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ORIGINAL ARTICLE

The effect of selective oestrogen receptor antagonists in an in vitro model of growth plate chondrogenesis

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Abstract While oestrogen is recognized to play a key role in regulating growth, particularly in relation to epiphyseal fusion, the mechanisms that mediate its effects are still unclear. We utilized an in vitro model of chondrogenesis, the RCJ3.1C5.18 cell line, to explore the effect of oestrogen on this process. We demonstrated the presence of oestrogen receptors (ER) α and β in these cells, with increased abundance of both receptor sub-types evident as the cells differentiated. ERα localized to the nucleus, suggesting it was signalling by genomic pathways, while ERβ was seen predominantly in the cytoplasm, suggesting it may be utilizing non-genomic signalling. While exogenous oestrogen had no effect on proliferation or differentiation, we found some evidence for the endogenous production of oestrogen (intracrinology), as suggested by the expression of aromatase in these cells. Selective ERα blockade with methyl piperidinopyrazole (MPP) led to a significant reduction in both proliferation and differentiation, while ERβ blockade with R,R tetrahydrochrysene (THC) led to an increase in these parameters. This is in keeping with results from mouse knockout models suggesting that unopposed ERβ signalling leads to an inhibition of skeletal growth. Our results are further evidence for the importance of differential ER signalling in regulating chondrogenesis. Future studies examining in vivo effects of these agents are required to extrapolate these findings to a mammalian model.

Keywords Oestrogen · Growth plate · RCJ3 cells · Aromatase

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Introduction

Oestrogen is critical in the regulation of linear growth in humans. It is a key molecule in both stimulation of the pubertal growth spurt and ultimately in mediating epiphyseal fusion. The underlying mechanisms of oestrogen's actions in epiphyseal chondrocytes are still unclear. Oestrogen deficiency, either absolute or functional, leads to a delay in epiphyseal fusion, with subsequent increase in final height. Use of this mechanism to increase final height, however, may perturb critical actions of oestrogen in other systems. Therefore, a selective oestrogen receptor antagonist able to block only the oestrogen effect at the growth plate could achieve this outcome without adverse systemic effects. Further insights into the specific roles of oestrogen receptor (ER) α and β in regulating chondrogenesis are required before this is possible.

Both ERα and β are known to be expressed in the growth plate of rodents and rabbits [1] as well as humans [2]. The greatest expression of both receptors is in the resting and proliferative zone, however, expression in the hypertrophic zone of the growth plate is evident around puberty, raising the possibility of a direct effect of oestrogen on hypertrophic chondrocytes leading to epiphyseal fusion. There is also evidence that locally produced oestrogen affects growth, with enzymes responsible for sex

steroid metabolism present in the growth plate around sexual maturation in rodent models [3], as well as in human growth plates [4].

The differential role of ERα and β on skeletal growth has been explored using mouse knockout models. Essentially, ERα knockout (ERKO) mice show decreased femur length [5], with a greater effect in females than males [6]. Importantly, this same study showed that female ERKO mice had fused epiphyses, not usually seen in rodents. The ERβ knockout (BERKO) female mouse showed increased axial and appendicular growth [7]. These observations led to an hypothesis that ERβ signalling led to epiphyseal fusion as well as inhibiting skeletal growth [8], with the higher levels of oestrogen in the ERKO mouse leading to greater activation of ERβ receptors unopposed by ERα activity and therefore epiphyseal fusion. This is in conflict with the male human model [9], where loss of ERα activity leads to loss of fusion. It has, however, been proposed that the truncated protein product produced by this mutation has a dominant negative effect and leads to effective blockade of ERβ also [8].

These studies all intriguingly point to an important role in differential signalling of ERα and β leading to regulation of linear growth and epiphyseal fusion, however, further evidence is required across species to determine the respective roles of the oestrogen receptor sub-types. Experiments using selective oestrogen receptor agonists and antagonists will assist in delineating the roles of the ER subtypes.

The RCJ3.1C5.18 chondrocyte is a modified rat calvaria cell that provides a validated model of in vitro chondrogenesis [10]. Over 14 days of cell culture with differentiation media, the cells undergo differentiation from proliferative to hypertrophic chondrocytes, with nodules of new bone formation. This provides a model of the progression of chondrocyte development. Our study aimed to explore the oestrogen receptor phenotype of this cell line, and then, in order to provide further insights into mechanisms of oestrogen-dependent regulation of chondrocyte fate, examine the effects of oestrogen and selective oestrogen receptor antagonists in regulating proliferation and differentiation of these cells.

Materials and methods

Reagents

17β oestradiol was purchased from Sigma and made up in 100% ethanol (ANALAR). ICI 182,780, methylpiperidinopyrazole (MPP) [11] and R,R tetrahydrochrysene (THC) [12], were all purchased from TOCRIS (St. Louis MO, USA). Concentrations used for these agents were based on previously reported cell culture doses [11–13], with ICI treatment (10^{-5} M), MPP treatment (10^{-6} M) or THC treatment (10^{-6} M). Experimental arms with exogenous oestrogen utilized 10^{-7} M, considered to be a pharmacological dose in cell culture. Samples labelled (N) were treated with standard media with no exogenous oestrogen.

RCJ3.1C5.18 cells were kindly supplied by Dr Anna Spagnoli (University of North Carolina, Chapel Hill, NC, USA). Cells were grown in standard media (MEMα with 15% Fetal Bovine Serum (FBS)), with ascorbic acid and β-glycerophosphate added to induce differentiation as described previously [10]. For experiments looking at oestrogen effects, we used phenol red free media and charcoal stripped FBS (Gibco, Invitrogen, Carlsbad CA, USA). Preliminary experiments undertaken confirmed that this cell line was undergoing in vitro chondrogenesis as reported previously (data not shown).

Immunocytochemistry

RCJ3.1C5.18 cells were grown in poly-L-lysine (0.01%) coated 4 well chamber slides as above. Cells were fixed with 4% paraformaldehyde (20 min) followed by glycine 100 mM in PBS pH 7.4 (10 min) and washed with PBS. One slide was prepared for each time point. For either immunoperoxidase (DAB; diaminobenzidine tetrahydrochloride; Sigma Chemical Co., St. Louis, MO) or immunofluorescence (IF), cells were blocked with 1% denatured BSA (DBSA) in PBS pH 7.4 prior to permeabilising cells with 0.1% Triton-X 100/1% DBSA in PBS pH 7.4.

Anti-ERα (sc-542) and anti-ERβ (Y-19; sc-6821) were purchased from Santa Cruz (Biotechnology, Santa Cruz, CA) and were all applied at 1:50. The primary antibodies were applied in 1% DBSA in PBS and incubated overnight at 4°C as indicated. DAB staining was performed with Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingham, CA) following the manufacturer's instructions. Representative photos were taken of the slides.

Semi-quantification of the immunocytochemistry slide images was performed by a single, blinded observer who was not otherwise involved in the study. The observer graded the slides according to the overall impression of staining using a one to three plus scale.

Immunofluorescence

RCJ3.1C5.18 cells were grown fixed, blocked and permeabilised as above. Antibodies, as for immunocytochemistry above, were used at dilution 1:100. Secondary antibodies used were Alexa 488 goat anti-rabbit IF antibody at 1:200 for ERα and Alexa 488 donkey anti-goat IF antibody at 1:200 for ERβ (Molecular Probes, Leiden, The Netherlands). Nuclear counterstaining was undertaken with 4,6′-diamidino-2-phenylindole (DAPI) stain (Chemicon, Temecula, CA).

DAB or IF was performed three times, and samples were run in duplicate. Omission of primary antibody or mouse IgG was used as negative controls (data not shown). Images were taken at a 10 or 40 magnification objective (inverted 1 _ 70 UV microscope; Olympus, Tokyo Japan) as indicated.

RNA extraction and real time PCR

For total RNA extraction at days 3 and 10 of cell culture in differentiation media we used the RNeasy Minikit (Qiagen, Valencia, CA) as per manufacturer's instructions. DNase digestion was performed using RNase free DNase kit (Ambion, Austin, Texas).

Aromatase real time PCR analysis was performed using primers: 5′-TTTACCCTTGAAAACTTTGAGAAGAAC-3′ (forward), 5′-GTAACCAGGACAACTTTCATCATCAC-3′ (reverse). Rat porphobilinogen deaminase (PBGD) was used as housekeeping gene to ensure the reaction was working effectively and provide a baseline for quantification [14]. This enables the data to be analysed using the ΔΔCt method (see below). Samples were run on the ABI-7300 real time thermal cycler, under the following conditions: 1 cycle of 95°C 10 min and then 40 cycles of 95°C for 15 s, 60°C for 1 min. A further dissociation step was undertaken after these 40 cycles.

Proliferation and differentiation studies

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide; Sigma) assay was performed, as previously described [15], to assess cell viability. Cells were grown in 24 well plates. At the timepoint of interest, 750 µl of media was removed and 250 µl of MTT solution (2 mg/ml in sterile PBS) was added to make a 1 mg/ml solution in the wells. The cells were incubated at 37°C for 2 h, then 375 µl MTT extraction buffer (10 gm SDS, 25 ml mQH_2O, 25 ml of dimethyl formamide, 1.25 ml 80% acetic acid, 1 N HCl then add I M NaOH until pH 4.7) was added and the cells incubated overnight at 37°C. 200 µl was extracted from each well and placed in a 96 well plate, with absorbance measured by plate reader at 540 nm.

Alkaline phosphatase activity and chondrocyte differentiation

Alkaline phosphatase activity demonstrates capacity for mineralization, a function of differentiated chondrocytes. The alkaline phosphatase activity assay was undertaken by growing cells in differentiation media in 24 well plates. 500 µl of lysis buffer (100 mM Tris–HCl, pH 9.0, 200 mM NaCl, 0.2% Nonidet P-40, 0.2% Triton X-100, 1 mM Mg SO4, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin) was added to each well. The plate was then placed on a plate shaker for 30 min at room temperature. The lysate was collected from 3 wells and combined. It was then stored at 4°C until all time points were completed. At this time, 50 µl of extract was combined with 200 µl of lysis buffer and 250 µl of phosphatase substrate (1 mg/ml in 20% diethanolamine). This preparation was incubated at 37°C for 30 min, with the absorbance then determined at 405 nm for duplicate samples.

Statistics

For results of assays such as MTT and ALP activity assays, statistical analysis was undertaken using the Prism data analysis software. Means and standard error of the mean (SEM) were calculated by the Prism program. The mean of the two groups of interest were compared using an unpaired Student's t test with a two-tailed P value. P value of <0.05 was considered to be statistically significant.

The RT^2-PCR data analysis was performed using the ΔΔCt method which was recently published [16].

Results

The presence of oestrogen receptor α and β demonstrated by immunocytochemistry

The RCJ3 cells showed positive staining for both ERα and β (Fig. 1a, b). Analysis by an independent blinded observer confirmed that there was an increase in staining evident as the cells differentiated (Table 1).

Intracellular location of ER subtypes as shown by immunofluorescence

Immunofluorescence was utilized to confirm appearance of ERα and β as well as to localize these receptors within the cells. The ERα staining was seen both at the early stage of chondrocyte development before significant differentiation had occurred (day 3) as well as at a late stage of development with significant differentiation (day 14). As was shown with immunocytochemistry, there was an apparent increase in ERα protein with increasing differentiation of cells as evidenced by far greater staining on day 14 as compared with day 3 (see Fig. 2a). DAPI staining was used to counterstain the nucleus and provide information about localization. The degree of superimposition of ERα and DAPI stains suggests that ERα is localized to the nucleus, particularly at day 14.

Immunofluorescent staining for ERβ again showed greater signal on day 14 compared with day 3, providing further evidence for increased abundance of ERβ with increasing cell differentiation (see Fig. 2b). However, nuclear counterstain showed little superimposition of the

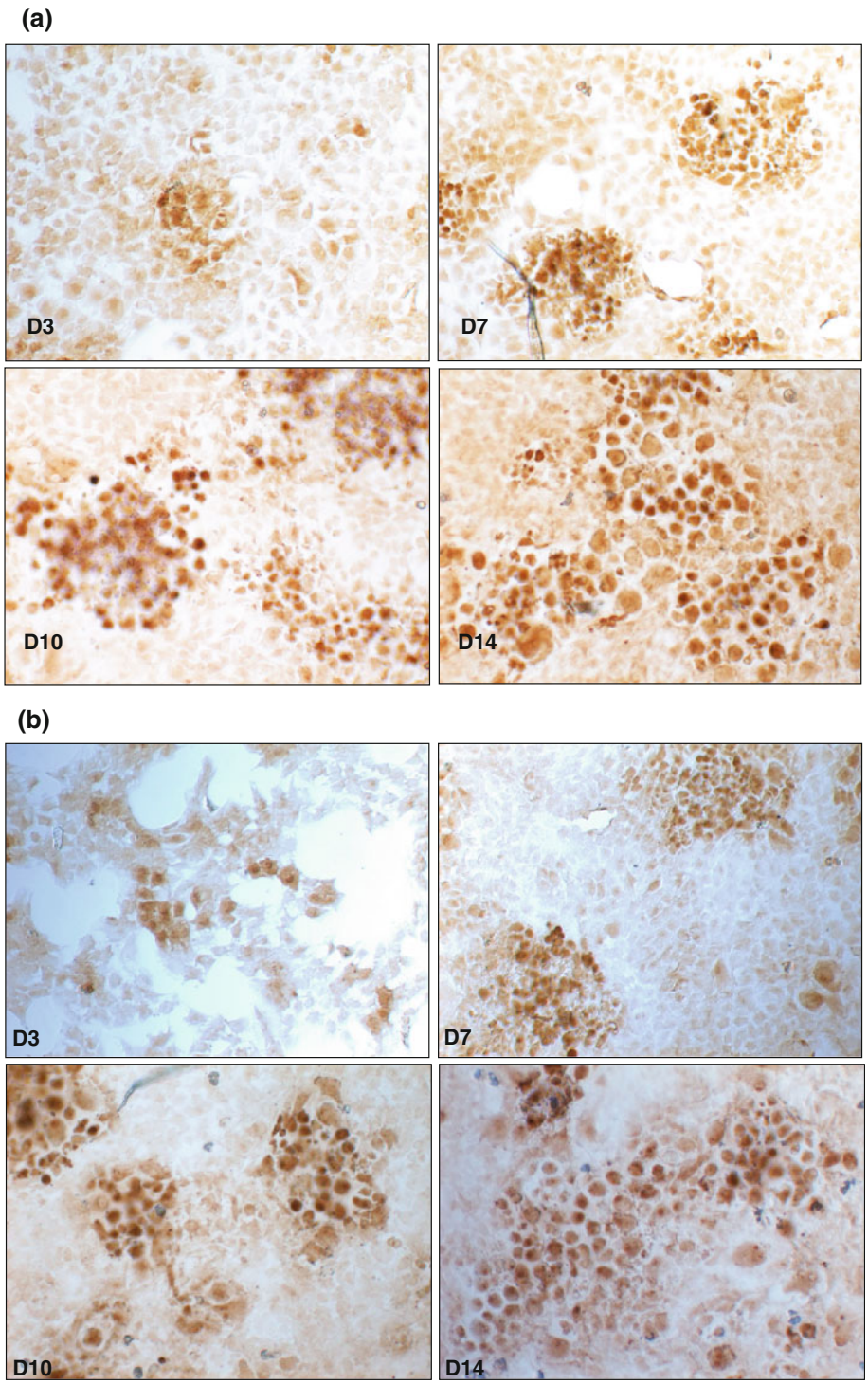

Fig. 1 **a** Immunocytochemistry demonstrating ERα protein in RCJ3 cells. **b** Immunocytochemistry demonstrating ERβ protein in RCJ3 cells

two signals, suggesting that ERβ is located predominantly in the cytoplasm.

Aromatase expression in RCJ3 cells

Analysis of conditioned media (phenol red free and charcoal stripped FBS) failed to detect oestradiol (results not shown), indicating no significant oestrogen from intracellular production into the extracellular medium. Messenger RNA from RCJ3 cells, analysed by real time PCR, however, showed expression of aromatase, the key enzyme involved in the synthesis of oestrogen (see Fig. 3). The presence of oestrogen led to a slight increase in aromatase production of no functional significance. Cells treated with

Table 1 Semi-quantitation of antibody staining assessed by blinded observer

	ERα ($n = 2$)	ERβ ($n = 2$)
D3	+ [25%]; ++ [1%]; +++ [5%];	+ [13%]; ++[1%]; +++[3%]
D7	+ [1%]; ++ [10%]; +++ [15%]	+ [3%]; ++ [5%]; +++ [15%]
D10	+ [15%]; ++ [5%]; +++ [25%]	+ [5%]; ++ [10%]; ++ [23%]
D14	+ [5%]; ++ [13%]; +++ [28%]	+ [1%]; ++ [15%]; +++ [21%]

+: low intensity DAB stain but significant above background (between areas of condensation and peri-nodules), ++: medium intensity DAB stain (mostly within focus of condensation and peri-nodules), +++: strong intensity DAB stain (mostly within focus of condensation or nodules)

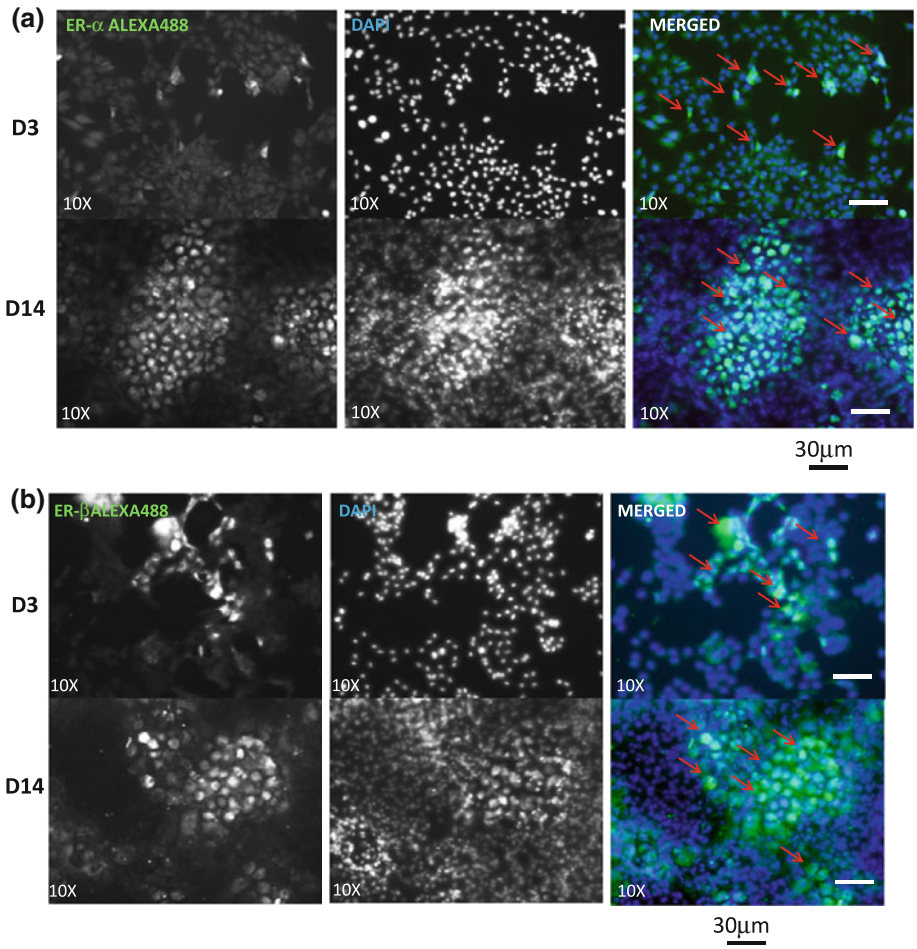

Fig. 2 **a** Immunofluorescence staining at 10× power demonstrating ER alpha antibody at days 3 and 14: antibody stain alone (*left panel*), DAPI staining of nucleus (*middle panel*) and superimposed figure of both stains (*right panel*). *Arrows* highlight areas of superimposed stain. **b** Immunofluorescence staining at 10× power demonstrating ER beta antibody at days 3 and 14: antibody stain alone (*left panel*), DAPI staining of nucleus (*middle panel*) and superimposed figure of both stains (*right panel*). *Arrows* highlight areas with no superimposition of stains

oestrogen alone showed no effect on either proliferation or differentiation (data not shown).

Effects of selective oestrogen receptor blockade on proliferation and differentiation

To explore the potential differential roles for ERα and β in this cell line, cells were treated with selective ER antagonists. MTT assay for cell viability showed a significant increase in proliferation with the non-selective ER blocker ICI 182,780 ($P < 0.0001$ at day 10). Treatment with the selective ERα antagonist MPP showed a significant decrease ($P = 0.0157$ at day 10) while the selective ERβ antagonist THC led to a significant increase ($P = 0.001$ at day 10) (Fig. 4a).

Using ALP activity as a marker of chondrocyte differentiation, there was no effect of the non-selective ER antagonist ICI on differentiation. However, the selective ERα antagonist MPP caused a significant decrease in differentiation at day 7 ($P = 0.0165$), day 10 ($P = 0.0167$) and day 14 ($P = 0.0083$). Selective ERβ antagonism with THC led to a significant increase in differentiation at day 3 ($P = 0.0069$), day 7 ($P = 0.0281$) and day 10 ($P = 0.0419$) (Fig. 4b).

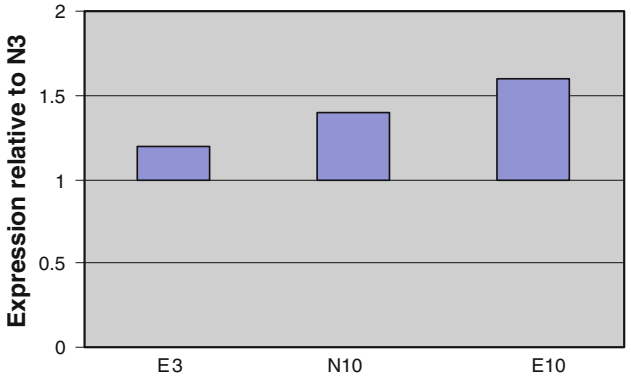

Fig. 3 Real time PCR results for aromatase mRNA expression in RCJ3 cells. Expression is shown as fold change compared to N3 (cells treated in oestrogen-free media for 3 days). E3—cells treated with oestrogen for 3 days, N10—cells treated in oestrogen-free media for 10 days, E10—cells treated with oestrogen for 10 days

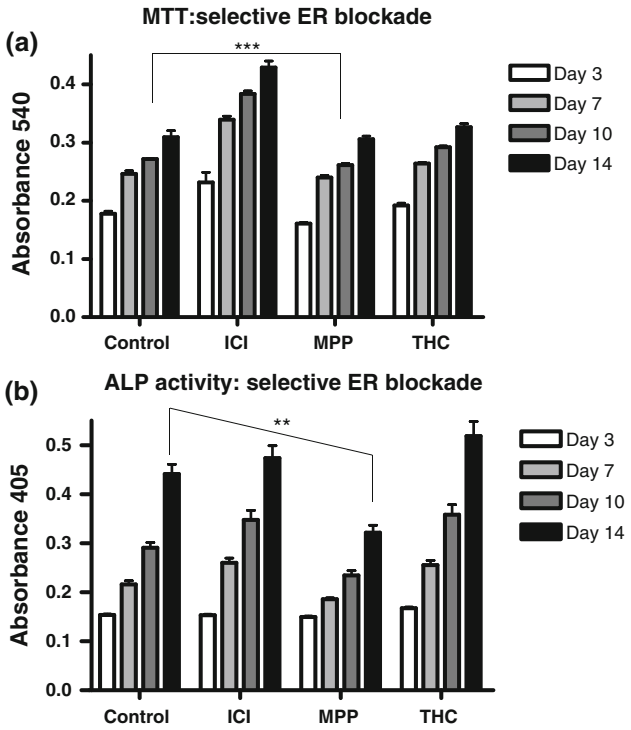

Fig. 4 **a** The effects of ER antagonists on proliferation of RCJ3 cells as shown by MTT assay. Non-selective ER antagonist ICI 182,780 (10-5 M) significantly increased proliferation as did selective ERβ antagonist THC (10-6 M), while selective ERα antagonist MPP (10-6 M) led to a significant decrease in proliferation. Results are mean of 3 samples with *error bars* representing SEM. **b** The effects of ER antagonists on differentiation of RCJ3 cells as shown by ALP activity assay. Results show a significant decrease in RCJ3 cell differentiation with selective ERα antagonism (MPP—10-6 M). Selective ERβ antagonism (THC—10-6 M) shows a significant increase in differentiation. The non-selective ER antagonist ICI (10-5 M) did not affect cell differentiation. Results are pooled replicates from 3 samples with absorbance read in duplicate and *error bars* indicating SEM for these readings

Discussion

This study shows for the first time the presence of ERα and β in the well-validated rat chondrocyte cell line RCJ3.1C5.18. This allows for further studies exploring the effects of oestrogen on chondrogenesis to be undertaken on this cell line. Both the immunocytochemistry and immunofluorescence results showed evidence of an increase in staining for both receptor sub-types as the cells differentiated. Previous studies have shown that both ERs are found across the growth plate in peripubertal humans, with slightly greater number of positive cells in the resting and proliferative zones as compared with the hypertrophic zones [2]. Rabbit and rodent studies suggest that hypertrophic zone expression of ERs is only seen close to puberty and therefore may be related to epiphyseal fusion [1]. Therefore, our finding of an apparent increasing abundance of ERs during differentiation suggests that this cell line may provide a useful model of epiphyseal fusion, with ER expression in hypertrophic chondrocytes as seen in peripubertal mammals.

Our data suggesting differential cellular localization of these receptors raises the possibility that they may activate different signalling cascades within the cell. Oestrogen receptor signalling is an extremely complex process, which is yet to be fully elucidated. The marked nuclear localization seen on immunofluorescence staining for ERα strongly point towards a key role for classical nuclear receptor signalling. The predominantly cytoplasmic location of ERβ, however, points to likely alternative signalling pathways involved in its signal transduction. Whether this non-genomic signalling involved pathways such as MAPKs, PI3K, protein kinase C as well as cAMP and calcium as described previously [17] remains unclear. The differential location in the RCJ3 cells of the receptors and therefore the possible differential signalling is a new finding which may help unlock some of the mechanisms involved in mediating oestrogen's effects on chondrogenesis.

There was no effect seen on either proliferation or differentiation for the RCJ3 cell line when treated with exogenous oestrogen despite the presence of oestrogen receptors, and the oestrogen being administered at a pharmacological dose (10^{-7} M). The response to oestrogen in chondrocyte cell lines is variable, although a direct stimulatory effect of oestrogen has been reported previously [18]. It is possible that oestrogen mediated other effects in the RCJ3 cells that were not demonstrated by MTT assay or ALP activity assay.

The other possibility is that the ERs did not respond to exogenous oestrogen as they were already utilized by endogenous oestrogens. The presence of mRNA in this cell line for the enzyme aromatase, the terminal step in the biosynthetic pathway of oestradiol, suggests that the RCJ3

cell may possibly produce its own oestrogen. This is consistent with previous reports of chondrocytes producing endogenous oestrogen, a process termed "intracrinology" [19]. It is thus possible that the observed effects following oestrogen receptor blockade are most likely the result of blocking the activity of endogenous oestrogen.

Selective estrogen receptor modulators (SERMs) have been developed in order to function as ER antagonists in some tissues but agonists in others, for example allowing ER antagonist action on breast tissue while having ER agonist action on bone. There is currently no clear mechanism to explain this tissue-specific differential action of ERs. They have varying actions on the growth plate, with raloxifene acting as an oestrogen agonist, leading to an acceleration of growth plate senescence and early epiphyseal fusion [20], while the SERM tamoxifen has been used in male human subjects to delay epiphyseal fusion, suggesting an oestrogen antagonist effect [21].

Other compounds that selectively inhibit ERα (MPP) and ERβ (THC) have been developed. Using these compounds, we demonstrated that ERα antagonism in this cell line leads to a moderate (but significant) decrease in proliferation and a marked decrease in differentiation. This is in keeping with reported studies for knockout mice models, where ERKO mice of both genders have decreased femur lengths [5, 6]. In contrast, treatment with ERβ antagonist led to the opposite effect, with increased proliferation and differentiation, which is consistent with the findings that female BERKO mice show an increase in skeletal growth at young adult age [6]. Therefore, our cell model is consistent with the hypothesis from the knockout mouse models that ERα signalling encourages proliferation and differentiation while ERβ signalling inhibits these processes. Further studies using agents to knock-down the effect of an oestrogen receptor subtype such as ERα siRNA would be of great benefit in further elucidating the role of the oestrogen receptor subtypes in chondrogenesis.

Our results provide insights into the potential effects of differential oestrogen signalling in chondrocytes, however, the limitations of a cell-based system, especially for an entity as complex as the growth plate, mean that in vivo experiments are required to fully determine their role. Using appropriate animal models such as rabbits, which undergo epiphyseal fusion, the effects of selective oestrogen receptor antagonists on growth and the growth plate will be of great interest in order to gain further insights into the mechanisms underpinning oestrogen's effects on the growth plate.

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