

# Tissue-Dependent Expression of Estrogen Receptor $\beta$ in $17\beta$ -Estradiol-Mediated Attenuation of Autoimmune CNS Inflammation

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**Abstract:** Treatment strategies using therapeutic estrogen are being developed and tested for multiple sclerosis (MS). MS is an autoimmune inflammatory disease that attacks the central nervous system, damages myelin and produces neurodegenerative changes associated with periodic and chronic progression of functional neurological deficit. Experimental studies in chimeric bone marrow transplant mice treated with  $17\beta$ -estradiol (E2) have revealed that the estrogen receptor-1 (Esr-1, or -alpha) expressed exclusively within the non-hematopoietic tissue compartment is sufficient for mediating a beneficial neuroprotective therapeutic response in mice lacking Esr-1 expression on T lymphocytes or other bone marrow-derived cells. Less is known regarding requirements for estrogen receptor-2 (Esr-2, or -beta) expression in E2-mediated therapy. Here, we tested and compared requirements for Esr-2 expression within distinct tissue compartments in bone marrow transplant mice. Our studies support a crucial role for Esr-1 in E2 treatment and demonstrate that Esr-2 expressed by non-bone marrow-derived cells plays a role in sustaining the neuroprotective response mediated through Esr-1.

**Keywords:** Autoimmune CNS inflammation, EAE, estrogen, gender.

## INTRODUCTION

Immunomodulatory and neuroprotective effects of estrogens have been recognized and are being exploited to develop effective strategies for treating the T cell-mediated autoimmune disease, multiple sclerosis (MS) [1, 2]. Distinct receptor subtypes mediate distinct and tissue specific effects to produce multiple biological responses such as estrogen-regulated immunosuppression and functional neuroprotection [3, 4].

Gender differences in experimental autoimmune encephalomyelitis (EAE, the experimentally-induced T cell-mediated autoimmune attack against the central nervous system (CNS) in mice) and in the epidemiology of MS have suggested that sex hormones play a role in disease pathogenesis [5, 6] and treatment studies have demonstrated that estrogen (E2) reduces pathology in EAE [7-9]. The use of targeted knockout (KO) mouse strains lacking estrogen receptor-1 (Esr-1) [10] and/or estrogen receptor-2 (Esr-2) [11] and the use of specific receptor ligands in mice with EAE have been effective strategies for investigating the differential therapeutic effects of E2 mediated through the distinct receptor types [12]. Thus, requirements for Esr-1 or Esr-2 have been demonstrated using the respective Esr KO strains treated with E2. Distinct E2 effects have also been attributed to Esr-1 using selective Esr-1-binding ligands such as propyl pyrazole triol (PPT) and selective Esr-2-binding ligands such as diarylpropionitrile (DPN) have been shown to induce selective effects attributable to Esr-2.

In selective Esr KO mouse strains, estrogen treatment was rendered ineffective in the absence of the relevant receptor. Thus, Esr-1 was shown to be required for E2-mediated inhibition of EAE [1, 10] but Esr-2 was not [12]. A role for Esr-1 was explored further using chimeric bone marrow mice that lacked Esr-1 expression on transplanted bone marrow-derived hematopoietic cells. Esr-1 was not required on transplanted bone marrow cells for the therapeutic response to E2 [10], indicating that the Esr-1 mediated response to E2 operated through effects on non-hematopoietic tissues such as the CNS.

Using the selective Esr ligands, functional neuroprotection was an indirect downstream consequence of early immunosuppression mediated exclusively through Esr-1 and delayed neuroprotective effects were mediated directly through Esr-2, bypassing immunosuppression [12]. Studies of passive EAE induced with adoptively transferred encephalitogenic T cells from Esr-1 KO donor mice showed that the therapeutic response to E2 did not depend upon signaling through Esr-1 expressed by T cells [13]. Thus, the acute therapeutic response to E2 (i.e. E2-induced reduction in paralytic severity during the acute onset phase of paralytic disease) was not mediated through Esr-2 [12] nor through direct effects on Esr-expressing T cells or hematopoietic-derived antigen presenting cells (APC) [10, 13]. Taken together, the results indicated that E2 acts through Esr-1 expressed within a non-bone marrow, non-hematopoietic compartment such as the CNS.

The involvement of Esr-2 expressed in distinct tissue compartments has also been examined in EAE studies using engineered male mice with a partial knockout phenotype (Esr +/-) which possessed variable expression of Esr-2 [11]. In those studies, EAE susceptible bone marrow chimeric mice varied in their expression of variably disrupted Esr-2

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on hematopoietic versus non-hematopoietic tissues (using chimeras constructed between WT donors or recipients and *Esr-2* +/- recipients or donors, respectively). Such mice exhibited a heterotic effect of *Esr-2* genotype on elevated EAE severity mediated through the non-hematopoietic compartment [11]. These results suggested that development within the non-hematopoietic compartment may be subject to regulation by *Esr-2* with relevant downstream consequences for effects on EAE [11]. However, due to the known direct involvement of hematopoietic-derived myelomonocytic cells such as antigen-presenting macrophages, dendritic cells or microglia in EAE, possibilities remain for E2 effects mediated through *Esr-2* expressed on bone marrow-derived hematogenous cells [14-17]. Moreover, acute effects of E2 acting through *Esr-2* expressed exclusively within distinct hematopoietic versus non-hematopoietic tissue compartments have not been examined in EAE. Here we investigate *Esr-2* in the therapeutic response to E2 using bone marrow chimeric mice lacking *Esr-2* in the bone marrow versus non-bone marrow compartments.

## MATERIALS AND METHODOLOGY

### Mice

Female B6.129-*Esr1*<sup>tm1Unc</sup> (*Esr1* KO, *Esr1*<sup>-/-</sup>, ERKO); and B6.129-*Esr2*<sup>tm1Unc</sup> (*Esr2* KO, *Esr2*<sup>-/-</sup>, BERKO) mice were a gift from Patricia Hurn (Department of Anesthesiology and Perioperative Medicine, Oregon Health & Science University, Portland, OR, USA). C57BL/6 wild-type (WT) control mice (C57BL/6-Tg(UBC-GFP)30Scha/J, stock number 004353 from Jackson Labs) expressing green fluorescent protein (GFP) were obtained from a breeding colony at the Veterinary Medical Unit, Portland VA Medical Center. All animal protocols were approved by the Portland VA IACUC.

### Bone Marrow Chimera Construction

Host mice (bone marrow recipients) were lethally irradiated and reconstituted by the intravenous injection of  $1 - 2 \times 10^7$  T-cell-depleted donor bone marrow cells (BMC). In this way the following three donor → recipient combinations were used to generate bone marrow chimeric mice: 1) *Esr2*<sup>-/-</sup> → WT; 2) WT → *Esr2*<sup>-/-</sup>; and 3) WT → *Esr1*<sup>-/-</sup>. Chimerism was assessed by flow cytometric analysis of green fluorescent protein in cells isolated from peripheral blood approximately 6 – 8 weeks after bone marrow transplant. Mean percent chimerism was calculated as the mean percent donor cells for the group ± standard deviation. Chimeric mice were implanted with E2 slow release pellets or control implant (placebo) one week prior to disease induction, approximately 8 – 12 weeks after bone marrow reconstitution.

### E2 Treatment

For treatment of mice with 17β-estradiol, a single 3-mm pellet containing 2.5 mg of E2 (Innovative Research of America, Sarasota, FL), providing a constant continuous controlled release of hormone over a period of 60 days, was implanted subcutaneously at the dorsal back of each mouse 7 days before immunization for induction of EAE. Control animals were implanted with placebo pellets containing vehicle. An acute therapeutic response to E2 was detected as the difference in clinical severity comparing E2-treated ver-

sus placebo-treated groups during the acute onset phase of paralytic disease.

### Disease Induction

Mice were immunized on experimental day 0 with 400 μg of myelin basic protein acetylated peptide Ac1-11 (MBP<sub>Ac1-11</sub>) (Beckman Institute, Palo Alto, CA) emulsified in complete Freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) by subcutaneous injection over four sites on the flank. On the day of immunization mice received by intraperitoneal injection 75 ng of pertussis toxin (PTX) (List Biological Laboratories Inc., Campbell, CA). Forty-eight hours later each mouse received an additional 200 ng of PTX by intraperitoneal injection. Mice were examined daily for clinical signs of EAE according to the following scale: 0, no signs; 1, limp tail; 1.5, moderate hind limb weakness with difficulty in righting; 2, moderate hind limb weakness without ability to right itself; 2.5, moderate hind limb weakness (waddling gait) without ability to right itself; 3, moderately severe hind limb weakness with the ability to walk upright for only a few steps; 3.5, moderately severe hind limb weakness with paralysis of one limb; 4, severe hind limb weakness; 4.5, severe hind limb weakness with mild forelimb weakness; 5, paraplegia with no more than moderate forelimb weakness; 5.5, paraplegia with severe forelimb weakness (quadriplegia); and 6, moribund condition. Error bars for mean disease severity of each group show standard deviation. Mann-Whitney U test was used for statistical comparison.

### Cytokine MultiPlex Assay

Single cell splenocyte suspensions from mice were prepared and suspended at  $4 \times 10^6$  cells/ml in stimulation media with 20 μg/ml of MBP<sub>Ac1-11</sub>. Cell culture supernatants were recovered at 72 hours and stored, frozen at -70°C. Cytokines were quantified using a Luminex BioPlex kit and analyzer (Bio-Rad, Richmond, CA) according to the manufacturer's protocol. IL-1-β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, and IFN-γ, were simultaneously detected. Standard curves were generated for each cytokine and the concentration of cytokine in the cell supernatant was determined by interpolation from the appropriate standard curve. Means and standard deviations were determined using assay replicates from individual animals and Student's T test was used for statistical comparison.

### T Cell Proliferation Assay

A single cell suspension of splenocytes ( $4 \times 10^5$  cells/well) were plated on standard 96-well flat-bottom tissue culture plates for 72 hours at 37°C and 7% CO<sub>2</sub> with and without antigen and in the presence of 0.5 μCi of <sup>3</sup>H-thymidine during the last 18 hours. Cells were harvested onto glass fiber filters, and thymidine uptake was determined by liquid scintillation. Data are expressed as corrected triplicate mean counts per minute ± standard deviation. Student's T test was used for statistical comparison.

## RESULTS

Three groups of chimeric mice were constructed by injecting *Esr2*<sup>-/-</sup> BMC into WT mice expressing GFP (designated *Esr2*<sup>-/-</sup> → WT); by injecting GFP-positive WT bone

**Table 1. Percentages of Donor Cells in Blood as an Indication of Transplant Engraftment and Chimerism**

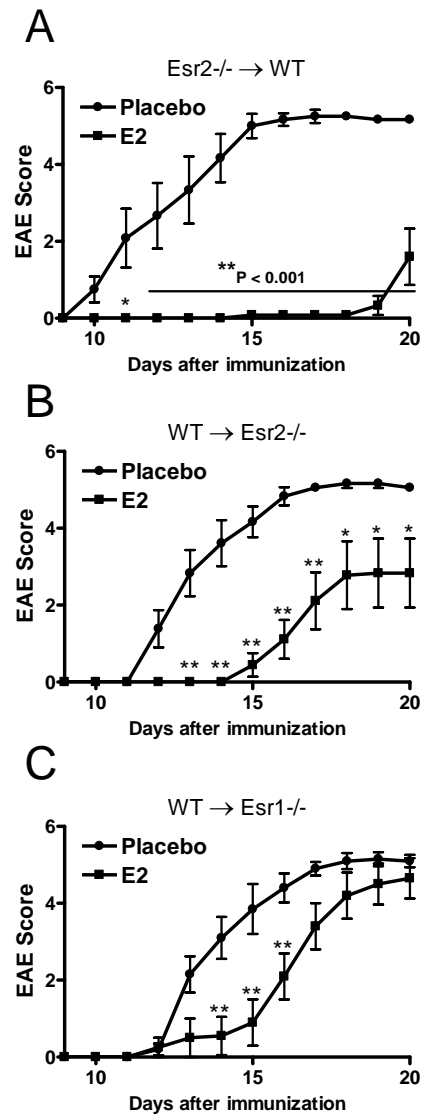
Donor → (BMC)	Recipient (non-BMC)	% chimerism E2	% chimerism Placebo
Esr2 <sup>-/-</sup>	WT	83 ± 6	83 ± 9
WT	Esr2 <sup>-/-</sup>	86 ± 11	84 ± 14
WT	Esr1 <sup>-/-</sup>	83 ± 14	82 ± 14

Percent chimerism was calculated from engraftment of transplanted donor cells isolated from peripheral blood 6 – 8 weeks after bone marrow transplant by quantifying the percentage of non-fluorescent donor cells in Esr2<sup>-/-</sup> → WT chimeras, and the percentages of fluorescent donor cells in WT → Esr2<sup>-/-</sup> and WT → Esr1<sup>-/-</sup> chimeras.

marrow into Esr2<sup>-/-</sup> mice (WT → Esr2<sup>-/-</sup>); and by injecting WT (GFP) bone marrow into Esr1<sup>-/-</sup> mice (WT → Esr1<sup>-/-</sup>). The resulting groups of bone marrow chimeric mice were evaluated for the level of hematopoietic engraftment by quantifying the percentage of GFP-positive cells in the circulation. Replacement of recipient-derived WT (GFP-positive) cells with Esr2<sup>-/-</sup> (GFP-negative) donor cells generated Esr2<sup>-/-</sup> → WT chimeric mice with relatively few GFP-positive host cells in the circulation. Similarly, transfer of WT (GFP-positive) donor bone marrow into Esr2<sup>-/-</sup> (GFP-negative) recipients generated WT → Esr2<sup>-/-</sup> chimeras with a high percentage of donor-derived GFP-positive cells in the circulation. The level of chimerism six - eight weeks after bone marrow transplant ranged from 82 – 86% in chimera groups selected for further study (donor cells/ total cells X 100) (Table 1). Disease was induced in chimeric mice by active immunization with myelin peptide in CFA approximately two to three months after bone marrow transplantation. Mice were followed daily and scored for clinical disease severity. Groups of mice were treated with 2.5 mg E2 or vehicle administered by a subcutaneous 60 days time release pellet surgically implanted 7 days prior to disease induction.

Esr2<sup>-/-</sup> → WT chimeras possessed the targeted deletion of Esr-2 on transplanted bone marrow-derived cells exclusively, thus leaving Esr-1 as the sole classical Esr on transplanted bone marrow-derived cells. Neither Esr-1 nor Esr-2 expression was manipulated by genetic knockout on non-bone marrow tissues such as the CNS in Esr2<sup>-/-</sup> → WT chimeric mice. As seen in Fig. (1A), Esr2<sup>-/-</sup> → WT mice treated with E2 had a significant reduction in disease severity ( $p < 0.001$  on days 12 – 20), compared to placebo-treated mice, that lasted nearly three weeks after disease induction. Therefore, Esr-2 expression on donor-derived bone marrow cells was not required for the early or sustained therapeutic response to E2.

In order to further examine requirements for Esr-2 expression, we evaluated the clinical response to E2 in Esr2<sup>-/-</sup> mice reconstituted with WT bone marrow (WT → Esr2<sup>-/-</sup>). WT → Esr2<sup>-/-</sup> chimeric mice possessed the targeted deletion of Esr-2 exclusively within the non-bone marrow-derived host tissues such as the CNS. As seen in Fig. (1B), WT → Esr2<sup>-/-</sup> mice showed a strong E2 response with significantly reduced disease severity early in the course of disease ( $p < 0.01$  on days 13 – 17 compared to placebo-treated), consistent with an early immunosuppressive response mediated through Esr-1. This was followed by mild-moderate disease that was less severe than that in the vehicle treated control mice ( $p < 0.05$  on days 18 – 20). Considered together, these results in chimeric mice indicate that early therapeutic control of acute disease was mediated through Esr-1 and did not



**Fig. (1).** Paralytic disease severity and course in placebo- and E2-treated bone marrow chimeric mice with EAE. (A) Esr2<sup>-/-</sup> → WT, n = 6 placebo, n = 6 E2; (B) WT → Esr2<sup>-/-</sup>, n = 9 placebo, n = 9 E2; (C) WT → Esr1<sup>-/-</sup>, n = 10 placebo, n = 10 E2. Chimeras were evaluated for engraftment 6 – 8 weeks after bone marrow transplant and selected for further study. E2 or placebo pellets (60 days time-release) were implanted one week prior to disease induction, 8 – 10 weeks after bone marrow transplant. Mice were followed daily for 20 days after immunization with MOG peptide.

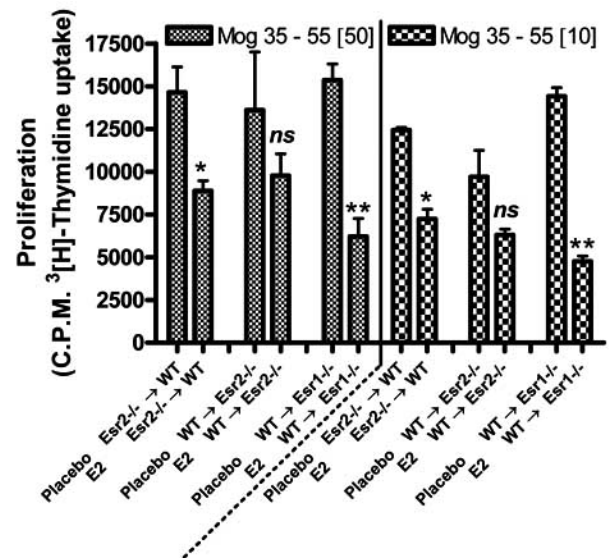
\*  $p < 0.01$ ; \*\*  $p < 0.001$ .

require Esr-2 expression within either the bone marrow or non-bone marrow compartments, while the full, lasting residual neuroprotective effect depended on signaling through Esr-2 within the non-bone marrow compartment (e.g. CNS cells).

WT  $\rightarrow$  Esr1 $^{-/-}$  mice expressed Esr-2 in both the bone marrow and non-bone marrow compartments and lacked Esr-1 in the non-bone marrow compartment. As expected, the response to E2 in WT  $\rightarrow$  Esr1 $^{-/-}$  mice was somewhat similar to that in non-chimeric Esr1 $^{-/-}$  mice [1], showing only a very short-lived (i.e. very early) E2 response that quickly became indistinguishable from no E2 treatment (placebo), thus demonstrating that the early and sustained E2 responses, such as achieved in Esr2 $^{-/-}$   $\rightarrow$  WT mice (with both early and sustained responses) and in WT  $\rightarrow$  Esr2 $^{-/-}$  (early response only), depended upon Esr-1 expression in non-hematopoietic tissues. Moreover, expression of Esr-2 on non-hematopoietic tissues in the absence of Esr-1 expression in WT  $\rightarrow$  Esr1 $^{-/-}$  mice was not sufficient for eliciting the residual Esr-2-mediated therapeutic E2 response observed in WT  $\rightarrow$  Esr2 $^{-/-}$  mice. Our results are consistent with previous reports using receptor specific ligands in which the Esr-1 mediated response was immunosuppressive early and conferred a lasting neuroprotective effect, while the Esr-2 mediated response, although lacking an early immunosuppressive component, was nevertheless potently neuroprotective at later time points when co-expressed with Esr-1 in the non-hematopoietic compartment [12, 18].

E2 treated and untreated myelin oligodendrocyte glycoprotein (MOG) peptide-immunized chimeric mice were tested for splenic T cell proliferative responses to MOG peptide to evaluate E2-mediated effects on systemic immune status (Fig. 2). While E2 treated mice uniformly expressed reduced proliferation compared to placebo-treated mice, the magnitude of these effects on proliferation varied among the E2-treated chimera types examined. For instance, those mice lacking Esr-1 within the non-hematopoietic compartment (WT  $\rightarrow$  Esr1 $^{-/-}$ ) showed the greatest reduction in proliferation due to E2 (\*\*  $p < 0.01$ ), while chimeric mice lacking Esr-2 in the non-hematopoietic compartment (WT  $\rightarrow$  Esr2 $^{-/-}$ ) displayed the least E2 mediated reduction in proliferation ( $p = ns$  compared to placebo treated). Such differences in the effect of E2 on proliferation *in vitro* due to the genotype of the recipient (comparing WT  $\rightarrow$  Esr1 $^{-/-}$  versus WT  $\rightarrow$  Esr2 $^{-/-}$  chimeras) demonstrate that systemic cellular immune responses are subject to upstream influences of recipient genotype on the function of the donor derived cells. Mice lacking Esr-2 on the hematopoietic cells (Esr2 $^{-/-}$   $\rightarrow$  WT) were intermediate in the magnitude of the E2-induced reduction in proliferation (\*  $p < 0.05$ ). These results were somewhat surprising because among the E2 treated chimeras, WT  $\rightarrow$  Esr1 $^{-/-}$  mice developed the most severe disease overall in spite of appearing to have the greatest E2 mediated reduction in the splenic proliferation response, while Esr2 $^{-/-}$   $\rightarrow$  WT developed the least severe disease in spite of not having the greatest E2-mediated reduction in splenic proliferation. These results demonstrate that E2-induced immunosuppression (as measured by antigen-specific T cell proliferation) was not tightly linked to the relative level of clinical benefit derived from E2 treatment and was not a precise predictor of overall clinical status. The results also indicate that immunosuppres-

sive pathways are not solely responsible for and/or directly linked mechanistically to the E2 clinical response.

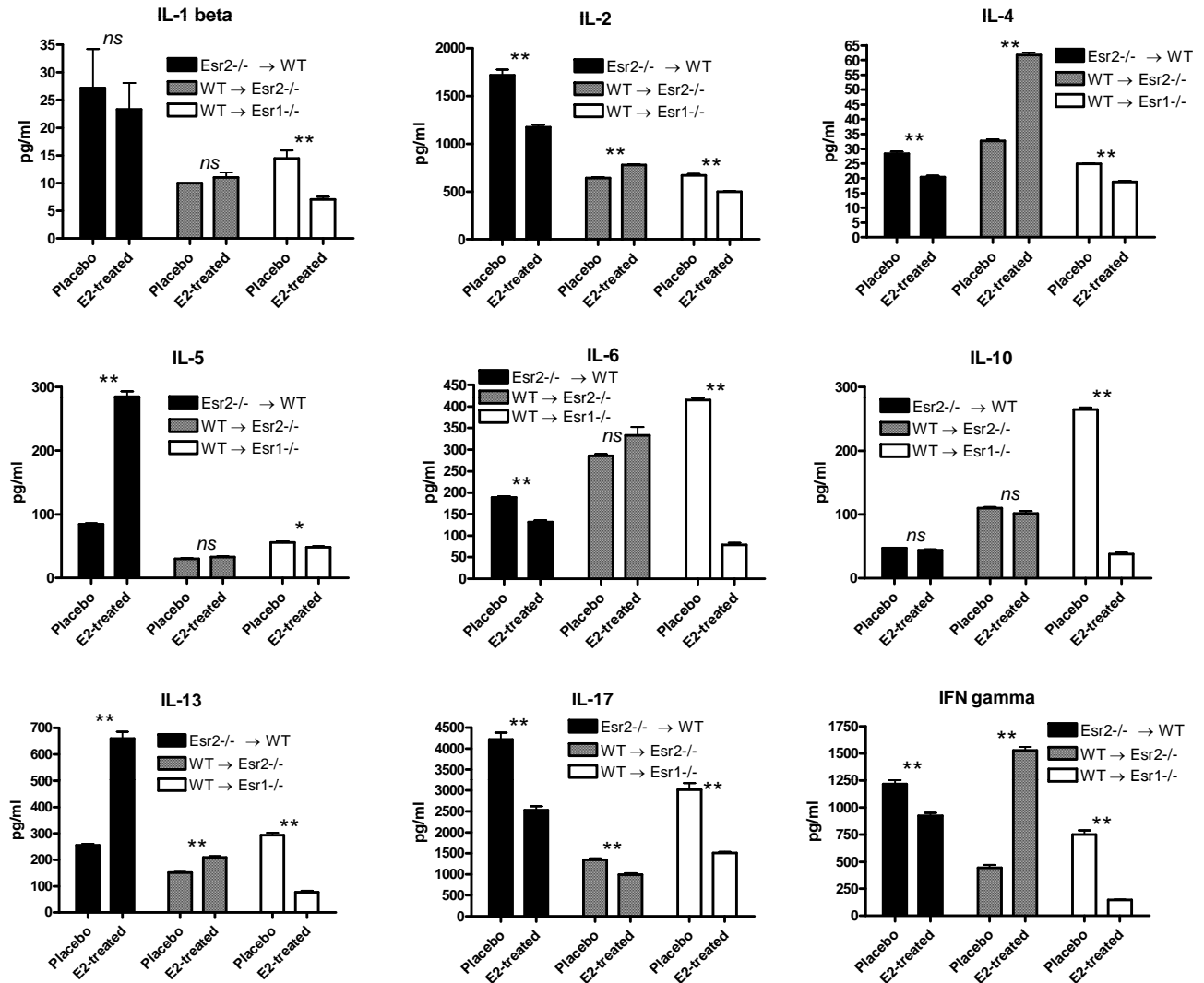


**Fig. (2).** *In vitro* proliferation of MOG peptide-specific T cells isolated from pooled spleens is presented as mean counts per minute (CPM)  $^3$ [H]-thymidine uptake  $\pm$  standard deviation (triplicate assay wells). Spleen cells were harvested on day 21 post immunization and tested *in vitro* for proliferation responses to stimulation with MOG peptide (WT  $\rightarrow$  Esr2 $^{-/-}$ : Placebo, n=3; E2, n=3. Esr2 $^{-/-}$   $\rightarrow$  WT: Placebo, n=5, E2, n=6. WT  $\rightarrow$  Esr1 $^{-/-}$ : Placebo, n=3; E2, n=3). \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

Fig. (3) shows *in vitro* cytokine expression by MOG peptide stimulated splenocytes harvested following experimental day 20 of clinical monitoring as a measure of residual systemic immune status. The comparison between E2 versus placebo treatment in chimeras with distinct patterns of tissue expression of Esr-2 demonstrated that Esr-2 plays a role in E2-mediated regulation of cytokine production. For instance, the Esr2 $^{-/-}$   $\rightarrow$  WT chimeras, lacking Esr-2 on transplanted bone marrow derived cells, had substantial E2-mediated elevations in regulatory Th2 cytokines, IL-5 and IL-13 (compared to placebo treated), and this cytokine response pattern (increase in both IL-5 and IL-13) did not occur in the other two chimera types in which Esr-2 was expressed on transplanted (i.e. WT) bone marrow cells.

E2 treatment induced a reduction in proinflammatory splenic IL-17 and IFN-gamma relative to placebo in Esr2 $^{-/-}$   $\rightarrow$  WT chimeras (Lacking Esr-2 on bone marrow cells), a response pattern shared with the E2-treated WT  $\rightarrow$  Esr1 $^{-/-}$  chimeras but absent or much less apparent in the WT  $\rightarrow$  Esr2 $^{-/-}$  chimeras. This pattern of E2 response (decreased IL-17 and IFN-gamma compared to placebo in both the Esr2 $^{-/-}$   $\rightarrow$  WT and WT  $\rightarrow$  Esr1 $^{-/-}$  chimeras, but not in the WT  $\rightarrow$  Esr2 $^{-/-}$  chimeras) was observed to varying degrees for several other cytokines (e.g. IL-2, IL-4, and IL-6), suggesting that signaling through Esr-2 expressed on non-bone marrow-derived cells may be important to immunosuppression by E2.

WT  $\rightarrow$  Esr2 $^{-/-}$  chimeras, lacking Esr-2 on recipient-derived cells, were unique in having substantial E2-mediated elevations in splenic IL-4 and IFN-gamma (compared to



**Fig. (3).** *In vitro* cytokine production of MOG peptide-specific T cells isolated from spleen. The indicated chimeric mice were treated with Placebo or E2 and immunized with MOG peptide. Spleen cells were harvested on day 21 post immunization and tested *in vitro* for cytokine responses to stimulation with MOG peptide (all groups, n = 3).

\* p < 0.05, \*\* p < 0.01.

placebo). This suggests the possibility that systemic activation of both the regulatory Th2 (e.g. IL-4) and proinflammatory Th1 (e.g. IFN $\gamma$ ) immune compartments occurred when Esr-2 was absent from the non-bone marrow compartment. In contrast, the WT  $\rightarrow$  Esr1<sup>-/-</sup> chimeras, lacking Esr-1 on recipient-derived cells (and used here as a chimeric control for Esr-2 expression within both donor and recipient tissues), had substantially decreased IL-6 and IL-10 (compared to placebo), two cytokines produced by myelomonocytic cells; monocytes, dendritic cells and activated macrophages. As noted above, such an E2-mediated decrease in cytokine production also occurred for some but not all cytokines in WT  $\rightarrow$  Esr1<sup>-/-</sup> and Esr2<sup>-/-</sup>  $\rightarrow$  WT chimeras but not in WT  $\rightarrow$  Esr2<sup>-/-</sup> chimeras, again suggesting that Esr-2 on non-bone marrow-derived cells was necessary and sufficient for this response.

## DISCUSSION

Previously we compared the early disease course in Esr-1<sup>+/+</sup> (WT) and Esr-1<sup>-/-</sup> (ERKO) mouse strains within 2 weeks of disease induction and showed that effective E2 treatment required Esr-1 [13]. That early Esr-1-mediated anti-inflammatory effect of E2 was validated in experiments comparing treatment with the Esr-1 versus Esr-2 selective ligands (PPT and DPN, respectively) [12]. Although a requirement for Esr-2 was not previously demonstrated using homozygous Esr-2<sup>-/-</sup> KO mice, roles for Esr-2 could not be ruled out since the Esr-2 selective ligand treatment produced a delayed therapeutic response that depended on Esr-2 expression and was attributed to an Esr-2 mediated neuroprotective effect at time points beyond 2 weeks [12]. An examination of EAE in heterozygous Esr-2 partial KO mice in the absence of E2 treatment suggested that non-hematopoietic

(e.g. non-bone marrow-derived) tissue development was subject to regulation by Esr-2 and influenced disease severity [11]. These results together predicted that Esr-1 but not Esr-2 would be required for an immunosuppressive E2 response early in the development of disease and that Esr-2 would be required for the later, neuroprotective response to E2. We have here sought to evaluate this prediction using the BERKO homozygous Esr-2  $-/-$  KO strain as either the bone marrow donor or the bone marrow recipient in chimeric EAE mice.

The results show that Esr-2 was not required in bone marrow-derived cells in Esr2 $-/-$   $\rightarrow$  WT chimeric EAE mice treated effectively with E2. Such E2-treated mice also exhibited the strongest E2-induced Th2 responses, and the weakest Th1 responses of the three chimera types tested. Our results demonstrated that E2 caused a decrease in splenic IL-17 and IFN-gamma relative to placebo treated Esr2 $-/-$   $\rightarrow$  WT chimeras, suggesting that the sustained delay in disease onset observed in mice lacking Esr-2 in the bone marrow compartment was accompanied by a systemic decrease in pathogenic Th17 and Th1 cells. It is worth mentioning that the timing of such a decrease is not known since the results did not provide an indication of cytokines and systemic immune status earlier following E2 treatment. Esr-2 was similarly not required in the bone marrow compartment for E2 mediated inhibition of antigen-specific T cell proliferation in Esr2 $-/-$   $\rightarrow$  WT mice. In addition to not being required for the clinical effect, when present in bone marrow cells in WT  $\rightarrow$  Esr2 $-/-$  mice for example, Esr-2 may have inhibited the Esr-1 mediated anti-inflammatory component of the E2 response.

Expression of Esr-2 by non-bone marrow-derived cells (such as CNS cells) was not required for the earliest portion of the E2-mediated inhibition of disease in WT  $\rightarrow$  Esr2 $-/-$  chimeras. However, the absence of Esr-2 from the non-bone marrow compartment resulted in eventual onset of disease, demonstrating that Esr-2 is important for maintaining the full sustained neuroprotective E2 response. Thus, the duration of the E2-mediated reduction in disease severity was shorter in WT  $\rightarrow$  Esr2 $-/-$  mice lacking Esr2 in the non-bone marrow compartment, suggesting, by comparison, that the neuroprotective response to E2 in Esr2 $-/-$   $\rightarrow$  WT mice was mediated, at least in part, through Esr-2 in the CNS. Esr-1 expression in both compartments (bone marrow and non-bone marrow) was sufficient to sustain the full E2-mediated neuroprotection in the presence but not absence of Esr-2 on non-bone marrow cells. However, the severity of disease at later time points (e.g. day 18 – 20) in WT  $\rightarrow$  Esr2 $-/-$  mice was significantly milder than in placebo treated mice, indicating that a partial or residual neuroprotective effect was likely operating through Esr-1 independent of Esr-2, in agreement with previous studies using Esr-1 and Esr-2 specific ligands [10, 12]. Both the regulatory Th2 cytokine, IL-4 and the proinflammatory Th1 cytokine, IFN-gamma were uniquely upregulated by E2 in WT  $\rightarrow$  Esr2 $-/-$  chimeras, in contrast to Esr2 $-/-$   $\rightarrow$  WT and WT  $\rightarrow$  Esr1 $-/-$  chimeras in which E2 caused a decrease in both IL-4 and IFN-gamma, compared to placebo. These elevated IL-4 and IFN-gamma responses were accompanied by an intermediate clinical response compared to the other two chimera types, perhaps reflecting contrasting functionalities of IL-4 versus IFN-gamma.

Among the three chimera types tested, mice lacking Esr-1 in the non-bone marrow compartment had the weakest (and shortest duration) therapeutic response to E2 and thus the most severe disease overall, providing yet one more indication that signaling through Esr-1 on non-bone marrow cells is important. Unlike the case of Esr-1 in the absence of Esr-2, Esr-2 in the absence of Esr-1 (Esr1 $-/-$ ) in the non-bone marrow compartment was not sufficient for sustaining even a partial neuroprotective response to E2 since the severity of disease was not significantly different than placebo at the later time points in WT  $\rightarrow$  Esr1 $-/-$  mice. Thus, while signaling through Esr-1 expressed in the non-bone marrow compartment was not needed for the earliest neuroprotective response (days 14 – 16), it was crucial to the sustained neuroprotective response at later time points (days 17 - 20). Decreased production of the cytokines IL-6 and IL-10 by Esr-1-expressing myelomonocytic lineage cells in E2-treated WT  $\rightarrow$  Esr1 $-/-$  chimeras raises the possibilities that disease onset was only slightly delayed in these mice due to E2-mediated effects on APC such as reduced antigen presentation activity and delayed immune activation by APC, and/or E2-induced enhancement of programmed death -1 (PD-1) expression [19], leading in turn to early downstream reductions in proinflammatory cytokines IL-17 and IFN-gamma. Again, these results are consistent with prior reports in which receptor specific ligands signaling through Esr-1 induced early immunosuppression, and signaling through Esr-2 extended neuroprotection [10, 12].

Interpretation of previous studies using selective Esr-binding ligands or selective Esr KO mouse strains in EAE have been complicated by reports implicating Esr-2 in regulation of Esr-1 activity. For instance, Esr-2 is capable of mediating an inhibitory activity on Esr-1-induced transcription regulation when the two Esr are co-expressed within a single cell [20-25]. Such an inhibitory regulatory function of Esr-2 would be expected to be absent in cases where Esr-2 was not expressed (e.g. in cells from Esr-2 KO strains such as Esr2 $-/-$ ). Elevated expression of the Th2 cytokines, IL-5 and IL-13 by Esr2 $-/-$  -derived splenocytes obtained from E2-treated Esr2 $-/-$   $\rightarrow$  WT chimeras may be such a case since it occurred in the absence of Esr-2 mediated signaling, whereas IL-5 and IL-13 were not upregulated by splenocytes from E2 treated chimeras expressing Esr-2 in the hematopoietic compartment (e.g. WT  $\rightarrow$  Esr2 $-/-$  and WT  $\rightarrow$  Esr1 $-/-$  chimeras). These results suggest that production of IL-5 and IL-13 by Th2 cells was enhanced by E2 acting through Esr-1, and that the enhanced production of these cytokines may have been subject to downregulation through Esr-2 expressed in the hematopoietic compartment. In this case, signaling through Esr-2 would be expected to reduce downstream immunosuppressive effects of these Th2 cytokines acting on encephalitogenic Th1 cells, thereby resulting in an indirect proinflammatory effect. Such a proinflammatory effect would be expected to increase clinical disease severity as we observed in E2 treated chimeras expressing Esr-2 within the hematopoietic compartment (i.e. WT  $\rightarrow$  Esr2 $-/-$  and WT  $\rightarrow$  Esr1 $-/-$  chimeras).

In addition to mediating acute responses to E2, Esr within the non-bone marrow compartment mediate development and downstream effects on sensitivity to E2 induced immunosuppression and/or neuroprotection. The two chimera types constructed here in knockout recipient strains

(WT → Esr2<sup>-/-</sup> and WT → Esr1<sup>-/-</sup>) may be subject to these considerations due to the presence of the knockout phenotype throughout early development in these strains. In contrast, such a developmental influence may be absent from Esr2<sup>-/-</sup> → WT chimeras due to the relatively late experimental introduction of Esr-2 KO cells into these mice. Moreover, the potential for regulatory interaction between the two Esr, the presence of multiple downstream signaling pathways (mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI3-K)/Akt) [26], the potential for heterotic developmental effects of Esr-2 [1], indirect roles for astrocytes and non-classical estrogen receptor [27] together with the complexities of Esr expression across CNS regions and during development (reviewed in [23]), collectively imply that the neuroprotective consequences of signaling through Esr may be subject to unnatural influences in the absence of one or multiple receptors in genetically modified strains, or in the presence of a single selective binding agent. Moreover, the potential multiple effects of E2 signaling through membrane G protein-coupled receptor 30 (GP30) [28] demonstrate that additional therapeutic pathways may operate in the presence and/or absence of Esr-mediated effects. Such influences may have a natural counterpart in outbred populations such as human where heterozygous Esr allelic forms may be common [29].

## CONCLUSION

In summary, our results show that Esr-1 signaling is not sufficient to sustain the full E2 response in the absence of Esr-2 in the non-bone marrow compartment, thus demonstrating that signaling through Esr-2 on non-bone marrow-derived cells (e.g. CNS) is important to the neuroprotective response to E2. Esr-2 signaling in the bone marrow compartment did not appear to be at all necessary for the full response to E2 when signaling through Esr-1 was intact and the early response to E2 (corresponding to early immunosuppression) seemed to be entirely independent of a requirement for Esr-2, instead depending on Esr-1. Systemic immunosuppression of proliferation was a shared consequence of E2 treatment independent of Esr-2, and did not appear to be finely tuned to clinical severity. Esr-2 was not required in the hematopoietic compartment for E2-induced up-regulation of IL-5 and IL-13, nor for the E2-induced down-regulation of IL-2, IL-4, IL-6, IL-17 and IFN-gamma. However, when present with Esr-1 in the hematopoietic compartment, Esr-2 may inhibit certain Esr-1 mediated anti-inflammatory cytokine responses to E2.

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## REFERENCES

- Polanczyk M, Zamora A, Subramanian S, *et al.* The protective effect of 17beta-estradiol on experimental autoimmune encephalomyelitis is mediated through estrogen receptor-alpha. *Am J Pathol* 2003; 163: 1599-605.
- Vegeto E, Belcredito S, Etteri S, *et al.* Estrogen receptor-alpha mediates the brain antiinflammatory activity of estradiol. *Proc Natl Acad Sci USA* 2003; 100: 9614-9.
- Weiss DJ, Gursipide E. Non-genomic effects of estrogens and anti-estrogens. *J Steroid Biochem* 1988; 31: 671-6.
- Enmark E, Gustafsson JA. Oestrogen receptors - an overview. *J Intern Med* 1999; 246: 133-8.
- Papenfuss TL, Rogers CJ, Gienapp I, *et al.* Sex differences in experimental autoimmune encephalomyelitis in multiple murine strains. *J Neuroimmunol* 2004; 150: 59-69.
- Whitacre CC. Sex differences in autoimmune disease. *Nat Immunol* 2001; 2: 777-80.
- Jansson L, Olsson T, Holmdahl R. Estrogen induces a potent suppression of experimental autoimmune encephalomyelitis and collagen-induced arthritis in mice. *J Neuroimmunol* 1994; 53: 203-7.
- Kim S, Liva SM, Dalal MA, Verity MA, Voskuhl RR. Estriol ameliorates autoimmune demyelinating disease: Implications for multiple sclerosis. *Neurology* 1999; 52: 1230-8.
- Matejuk A, Adlard K, Zamora A, Silverman M, Vandenbark AA, Offner H. 17 beta-estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nervous system of female mice with experimental autoimmune encephalomyelitis. *J Neurosci Res* 2001; 65: 529-42.
- Garidou L, Laffont S, Douin-Echinard V, *et al.* Estrogen receptor alpha signaling in inflammatory leukocytes is dispensable for 17beta-estradiol-mediated inhibition of experimental autoimmune encephalomyelitis. *J Immunol* 2004; 173: 2435-42.
- Polanczyk M, Yellayi S, Zamora A, *et al.* Estrogen receptor-1 (esr1) and -2 (esr2) regulate the severity of clinical experimental allergic encephalomyelitis in male mice. *Am J Pathol* 2004; 164: 1915-24.
- Tiwari-Woodruff S, Morales LB, Lee R, Voskuhl RR. Differential neuroprotective and antiinflammatory effects of estrogen receptor (er)alpha and erbeta ligand treatment. *Proc Natl Acad Sci U S A* 2007; 104: 14813-8.
- Polanczyk MJ, Jones RE, Subramanian S, *et al.* T lymphocytes do not directly mediate the protective effect of estrogen on experimental autoimmune encephalomyelitis. *Am J Pathol* 2004; 165: 2069-77.
- Aghdami N, Gharibdoost F, Moazzeni SM. Experimental autoimmune encephalomyelitis (eae) induced by antigen pulsed dendritic cells in the c57bl/6 mouse: Influence of injection route. *Exp Anim* 2008; 57: 45-55.
- Hinrichs DJ, Wegmann KW, Dietsch GN. Transfer of experimental allergic encephalomyelitis to bone marrow chimeras: Endothelial cells are not a restricting element. *J Exp Med* 1987; 166: 1906-11.
- Hickey WF. Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. *Brain Pathol* 1991; 1: 97-105.
- Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen *in vivo*. *Science* 1988; 239: 290-39.
- Tiwari-Woodruff S, Voskuhl RR. Neuroprotective and anti-inflammatory effects of estrogen receptor ligand treatment in mice. *J Neurol Sci* 2009; 286: 81-5.
- Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Estrogen-mediated immunomodulation involves reduced activation of effector T cells, potentiation of Treg cells, and enhanced expression of the pd-1 costimulatory pathway. *J Neurosci Res* 2006; 84: 370-8.
- Nilsson S, Makela S, Treuter E, *et al.* Mechanisms of estrogen action. *Physiol Rev* 2001; 81: 1535-65.
- Zhao C, Matthews J, Tujague M, *et al.* Estrogen receptor beta2 negatively regulates the transactivation of estrogen receptor alpha in human breast cancer cells. *Cancer Res* 2007; 67: 3955-62.
- Matthews J, Wihlen B, Tujague M, Wan J, Strom A, Gustafsson JA. Estrogen receptor (er) beta modulates eralpha-mediated transcriptional activation by altering the recruitment of c-fos and c-jun to estrogen-responsive promoters. *Mol Endocrinol* 2006; 20: 534-43.
- Morissette M, Le Saux M, D'Astous M, *et al.* Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. *J Steroid Biochem Mol Biol* 2008; 108: 327-38.
- Pettersson K, Delaunay F, Gustafsson JA. Estrogen receptor beta acts as a dominant regulator of estrogen signaling. *Oncogene* 2000; 19: 4970-8.
- Delaunay F, Pettersson K, Tujague M, Gustafsson JA. Functional differences between the amino-terminal domains of estrogen receptors alpha and beta. *Mol Pharmacol* 2000; 58: 584-90.

- [26] Dhandapani KM, Brann DW. Protective effects of estrogen and selective estrogen receptor modulators in the brain. *Biol Reprod* 2002; 67: 1379-85.
- [27] Dhandapani KM, Brann DW. Role of astrocytes in estrogen-mediated neuroprotection. *Exp Gerontol* 2007; 42: 70-5.
- [28] Wang C, Dehghani B, Li Y, *et al.* Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. *J Immunol* 2009; 182: 3294-303.
- [29] Figtree GA, Noonan JE, Bhindi R, Collins P. Estrogen receptor polymorphisms: Significance to human physiology, disease and therapy. *Recent Pat DNA Gene Seq* 2009; 3: 164-71.

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