabbit cartilage cells during skeletal growth: influence of age and sex.** *Endocrinol.* 1987, **120**:1422-1429
13. Sömjen D, Weisman Y, Mora M, Harell A, Kaye AM: **Regulation of proliferation of rat cartilage and bone by sex steroid hormones.** *J. Steroid Biochem. Mol. Biol.* 1991, **40**:717-712
14. Itagane Y, Inada H, Fujita K, Isshiki G: **Interactions between steroid hormones and insulin-like growth factor-I in rabbit chondrocytes.** *Endocrinol.* 1991, **128**:1419-1424
15. Nasu M, Sugimoto T, Kaji H, Chihara K: **Estrogen modulates osteoblast proliferation and function regulated by parathyroid hormone in osteoblastic SaOS-2 cells: role of insulin-like growth factor (IGF)-1 and IGF-binding protein-5.** *J. Endocrinol.* 2000, **167**:305-313
16. Gruber HE, Norton HJ, Hanley EN Jr: **Anti-apoptotic effects of IGF-1 and PDGF on human intervertebral disc cells in vitro.** *Spine* 2000, **25**:2153-2157
17. Gruber HE, Fisher EC Jr, Desai B, Stasky AA, Hoelscher G, Hanley EN: **Human intervertebral disc cells from the annulus: Three-dimensional culture in agarose or alginate and responsiveness to TGF-β1.** *Exp. Cell Res.* 1997, **235**:13-21
18. Gruber HE, Stasky AA, Hanley EN Jr: **Characterization and phenotypic stability of human disc cells in vitro.** *Matrix Biol* 1997, **16**:285-288
19. Gruber HE, Hanley EN Jr: **Analysis of aging and degeneration of the human intervertebral disc – Comparison of surgical specimens with normal controls.** *Spine* 1998, **23**:751-757
20. Li X, Schwartz PE, Rissman EF: **Distribution of estrogen receptor-b-like immunoreactivity in rat forebrain.** *Neuroendocrinology* 1997, **66**:63-67
21. Alves SE, Lopez BS, McEwen BS, Weiland NG: **Differential colocalization of estrogen receptor beta (ERbeta) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: An immunocytochemical study.** *Proc. Natl. Acad. Sci. USA* 1998, **95**:3281-3286