Endometrial expression of cellular protooncogene c-*ski* and its regulation by estradiol-17β

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Received 1 February 1999; received in revised form 19 March 1999

Abstract The expression of the cellular protooncogene c-*ski* was examined in the rat uterus. In situ hybridization revealed that c-*ski* mRNA was expressed in the uterus of the adult rat on the day of estrous and localized mainly in the luminal and glandular epithelia. To test the possibility that the expression of c-*ski* mRNA is induced by estrogen, rats were ovariectomized and estradiol-17β (E_2) was injected. The expression of c-*ski* mRNA was upregulated 3 h after E_2 treatment, reaching the highest level at 6 h and this persisted until 24 h; the E_2-induced expression of c-*ski* mRNA was restricted to the luminal and glandular epithelia. These results suggest that the c-*ski* gene plays a role in uterine epithelial cell proliferation and mediates the proliferative action of E_2.

Key words: C-ski; Protooncogene; Uterine epithelial cell; Proliferation; Estradiol

1. Introduction

C-ski is a cellular protooncogene, which was originally shown to induce myogenesis when transfected to non-myogenic quail embryo cells in vitro [1]. Transgenic mice expressing the c-*ski* gene under the control of a murine sarcoma virus long terminal repeat showed large increases in their skeletal muscle mass [2], while c-*ski* deficient mice have defects in their skeletal muscle development [3]. These results suggested that the c-*ski* gene product (Ski) is involved in both the proliferation and differentiation of skeletal muscle cells during development [2,3]. However, little is known about the pathways through which Ski expresses its disparate activities.

The uterus comprises several cell types including epithelial, stromal and myometrial cells. Of these cells, the luminal and glandular epithelial cells proliferate in response to estrogen treatment in the rodent [4]. The presence of estrogen receptors in the uterine epithelial cells has been shown and a number of regulatory factors that mediate uterine responses to estrogen have been identified. Among these factors are nuclear protooncogenes such as c-*fos* and c-*jun* [5]. Transient expression of these protooncogenes is rapidly induced by estrogen preceding the initiation of proliferation of uterine epithelial cells [5–7]. Recently, it was shown that Ski exerts its effect by modulating retinoic acid receptor signaling [8] and it was suggested that some of the biological activities of nuclear hormone receptors are in part modulated by Ski [8]. Considering the interaction between Ski and nuclear receptors, it would be of value to examine if the c-*ski* gene is expressed in uterine epithelial cells and if so, these cells would be a good model to test the possibility that Ski mediates the proliferative effect of estrogen in these cells. In fact, the c-*ski* gene is known to be expressed in the uterus of adult mouse though its cellular localization is unknown [9]. The present study, therefore, was conducted to determine the cellular localization of the c-*ski* gene in the uterus of the rat and to examine whether the uterine expression of the c-*ski* gene is regulated by estrogen.

2. Materials and methods

2.1. Animals and tissues

Adult cycling female Wistar Imamichi rats were purchased from the Imamichi Institute of Animal Reproduction (Ibaraki, Japan). Rats were housed under controlled light (14 h light:10 h dark) and food and water were given ad libitum. They were ovariectomized (OVX) under light ether anesthesia and estradiol-17β (E_2, Sigma, St. Louis, MO, USA) dissolved in 0.2 ml of sesame oil was injected subcutaneously after 1 week or later. At 0 (before injection of E_2), 1, 3, 6, 12 or 24 h after E_2 treatment, rats were killed by cervical dislocation and uterine tissues were collected and snap frozen in liquid nitrogen or embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). The obtained samples were stored at −80°C until use.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Cellular RNA was extracted from each tissue using TRIzol reagent (Gibco BRL, New York). One microgram of the cellular RNA was reverse-transcribed into its cDNA using SuperScript II (Gibco BRL, New York) in a reaction volume of 20 μl according to the manufacturer's protocol. The reaction mixture contained 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.5 mM each of dATP, dTTP, dGTP and dCTP, 5 mM random hexamer and 200 U SuperScript II. Two microliters out of 20 μl was used as a template for further PCR. The primer set used for amplification of the partial rat c-*ski* cDNA fragment consists of the forward primer, 5′-ACC ATC TCG TGC TTC GTG GTG GGA-3′ and the reverse primer, 5′-CTC CTT GCC CGT GTA ATC CTG GCT-3′, and was designed on the basis of the DNA sequences of the mouse [10] and human [11] c-*ski* gene published in the literature [12]. The predicted PCR amplified sequence was set within exon 1 of the c-*ski* gene because of its high homology with the mouse [10] and human [11] c-*ski*, and a 561 bp product was expected to be generated [12]. Fifty microliters of the reaction mixture contained 2 μl of reverse transcription reaction as above, 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 200 μM each of dATP, dTTP, dGTP and dCTP, 10 pmol of each primer, and 1.25 U Taq polymerase (rTaq polymerase, Takara, Japan). PCR amplification was performed in a Perkin Elmer DNA thermal cycler model 480, under the following conditions: 1 cycle of 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; 1 cycle of 72°C for 10 min. After PCR, an aliquot of the reaction mixture was electrophoresed on 1% TAE agarose gel and the products were stained with ethidium bromide. The DNA sequence of the PCR product was determined and was judged to be a partial fragment of the rat c-*ski* cDNA since it showed high similarity (>93%) to that of the corresponding region of the mouse [10] and human [11] counterparts. The number of cycles used for PCR was chosen so that the amount of RNA used for RT-PCR versus the intensity of the ethidium bromide-

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PII: S0014-5793(99)00424-X

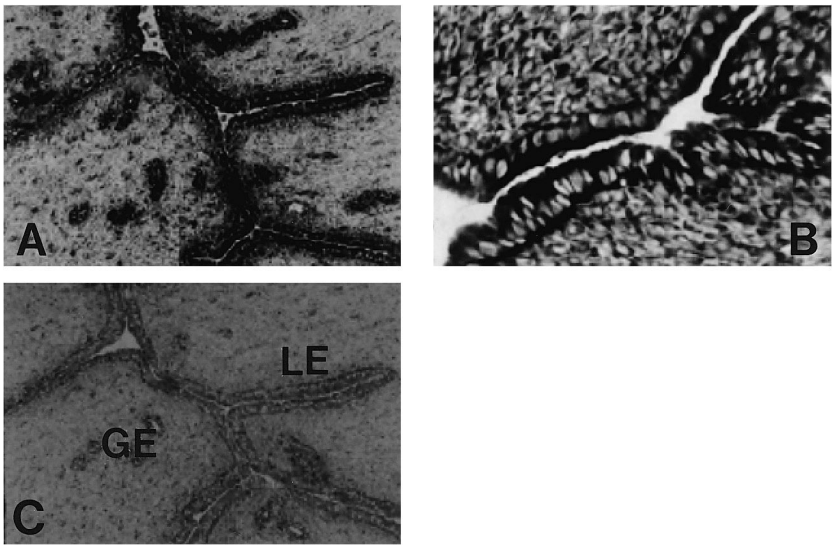

Fig. 1. In situ hybridization analysis on the expression of c-*ski* mRNA in the uterine tissues from adult female rats on the day of estrous. A and B: Antisense cRNA probe specific to rat c-*ski* was used. C: Sense probe (control). GE, glandular epithelium; LE, luminal epithelium. Magnification, ×220 (A and C), ×870 (B).

stained product on agarose gel was within a linear range. The equality of the amount of cDNA samples used for PCR of rat c-*ski* was verified by PCR using the primer set of rat G3PDH (Clontech, CA, USA). Quantitative analysis of the expression of c-*ski* mRNA was performed on the photo of the gel using NIH image software (NIH, ver. 1.62) and the data are expressed as 'pixel'.

2.3. In situ hybridization

The PCR product of rat c-*ski* cDNA (561 bp) was subcloned into a plasmid vector pGEM-T (Promega, Madison, WI, USA). The plasmid was linearized by an appropriate restriction enzyme and digoxigenin (DIG)-UTP labeled cRNA probes were synthesized using T7/SP6 RNA polymerase (DIG RNA labeling kit, Boehringer Mannheim, Germany).

Cryostat sections (5 μm thick) of the rat uterine tissues from 0 to 24 h were mounted on slides coated with 2.3% Vectabond reagent (Vector Laboratory, CA, USA) in acetone. The sections were fixed with 3% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 60 min at room temperature, washed for 10 min in PBS and then processed according to the method of Hirota et al. [13]. DIG-labeled cRNA in the tissue sections was detected after overnight incubation with an anti-DIG alkaline phosphatase-conjugated antibody (Nucleic Acid Detection Kit, Boehringer Mannheim, Germany) at a dilution of 1:500. The slides were mounted in glycerol gel and examined under bright-field microscopy.

2.4. Immunohistochemistry

Frozen tissue sections (5 μm thick) prepared from the rat uterine tissues (0, 6 and 24 h after E_2 treatment) were subjected to immunohistochemical analysis for the detection of proliferating cell nuclear antigen (PCNA). All procedures were performed at room temperature. The sections were fixed in 3% PFA in PBS for 30 min, followed by incubation in 0.1% Triton X-100 in PBS for 15 min. After washing in PBS, endogenous peroxidase activity was inactivated by incubation in 0.3% hydrogen peroxide in methanol for 30 min. Then the sections were immersed in blocking solution (8% skim milk in PBS) for 30 min. The primary antibody specific for PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilution 1:100 with 1% BSA in PBS) was applied and incubated for 90 min. As a secondary antibody, biotin-labeled goat anti-mouse IgG (Kirkegaard and Perry Laboratory, MD, USA; diluted at 1:100 with 1% BSA in PBS) was used and incubation was performed for 40 min. Detection of the signal for PCNA was performed using the avidin-biotin complex system (Vectastain ABC kit, Vector Laboratory, CA, USA). The specificity of the primary antibody used for the presence of PCNA has been confirmed using tissue sections from rat small intestine (see Fig. 4D).

2.5. Statistical analysis

The graphed data were analyzed by ANOVA followed by *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. The c-ski gene is expressed in the uterine luminal and glandular epithelial cells of the rat on the day of estrous

In situ hybridization was performed to determine the localization of c-*ski* mRNA in the uterine tissue sections of the rat obtained on the day of estrous. The DIG-labeled antisense c-*ski* cRNA probe was detected in the luminal and glandular

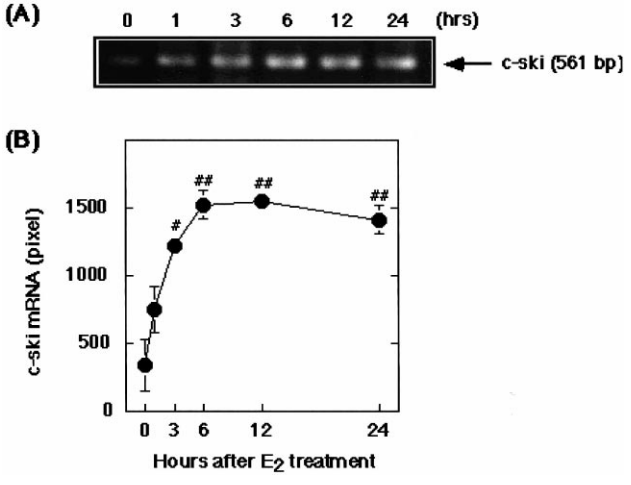

Fig. 2. Quantitative RT-PCR analysis of the temporal expression of c-*ski* mRNA in the uterus of OVX+E_2-treated rats. A: Representative photograph of ethidium bromide-stained gel. The PCR product of c-*ski* is indicated by an arrow. Numbers above the photograph show time (hours) after E_2 treatment. B: Changes in the expression of c-*ski* mRNA. Data are expressed as the mean ± S.E.M. Four to six samples were used at each time point, and similar results were obtained in several independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. 0 h.

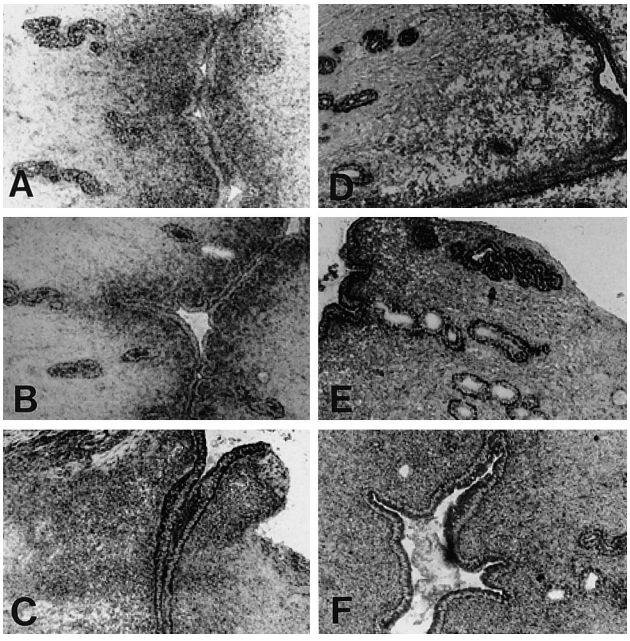

Fig. 3. In situ hybridization analysis to locate the expression of c-*ski* mRNA in the uterus of OVX+E_2-treated rats. A: 0 h (OVX but no E_2 injected). B, C, D, E and F: 1, 3, 6, 12 and 24 h after E_2 treatment, respectively. Magnification, ×140.

epithelial cells (Fig. 1A,B), while no signal was found when sense cRNA probe was used (Fig. 1C).

3.2. Administration of E_2 induces the expression of the c-ski gene in the uterus of OVX rats

To examine the effect of E_2 on the expression of c-*ski* mRNA in the rat uterus, OVX rats were treated with 1 μg of E_2 (s.c.) and the uterine tissues were collected. The relative expression level of c-*ski* mRNA at each time point was compared using quantitative RT-PCR analysis. The administration of E_2 caused a significant ($P < 0.05$) increase at 3 h, which maximized at 6 h and this level was maintained at 24 h (Fig. 2).

3.3. E_2 induces the c-ski gene specifically in the uterine luminal and glandular epithelium of OVX rats

The result of quantitative RT-PCR analysis revealed that expression of c-*ski* mRNA is induced by E_2 treatment in OVX rats. To determine the cellular localization of c-*ski* mRNA expression induced in this manner, in situ hybridization was performed. At 0 and 1 h, only a faint staining showing the presence of c-*ski* mRNA was detected in the subepithelial stromal and glandular epithelial cells (Fig. 3A,B). In the luminal epithelial cells, a significant message was first detected at 3 h and thereafter strong expression persisted both in the luminal and in the glandular epithelial cells up to 24 h (Fig. 3C–F). The relative cellular expression patterns during 0–24 h are summarized in Table 1.

Table 1
Changes in the distribution of c-*ski* positive cells in the uterus of ovariectomized rat after a single injection of estradiol-17β (E_2)

Time after E_2 injection (h)	Epithelium		Stroma
	Glandular	Luminal	
0	+	−	+
1	+	−	+
3	++	+	+
6	++	++	+
12	++	++	+
24	++	++	+

3.4. Proliferation of uterine epithelial cells is induced by E_2 treatment in OVX rats

Administration of E_2 to OVX rats is known to induce proliferation of uterine epithelial cells [7]. To confirm that this is the case in our study, immunohistochemical analysis of the expression of PCNA, a marker of proliferating cells, was performed. Immunohistochemical analysis of the expression of PCNA in the uterine tissues revealed that no positive staining was observed at 0 and 6 h (Fig. 4A,B), while specific staining was present in the luminal and glandular epithelial cells at 24 h (Fig. 4C). The specificity of the antibody used in the present work was verified by the application of this method on a tissue section from rat small intestine that resulted in positive staining in the actively dividing epithelial cells (Fig. 4D).

4. Discussion

The cellular protooncogene c-*ski* was originally found as a transcriptional regulatory factor which could induce differentiation of non-skeletal muscle cells to a skeletal muscle lineage [1], and most studies on the function of the c-*ski* gene have focused on its role in myogenesis (for review, see [14]). In addition to skeletal muscle, the expression of this gene is distributed to a variety of tissues and this was true in the reproductive tissues such as uterus, ovary and so on [9] though no study on the expression and role of this gene has been performed in these tissues. In this regard, the present study demonstrated for the first time the localization of c-*ski* message in the uterine tissues and that its expression is under the influence of E_2.

In our in situ hybridization analysis, the expression of c-*ski* message in both the glandular and luminal epithelium was first up-regulated 3–6 h after E_2 treatment. On the other hand, the result obtained by quantitative RT-PCR analysis also revealed that the c-*ski* message in the uterus significantly

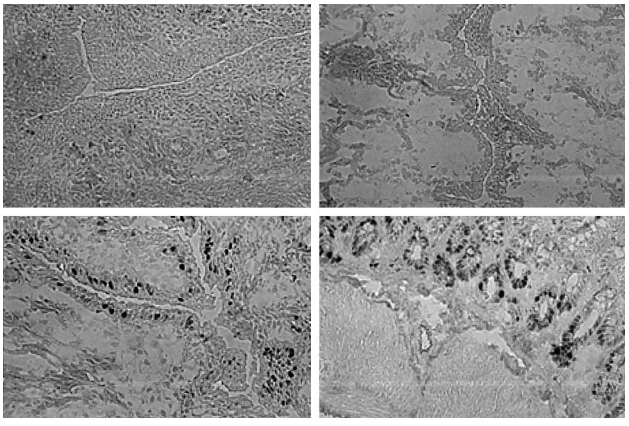

Fig. 4. Immunohistochemical analysis on the expression of PCNA in the uterus of OVX+E_2-treated rats. A: 0 h (OVX but no E_2 injected). B and C: 6 and 24 h after E_2 treatment, respectively. D: The same staining procedure was applied to the section from rat small intestine. Magnification, ×300.

($P < 0.05$) increased 3–6 h after the treatment. Therefore the increased expression of c-*ski* message observed in RT-PCR analysis may be concluded to represent its epithelial expression induced by E_2 treatment. Since uterine stromal cells lose their ability to proliferate in response to E_2 during the maturational process [15], this is in good agreement with our result that the relatively low expression in the subepithelial stromal cells was unchanged regardless of E_2 treatment.

The uterine epithelial cells are known to respond to in vivo E_2 treatment and proliferate as shown by several studies [7,16]. In the mature, ovariectomized rat model, estradiol treatment induces DNA synthesis 12–16 h after treatment, and mitosis follows at 18–24 h [17]. This was also confirmed in our study in which the expression of PCNA was evident in uterine epithelial cells of OVX rats 24 h after E_2 treatment. Although the precise function of c-*ski* expressed in the uterus is unknown at present, we would present one possibility that this gene is involved in mediating the proliferative effect of E_2 on uterine epithelial cells. The animal model used in the present experiment was according to the previous reports by several groups [6,7] so that we could directly compare the results obtained with theirs, i.e. they used ovariectomized adult female rats and treated them with a single injection (s.c., 1 μg in sesame oil) of E_2. Among these reports, the temporal and cellular expression patterns of c-*fos* mRNA [7] as well as its translated product Fos [6] were investigated and its role in the proliferation of uterine epithelial cells was suggested [5–7,18]. In these studies, the expression of c-*fos* message (and protein) was transient, and peaked earlier than that of c-*ski*. Comparing their results with ours, there was a 5 h interval in the period of its peak/maximum expression achieved between c-*fos* (1 h, by Papa et al. [6]) and c-*ski* (6 h, the present study). The regulatory mechanism of c-*ski* gene expression is currently unknown, however, it should be noted that the DNA sequencing analysis on the homologue of c-*ski* gene, *snoN*, revealed that this gene has an AP-1 binding site [19], a well known regulatory region to which Fos binds together with Jun [20], in its 5′ promoter region. This led us to the hypothesis that Fos, induced rapidly and transiently by E_2, sequentially acts on the regulatory region of the c-*ski* gene and promotes its transcription, although the possibility that E_2 directly induces c-*ski* gene expression cannot be excluded. To clarify this issue, more detailed study on the regulatory mechanism of c-*ski*/*sno* gene expression will be required in the near future.

In conclusion, our study clearly indicates that the expression of c-*ski* gene is specifically induced in the uterine epithelial cells of OVX rats by E_2 treatment, and suggests that c-*ski* is involved in mediating proliferative effect of E_2 in these cells.

Acknowledgements: The authors are grateful to the members of the Laboratory of Veterinary Pathology, the University of Tokyo for their generous help in this work. This work was supported in part by a grant-in-aid from the Ministry of Education, Sports and Culture of Japan.

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