

Regulation of the Ovine Interferon-Tau Gene by a Blastocyst-Specific Transcription Factor, Cdx2

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ABSTRACT Expression of ovine interferon-tau (oIFN τ), a factor essential for the process of maternal recognition of pregnancy in ruminant ungulates, is restricted to the trophoblast. However, the molecular mechanisms by which oIFN τ expression is restricted to the trophoblast have not been fully elucidated. The objective of this study was to determine whether oIFN τ gene transcription could be regulated through Cdx2 expression, a transcription factor implicated in the control of cell differentiation in the trophoblast. Human choriocarcinoma JEG3 cells were co-transfected with an oIFN τ (–654 base pair, bp)-luciferase reporter (–654-oIFN τ -Luc) construct and several transcription factor expression plasmids. Compared to –654-oIFN τ -Luc alone, transcription of the –654-oIFN τ -Luc increased more than 30 times when this construct was co-transfected with Cdx2, Ets-2, and c-jun. The degree of transcription decreased to 1/4 levels when the upstream region was reduced to –551 bp, and became minimal with further deletions; this was confirmed with the use of the reporter constructs with mutated c-jun, Ets-2, and/or Cdx2 sites. In trophoblast unrelated NIH3T3 cells, which do not support IFN τ gene transcription, the oIFN τ -Luc transcription was enhanced approximately eightfold when the cells were co-transfected with the Cdx2/Ets-2 or Cdx2/Ets-2/c-jun expression plasmids. These findings were confirmed by gel-shift assays examining Cdx binding site on the oIFN τ gene's upstream region, by immunohistochemical study identifying the presence of Cdx2 in day 15 and 17 ovine conceptuses, and by Western blot detecting Cdx2 in day 17 conceptuses. Our results indicate that oIFN τ gene transcription is regulated by Cdx2, and suggest that Cdx2 could be a key molecule in determining oIFN τ gene transcription by the trophoblast. *Mol. Reprod. Dev.* 73: 559–567, 2006. © 2006 Wiley-Liss, Inc.

Key Words: interferon- τ ; Cdx2; JEG3; NIH3T3; trophoblast; ovine

INTRODUCTION

Interferon-tau (IFN τ) is regarded as a major protein implicated in the process of maternal recognition of pregnancy in ruminant ungulates (Godkin et al., 1982; Imakawa et al., 1987; Roberts et al., 1992). IFN τ acts on the uterine epithelium and attenuates secretion of a luteolysin, prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$), resulting in the maintenance of corpus luteum (CL) function (Vallet et al., 1988). Based on cDNA and amino acid sequences, IFN τ is classified among the type I IFNs (Imakawa et al., 1987, 1989; Stewart et al., 1987; Charpigny et al., 1988). However, the expression of IFN τ is quite different from that of other type I IFNs such as IFN α and IFN β that are induced by viruses or double stranded RNA, and maintained for only a few hours (Pestka et al., 1987; Farin et al., 1991). The expression of IFN τ is subject to temporal and spatial limits since its production is restricted to trophoblast cells during peri-implantation period (Farin et al., 1989; Guillomot et al., 1990; Demmers et al., 2001). In fact, ovine interferon-tau (oIFN τ) production begins on day 8 of pregnancy (day 0 = first day of estrus). Its production increases as the conceptus elongates and reaches the highest production (up to 100 μ g/conceptus/24 hr) on day 16, just before the attachment of the conceptus to the uterine epithelium (Godkin et al., 1982; Ashworth and Bazer, 1989). Conceptus production of oIFN τ appears to decrease as trophoblasts attach to the uterine endometrium (Guillomot et al., 1990), and by day 22, when placenta formation is initiated, oIFN τ production is minimal (Godkin et al., 1982).

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So far, more than 10 *IFN τ* genes or cDNAs have been isolated and characterized for the ovine and bovine species, exhibiting a high degree of similarity within and among ruminants (Leaman and Roberts, 1992; Nephew et al., 1993; Ryan and Womack, 1993; Alexenko et al., 2000; Ealy et al., 2001). Because of massive amounts of *IFN τ* produced during peri-implantation period, these genes are expected to have comparable expression patterns; however, expression appeared to be bias toward certain gene(s) (Nephew et al., 1993; Ealy et al., 2001). In addition, the upstream region of the *IFN τ* gene, *oIFN τ -o10*, which has been extensively studied in our laboratory (Yamaguchi et al., 1999, 2001), was independently isolated and characterized by others (Charlier et al., 1991). By transient transfection of reporter constructs with deleted or mutated 5'-upstream regions of the *oIFN τ -o10* gene in human choriocarcinoma JEG3 cells, an AP-1 binding site in the distal enhancer region was shown to be effective in *oIFN τ* -reporter transactivation (Yamaguchi et al., 1999, 2001). Another regulatory domain exists at the proximal promoter region to which the transcription factor Ets-2 binds (Ezashi et al., 1998; Yamaguchi et al., 1999, 2001). Ezashi et al. (2001) found that the action of Ets-2 on bovine *IFN τ* (*bIFN τ*) was inhibited by Oct-4, a mammalian POU transcription factor expressed by early embryo cells and germ cells (Nichols et al., 1998). Once Oct-4 expression subsided, Ets-2 became effective in the activation of *bIFN τ* gene transcription. Recently, a transcription co-activator, cAMP-response element binding protein-binding protein (CBP), was shown to enhance *oIFN τ -o10* gene transcription (Xu et al., 2003). Both AP-1 and Ets-2 binding domains are located on the CBP polypeptide sequences, to which these transcription factors bind, possibly resulting in further activation of *oIFN τ -o10* gene transcription. Quite recently, the activation of intracellular signaling Ras/MAPK was found to substantially enhance *bIFN τ* gene transcription (Ezashi and Roberts, 2004). However, trophoblast cell-specific expression of the *IFN τ* gene has not been fully characterized.

In mice, Oct-4 is expressed in oogenesis and throughout early cleavage stages (Scholer et al., 1989) and becomes progressively restricted, first to the entire inner cell mass (ICM) and then to the epiblast as blastocyst development proceeds (Palmieri et al., 1994). Ablation of the *Oct-4* gene results in lethality at implantation because conceptuses fail to generate any ICM at the blastocyst stage (Nichols et al., 1998). In the absence of Oct-4, however, differentiation into the trophoblast lineage is not simply the developmental default of ICM. The homeobox gene *Cdx2*, a transcription factor and a homolog of the *Drosophila* gene *caudal*, has been implicated in the control of cell differentiation in the trophectoderm and the intestinal epithelium in mice (Beck et al., 1995). This factor shows a reciprocal pattern of its expression to Oct-4 in early embryos, becoming restricted to the trophoblast by the blastocyst stage (Beck et al., 1995). Consistent with the early developmental expression, *Cdx2* null mutant mice dis-

play preimplantation lethality (Chawengsaksophak et al., 1997). Because *IFN τ* production is restricted to the blastocyst, we hypothesized that in addition to AP-1, Ets-2, and Ras/MAPK so far examined, cell-specific expression of the trophoblast *IFN τ* gene might also be regulated through *Cdx2* expression. Objectives of this study were to demonstrate that *Cdx2* is involved in transcription of the *oIFN τ* gene, and to elucidate molecular mechanisms by which *oIFN τ* gene transcription could be regulated through *Cdx2*.

MATERIALS AND METHODS

Animals, Fixation of Uteri, and Sampling

Six-mature, crossbred ewes were maintained at the U.S. Meat Animal Research Center, Clay Center, NE and care was in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, the U.S. Department of Agriculture. Estrous synchronization and breeding procedures were performed as previously described (Imakawa et al., 2002). Pregnant ewes were slaughtered on days 15 and 17 of gestation, and three uteri each were immediately subjected to whole uterus fixation. The middle uterine artery associated with the left and right uterine horns was cannulated, each were infused with 60 ml phosphate buffered saline (PBS, pH 7.2), and 60 ml of 10% formalin (Polyscience, Inc., Warrington, PA) to initiate fixation. Whole uteri were submerged in 10% formalin for 24 hr, decanted and re-submerged in 10% formalin for another 24 hr period to complete fixation. Fixed whole uteri were serially dissected into 1.5 cm blocks (proximal to distal uterine horn origination, 10 blocks/uterine horn). Each block was sectioned (5 μ m), mounted on glass slides and evaluated microscopically for the presence of conceptus(es) after hematoxylin-eosin staining.

Immunohistochemistry

Serial sections (5 μ m) were cut, mounted on glass slides precoated with 3-aminopropyltriethoxysilane, deparaffined, rehydrated, washed in distilled water, and then immersed in methanol with 0.3% (v/v) H_2O_2 for 30 min. After a wash with PBS, normal horse serum (Vector Laboratories, Burlingame, CA) was placed on each section to block nonspecific protein binding, and the sections were incubated at room temperature for 1 hr with rabbit anti-human *Cdx2* antibody (CeMines, Evergreen, CO) or normal rabbit IgG (Sigma-Aldrich) diluted 1:500 with PBS. Subsequently, the sections were washed with PBS and incubated with biotinylated anti-rabbit IgG (1:200; Vector Laboratories). Immunoreactivity was visualized using a VectaStain avidin-biotin-peroxidase kit (Vector Laboratories) according to the manufacturer's directions. The sections were washed well with distilled water, counter-stained with methylgreen or hematoxylin, dehydrated, mounted with Entellan (Merck, Darmstadt, Germany), and then examined using light microscope (BX-51; Olympus, Tokyo, Japan).

Plasmid Construction

5'-deletion constructs representing various lengths of the upstream regions of *oIFN τ* gene (ovine IFN τ -o10, GenBank accession number M88773) had been prepared previously (Yamaguchi et al., 2001). These constructs were based on the pGL3 Basic Vector consisting of a firefly luciferase (*Luc*) gene (Promega, Madison, WI). Constructs with one point mutation at AP-1 and/or Ets-2 sites also had been prepared previously (Yamaguchi et al., 2001). Constructs containing mutated Cdx2 and mutated AP-1, Ets-2, and Cdx2 were prepared by inverse PCR procedure (Expanding long template PCR system, Roche Diagnostics, Mannheim, Germany) with appropriate primers including mutation; Cdx2 recognition site mutation, -581 to -575 bp, of -654-*oIFN τ* -*Luc* was point mutated as 5'-TTTACTG-3' \rightarrow 5'-TTTTTGTG-3'. One point mutation was also introduced into the Cdx expression plasmid (Suh et al., 1994) at the serine 60 residue to alanine, which had been demonstrated to cause a loss of Cdx2 effectiveness in its transactivation capacity (Rings et al., 2001). These constructs were each confirmed to have expected nucleotide sequence by dideoxy sequencing.

Cell Culture and Transient Transfection

NIH3T3 cells and human choriocarcinoma JEG3 cells (American Type Culture Collection, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 40 U/ml penicillin, and 40 μ g/ml streptomycin (Sigma-Aldrich). Both cells were incubated at 37°C CO₂ incubator (5% CO₂ in air). When cells were grown sufficiently, they were replated onto 24-well plastic plates (Nunc, Roskilde, Denmark) for subsequent transfection. At 60%–80% cell confluency, transient transfection was performed with TransFast (3 μ l, cationic lipids, Promega) according to the manufacturer's protocol, and plates were incubated in the 5% CO₂ incubator at 37°C for 48 hr. For one well, 1 μ g total plasmid DNA was prepared in 200 μ l DMEM containing 10% FBS. To normalize transfection efficiency, pRL-TK vector (containing herpes simplex virus thymidine kinase promoter and *Renilla* luciferase gene, Promega) along with each of pGL3 vector and a transcription factor expression plasmid without c-jun, Ets-2, or Cdx2 (empty vector, pRVS) was transfected into JEG3 or NIH3T3 cells. The ratio of pGL3 vector to pRL-TK vector, and to the empty vector was 8:1:1 (800:100:100 ng). When three kinds of expression vectors were co-transfected, the ratio of these plasmids was 1:1:1 (33.3:33.3:33.3 ng). Regardless of expression plasmid combination, the amounts of expression plasmids (total of 100 ng) were kept constant (33.3 ng each) with the inclusion of the empty vector. Expression of murine c-Jun is driven by the Rous Sarcoma Virus long terminal repeat (LTR) (Yamaguchi et al., 1999). The Ets-2 expression vector was a pSG5-based construct containing the SV40 promoter/enhancer (Wakiya et al., 1996). The Cdx2 expression plasmid

was a pRC-Cytomegalovirus (CMV) based construct containing the SV40 promoter/enhancer (Suh et al., 1994). After 48 hr incubation, transfected cells were lysed with Passive lysis buffer (Promega). Luciferase activity of these lysates was measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Signal intensity was obtained through the normalization of transfection efficiency after the luminescence of firefly luciferase had been divided by that of *Renilla* luciferase.

Preparation of Nuclear Extracts, Western Blot, and Electrophoretic Mobility Shift Assay

JEG3 cells, transfected or not transfected with Cdx2 expression plasmid, on three flasks (75 cm², BD Biosciences, Bedford, MA) were washed with PBS and scrapped off the plate, from which nuclear proteins were extracted using the method described previously (Matsuda et al., 2004) and concentrations of the nuclear extracts were determined by using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA).

Nuclear extracts (15 μ g) from JEG3 cells and day 17 conceptuses (Yamaguchi et al., 2001) were loaded into each lane and separated by 12% SDS-polyacrylamide gel electrophoresis, which were transferred onto PVDF membrane (Millipore, Bedford, MA) and then treated with rabbit anti-human Cdx2 antibody (CeMines) or normal rabbit IgG (Sigma-Aldrich) diluted 1:750 with PBS. The proteins were detected using the secondary antibody conjugated with horseradish peroxidase, ECL Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Binding of nuclear proteins to a potential Cdx2 binding site located on the upstream region of *oIFN τ* gene was examined by electrophoretic mobility shift assays (EMSA). In a preliminary experiment, the gel-supershift assay, which used the antibody that immunohistochemically detected Cdx2 in ovine conceptuses, was not successful. Specificity of protein binding to the Cdx region was determined using the following probes and competitors. Oligonucleotide probe for Cdx site was from -586 to -567 bp of the *oIFN τ* gene (5'-CCTGGTTTTACTGGATATGGCA-3'; Cdx binding site is underlined), and Cdx site was point mutated as 5'-TTTACTG-3' \rightarrow 5'-TTTTTGTG-3'. Oligonucleotide competitor containing AP-1 and Cdx2 binding sites (AP-1/Cdx), -597 to -574 bp, of the *oIFN τ* gene was 5'-GAATGTGTCATCCTGGTTTTACTGA-3' (binding sites of each are underlined). Oligonucleotide competitor representing consensus AP-1 binding site was 5'-CGCTTGATGAGTCAGCCGGAA-3' (AP-1C, binding site is underlined) (Yamaguchi et al., 2001), and the one with consensus Cdx2 sequence was 5'-GTGCAA-TAAAACTTTATGAGTA-3' (Cdx-C, binding site is underlined) (Suh et al., 1994). Oligonucleotide probes containing the Cdx site and mutated Cdx site were end-labeled with [γ -³²P] ATP (6,000 Ci/mmol, NEN, Boston, MA) and T4 polynucleotide kinase (Takara, Tokyo, Japan). End-labeled oligonucleotides were ethanol

precipitated and annealed with antisense oligonucleotide by cooling slowly from 95°C to room temperature. To prepare competitors, nonlabeled sense and antisense oligonucleotides were mixed and annealed by cooling slowly. For EMSA, 10 µg of nuclear extracts were placed in a binding buffer containing 100 µg/ml poly(dI-dC) poly(dI-dC), 50 µg/ml bovine serum albumin (BSA), 7 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.9), 0.5 mM MgCl₂, 35 mM KCl, 7% glycerol, 70 nM ethylenediamine tetraacetic acid (EDTA), 0.3 mM dithiothreitol (DTT) on ice with or without a 10- or 50-fold molar excess of unlabeled competitor. The ³²P-labeled probe (3,000 cpm, 1–3 ng DNA depending upon the specific activity) was then added and the mixture was incubated at room temperature for 20 min. Reaction mixtures were then applied to 5% polyacrylamide gel in 0.5× TBE buffer. After the electrophoresis, the gel was dried and autoradiographed.

Statistical Analysis

The results of luciferase assays were expressed as mean ± SEM. Differences in fold activation were examined by one-way ANOVA followed by Tukey's multiple comparison tests (Multistat; Biosoft, Cambridge, UK). All differences with a value of *P* < 0.05 were considered significant.

RESULTS

Localization and Detection of Cdx2 in the Ovine Conceptuses

Cdx2 was localized in day 15 and 17 conceptuses using a rabbit anti-human Cdx2 antibody (Fig. 1). It appeared that the staining intensity for Cdx2 expression was higher in day 15 conceptuses than in day 17 conceptuses. In addition, as conceptuses attached to the uterine endometrium, the intensity of Cdx2 staining appeared to be lower than that of unattached conceptuses (Fig. 1A vs. B vs. E and G).

Expression of Cdx2 in JEG3 and day 17 trophoblast cells was examined by using Western blot with the same antibody that detected trophoblast Cdx2 in the immunohistochemical analysis. High level of Cdx2 was found in JEG3 cells, which had been transfected with the Cdx2 expression plasmid (Fig. 2, Upper panel). In addition, Cdx2 was also detected in day 17 conceptuses.

Examination of the Role of Cdx2, Alone or in Combination With c-jun and/or Ets-2, in oIFN τ Gene Transcription

To investigate whether Cdx2 had an effect on *oIFN τ* gene transcription, the -654-*oIFN τ* -Luc construct along with expression plasmids, c-jun, Ets-2, and/or Cdx2, were transiently transfected into JEG3 or NIH3T3 cells, and luciferase activities were measured (Fig. 2, Lower panel). In JEG3 cells, the luciferase activity of -654-*oIFN τ* -Luc increased more than 30-fold when all three expression plasmids were co-transfected with the -654-*oIFN τ* -Luc plasmid. The luciferase activity of -654-

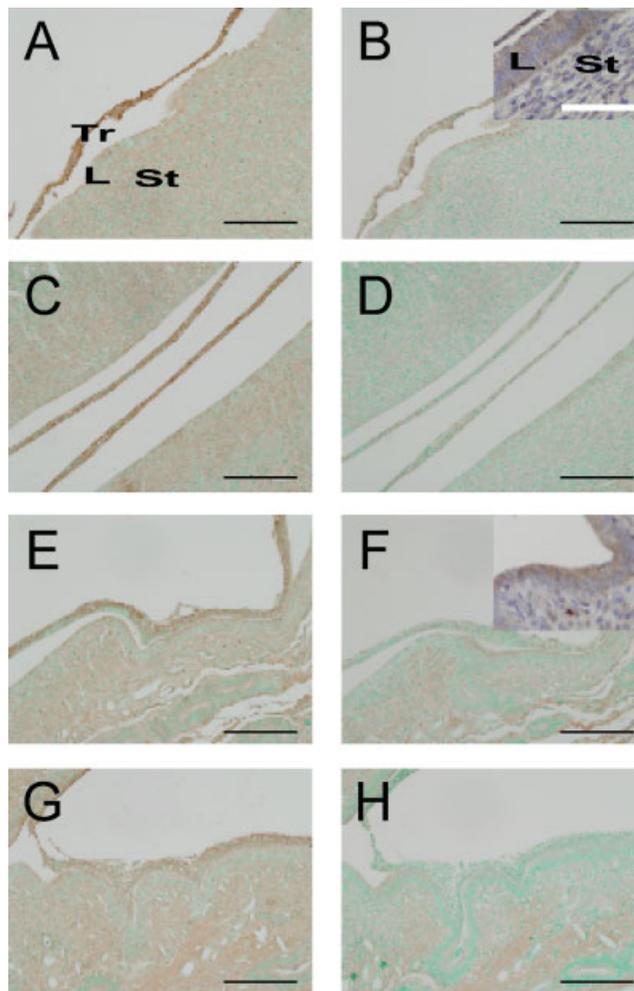


Fig. 1. Immunohistochemical localization of Cdx2 on ovine conceptuses. Whole uteri from days 15 (A, B) and 17 (C, D, E, F, G, and H) of pregnant ewes (*n* = 3 each) were fixed by infusion of 10% formalin solution into uterine artery, and were paraffin embedded. A, C, E and G: Detection of Cdx2 with rabbit anti-human Cdx2 antibody. B, D, F and H: Negative control with normal rabbit serum, all of which were counter-stained with methylgreen except that the ones shown in insets were counter-stained with hematoxylin. Tr, trophoblast; L, uterine luminal epithelium; St, stroma. A representative one from three independent experiments is shown. Black bar, 100 µm. White bar within the inset, 20 µm.

oIFN τ -Luc was low when any other combination of expression plasmids was examined. In NIH3T3 cells; however, results were quite different from those detected in JEG3. Although Cdx2 co-transfection alone elicited two- to fourfold increases, the combination of all three expression plasmids resulted only in an approximately eightfold increase (Fig. 2). However, to date this was the greatest increase elicited by the -654-*oIFN τ* -Luc construct in a cell type not related to trophoblast/placenta.

Degree of Transcription With Deletion Constructs

From the -654-*oIFN τ* -Luc, 30–150 bp of the upstream region were serially deleted and the resulting

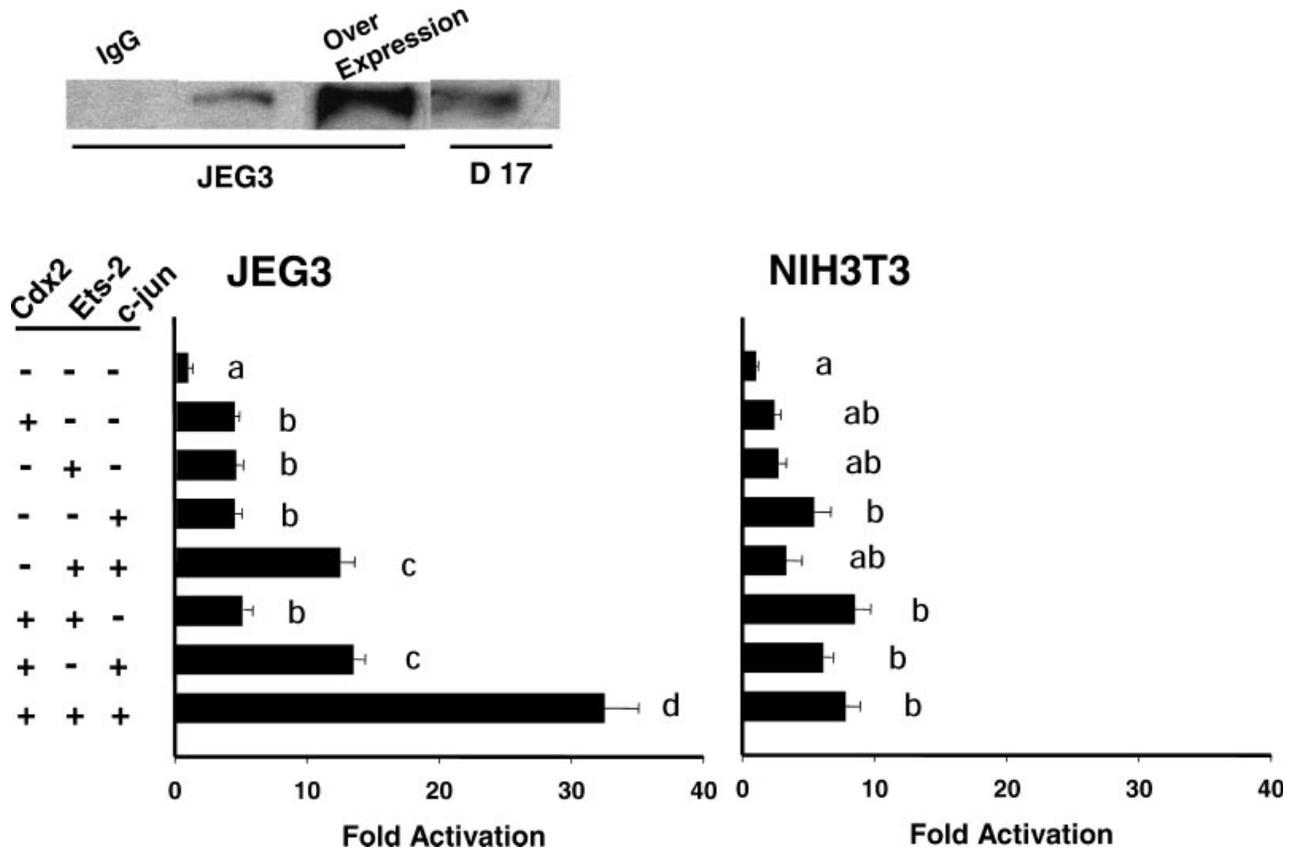


Fig. 2. Cdx2 expression and transcriptional activity of -654 -oIFN τ -Luc construct with Cdx2 expression plasmid. Upper: Western blot analysis. Fifteen micrograms nuclear proteins extracted from JEG3 or day 17 trophoblast cells were analyzed. A single band of approximately 34 kDa was found when the Cdx2 antibody, not normal IgG, was applied. Overexpression: proteins extracted from JEG3 cells that had been transfected with the Cdx2 expression plasmid, D 17: day 17 conceptuses. Lower Left: Effects of Cdx2, Ets-2, and/or c-jun expression

plasmids on the degree of -654 -oIFN τ -Luc transcription in JEG3 cells. Lower Right: Transcriptional activity of -654 -oIFN τ -Luc construct in 3T3 cells. Lower-Far left: Presence (+) or absence (-) of expression plasmid. Results were expressed as relative luciferase activity to that of the -654 -oIFN τ -Luc construct without any expression plasmid. Values represent mean \pm SEM. Results with different letters differ at $P < 0.01$ ($n = 4$ each).

fragments were inserted into the pGL3 Basic Vector. These constructs with or without three expression plasmids, c-jun, Ets-2, and Cdx2, were transiently transfected into JEG3 or NIH3T3 cells, and luciferase activities were measured (Fig. 3). In JEG3 cells, the -654 -oIFN τ -Luc construct with the expression plasmids caused a 30-fold increase in luciferase activity, but when shorter oIFN τ -Luc constructs were examined, activities declined rapidly. These results indicated that a potential Cdx2 binding site could be located between -654 and -550 bases of the oIFN τ gene. In NIH3T3 cells, however, results were again different from those detected in JEG3. Approximately, 13-fold increase in luciferase activity was found when -551 -oIFN τ -Luc and -274 -oIFN τ -Luc constructs with the expression plasmids were examined; in these cases, luciferase activity without the expression plasmids was 3–4 fold higher than that of the -654 -oIFN τ -Luc construct alone (Fig. 3).

Luciferase Activity With Mutation Constructs

To further investigate effects of Cdx2 on oIFN τ gene transcription, a potential Cdx2 binding site at -581 to -575 bp on the -654 -oIFN τ -Luc construct was point

mutated as $5'$ -TTTACTG- $3'$ \rightarrow $5'$ -TTTTGTG- $3'$, and transfected to JEG3 cells (Fig. 4). Compared with the wild type, mutation to Cdx2 site of the -654 -oIFN τ -Luc construct did not decrease luciferase activity. When this reporter construct along with c-jun, Ets-2, and Cdx2 expression plasmids was co-transfected into JEG3 cells, luciferase activity increased 5–6 folds (Fig. 4). However, the construct with mutation to all c-jun, Ets-2, and Cdx2 binding sites exhibited lowered luciferase activity in the presence or absence of the three expression plasmids. With the mutated Cdx2 expression plasmid at serine 60, luciferase activity was not completely abolished, rather it increased up to twofold versus the -654 -oIFN τ -Luc without the expression plasmid (Results not shown).

Analysis of DNA and Nuclear Protein Binding by Electrophoretic Mobility Shift Assay (EMSA)

Based on the deletion analysis, it appeared that among numerous potential Cdx binding sites located on the upstream region of the oIFN τ gene, the active Cdx2 binding site is likely located at -581 to -575 bp. Binding of nuclear proteins from JEG3 cells to upstream regions of the oIFN τ gene was examined by EMSA

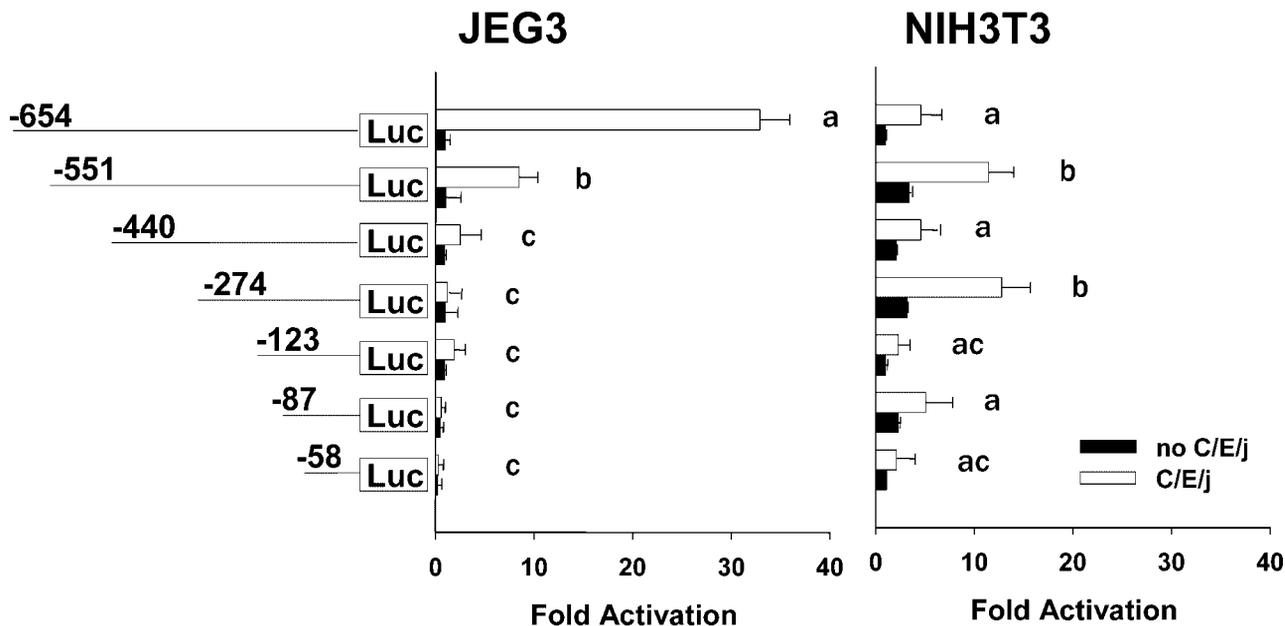


Fig. 3. Transcriptional activity of oIFN τ -Luc construct that had been deleted in its upstream region. Upstream regions of oIFN τ -o10 construct tested were -654, -551, -440, -274, -123, -87, or -58 bases. Each of these constructs in the presence or absence of Cdx2, Ets-2, and c-jun expression plasmids (C/E/j or no C/E/j, respectively) was

transfected into JEG3 or NIH3T3 cells, and luciferase activity was determined. Results were expressed as relative luciferase activity to that of the -654-oIFN τ -Luc construct without any expression plasmid. Values represent mean \pm SEM. Results with different letters differ at $P < 0.01$ ($n = 4$ each).

(Fig. 5). Specific binding with nuclear extracts from JEG3 cells was observed with the oIFN τ Cdx2 probe (oIFN τ Cdx), whereas the mutated Cdx2 probe did not exhibit any binding with nuclear extracts. Binding between the oIFN τ Cdx2 probe and nuclear extracts was abolished with the addition of 50-fold-excess unlabeled oIFN τ 's Cdx site sequence, the Cdx site with AP-1 sequence (AP-1/Cdx), or consensus Cdx2 sequence

(Cdx-C), but was not affected when a consensus AP-1 site sequence (AP-1C) was examined (Fig. 5).

DISCUSSION

Staining for Cdx2 found in day 15 and 17 ovine conceptuses agrees with the general notion that Cdx2 expression is associated with the trophoblast (Ralston and Rossant, 2005; Strumpf et al., 2005). Degree of staining, however, was not the same between day 15 and 17 conceptuses. Although the result from this study was qualitative, it appeared that as the conceptus attached to the uterine epithelium, the staining appeared to subside. Quantitative measurements are still required for definitive conclusion, but this pattern of Cdx2 expression resembles that of oIFN τ expression during the peri-implantation period (Guillomot et al., 1990). Guillomot et al. (1990) reported earlier that physical attachment between the conceptus and endometrium reduces oIFN τ expression. In addition, Nojima et al. (2004) demonstrated that DNA methylation to oIFN τ gene was initiated on day 17, which was associated with the reduction in oIFN τ gene transcription. Although the molecular mechanisms by which Cdx2 gene transcription subsides are unknown, similar regulatory mechanism might exist between oIFN τ and Cdx2 in the ovine conceptuses.

Studies of the 5'-upstream region regulating oIFN τ gene expression have been done previously utilizing transient transfection methods with human choriocarcinoma cell line, JEG3 (Yamaguchi et al., 1999, 2001). Others also have demonstrated an increase in transcriptional activity when the bIFN τ -reporter construct was

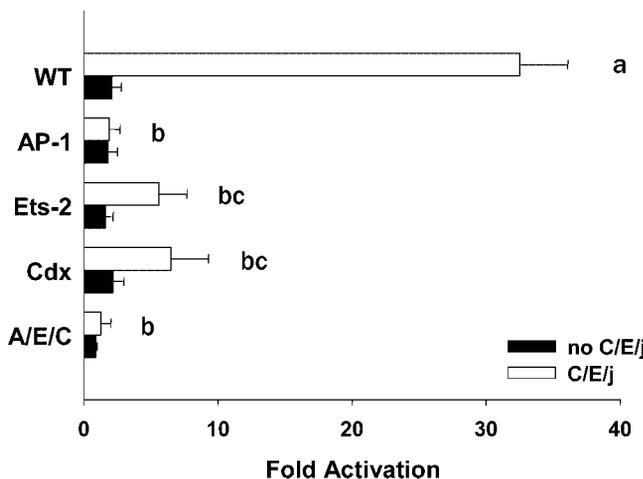


Fig. 4. Transcriptional activity of mutated -654-oIFN τ -Luc construct at AP-1, Ets-2, and/or Cdx. Results were expressed as relative luciferase activity to that of the -654-oIFN τ -Luc construct (WT) without any expression plasmid. Values represent mean \pm SEM. Results with different letters differ at $P < 0.01$ ($n = 4$ each). A/E/C: mutation to AP-1, Ets-2, and Cdx binding sites.

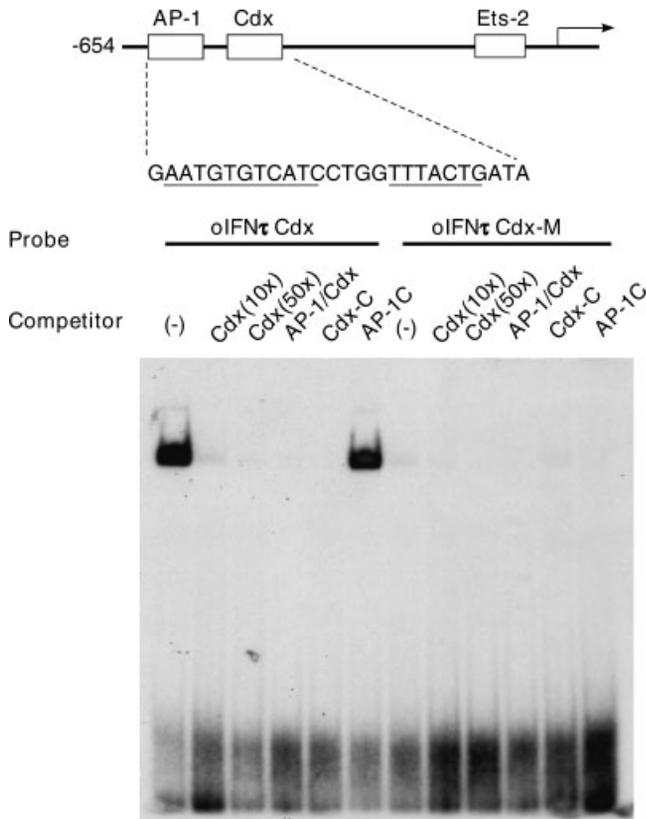


Fig. 5. Electrophoretic mobility shift assay (EMSA) analysis on the enhancer region containing Cdx binding site. Upper: The sequences of oIFN τ -o10's upstream region examined through EMSA. Lower: Binding of nuclear protein to the sequences. Binding of nuclear protein extract from JEG3 cells to γ -³²P labeled-oligonucleotide probes was examined. Specificities of the shifted bands were examined through the addition of molar excess nonlabeled oligonucleotide of the same sequences (Cdx, 10- or 50-fold), Cdx/AP-1 (50-fold), Cdx consensus (Cdx-C, 50-fold), and AP-1 consensus (AP-1C, 50-fold) sequences. A representative EMSA gel from three independent analyses is shown.

transiently transfected into choriocarcinoma JAR cells (Ezashi et al., 1998; Ezashi et al., 2001). However, trophoblast cell-specific expression of the IFN τ gene has not been definitively proven. This study represents the first experiments that utilized a trophoblast lineage-specific transcription factor, Cdx2, to study the molecular mechanisms regulating IFN τ gene expression. Observation in which the transient transfection studies using JEG3 cells showed strong luciferase activity in this study agreed with those of Yamaguchi et al. (1999, 2001). Deletion analysis of the oIFN τ gene's upstream region with transcription factor expression constructs revealed the importance of a Cdx2 binding site (-581 to -575) in the enhancer region. Yamaguchi et al. (1999) had postulated the existence of another transcription factor that would bind to the region next to the AP-1 binding site in the enhancer region. Within the region next to the AP-1 site, there are numerous binding sites for a transcription factor with zinc finger DNA-binding domain, GATA; however, co-transfection of GATA1 or GATA2 expression plasmid did not increase oIFN τ gene

transcription when examined in JEG3 cells (Yamaguchi et al., 1999). This postulate was supported by EMSA analysis, demonstrating the specific binding of JEG3 nuclear proteins to the Cdx recognition sequence located next to the AP-1 site in the oIFN τ enhancer region. A protein bound to the Cdx2 probe appeared to be Cdx2, because the binding was abolished with excess unlabeled oIFN τ 's Cdx2 or AP-1/Cdx2, or consensus Cdx2 sequence, but not with the consensus AP-1 sequence.

DNA transcription is regulated in complex manners, with numerous factors affecting a single gene transcription or a single transcription factor affecting transcription of many genes. In addition to relative quantity, activation or inactivation of transcription factors (e.g., via phosphorylation) may also be important in the regulation of IFN τ gene transcription. Mutation to the phosphorylation site at serine 60 residue within the Cdx2 activation domain reduced the effectiveness of Cdx on oIFN τ gene transcription. However, a loss of phosphorylation to the serine 60 residue did not abolish IFN τ -reporter transcription, suggesting that in addition to the serine 60, there are more phosphorylation sites on this protein, which could contribute to the upregulation of IFN τ -reporter transcription. Expression of Cdx along with c-jun and Ets-2 in NIH3T3 cells did not increase oIFN τ gene transcription to the degree exhibited in JEG3 cells, suggesting that Cdx alone is not sufficient to convert NIH3T3 cells to trophoblast-like cells. Considering that at an early embryonic stage Cdx2 expression was found only in trophoblast cells, and mutation to the Cdx2 gene resulted in embryonic lethality (Chawengsaksophak et al., 1997), activation of AP-1/Ets-2, Ras/MAPK (Ezashi and Roberts, 2004), unidentified factor(s), and/or their interactions with Cdx2 could be key elements that determine cell-specific activation of IFN τ gene expression. In fact, CREB binding protein (CBP), known as a transcriptional co-activator, was suggested to activate the oIFN τ gene transcription by connecting AP-1 and Ets-2 to the basal transcription apparatus (Xu et al., 2003). Another potential transcriptional regulator of IFN τ could be Oct-4, previously demonstrated by Ezashi et al. (2001). It was shown that Cdx2 turns off Oct-4 in trophectodermal cells (Strumpf et al., 2005), which would derepress IFN τ , resulting in upregulation of IFN τ with c-jun, Ets-2, and Cdx2. In addition to potential involvement of a co-activator and/or co-repressor, epigenetic regulation such as DNA methylation, histone acetylation, and related effects on chromatin structure are undoubtedly involved in oIFN τ gene transcription.

In addition to the proximal promoter located within -120 bases of IFN τ 's upstream region, whether the distal enhancer region (-654 to -551 bases) is required for IFN τ gene transcription has been debated (Yamaguchi et al., 1999, 2001; Ezashi and Roberts, 2004). Ezashi and Roberts (2004) have emphasized that the distal enhancer region of IFN τ genes is poorly conserved across ruminant species and is not even present in the bovine gene. The gene examined in our laboratory had been described by Nephew et al. (1993), and the

nucleotide sequence of the gene is identical to the one independently isolated and characterized by Charlier et al. (1991). Our experiments performed here and earlier (Yamaguchi et al., 1999, 2001; Matsuda et al., 2004) indicated that although the proximal promoter is undoubtedly required, the far upstream of the distal enhancer region is also necessary for the potentiation of *oIFN τ* gene transcription. Furthermore, AP-1-like and Cdx binding sites are also found in the proximal region of *oIFN τ* gene. However, luciferase activity from the constructs with mutated AP-1 like and/or Cdx binding sites of the proximal promoter region was similar to that of the -654-*oIFN τ* -Luc reporter construct (K. Imakawa, unpublished observation). Therefore, the AP-1 and Cdx binding sites functional for *oIFN τ* expression appear to be located in the distal enhancer region.

A cytokine, fibroblast growth factor (FGF), signaling is required for cell divisions leading to the blastocyst (Chai et al., 1998) and for proliferation of both trophoblast and primitive endoderm at the blastocyst stage (Rappolee et al., 1994). Ablation of Oct-4 results in the generation of only trophoblast cells (Nichols et al., 1998). In mice, trophoblast stem cell lines cannot be obtained from Cdx2 $-/-$ embryos, although homozygous ES cells are obtained (Ralston and Rossant, 2005). In addition to FGF, trophoblast formation thus requires loss of Oct-4 and gain of Cdx2 expression. Furthermore, overexpression of Oct-4 in transient transfection assays reduced the degree of IFN τ gene transcription (Ezashi et al., 2001; K. Imakawa, unpublished observations). It is not clear whether there is a direct network between FGF, Oct-4, and Cdx2 in the developing conceptuses of ruminant ungulates. It is still possible that Oct-4, Cdx2, FGF, and possibly other factor(s) are involved in determining temporal and spatial expression of IFN τ genes by the trophoblast. Moreover, it has been debated whether conceptus IFN τ production is regulated or influenced by endometrial factors. There is some indication that conceptus IFN τ expression is influenced by endometrial cytokines (Ko et al., 1991; Imakawa et al., 1993; Ezashi and Roberts, 2004), although some results have been disputed. Therefore, further investigations are required to determine whether the expression of conceptus transcription factors is regulated by maternal factors, and to elucidate molecular mechanisms by which IFN τ is expressed in a temporal and spatial manner.

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REFERENCES

- Alexenko AP, Ealy AD, Bixby JA, Roberts RM. 2000. A classification for the interferon- τ . *Interferon Cytokine Res* 20:817–822.
- Ashworth CJ, Bazer FW. 1989. Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone. *Biol Reprod* 40:425–433.
- Beck F, Erler T, Russell A, James R. 1995. Expression of Cdx-2 in the mouse embryo and placenta: Possible role in patterning the extra-embryonic membranes. *Dev Dyn* 204:219–227.
- Chai N, Patel Y, Jacobson K, McMahon A, Rappolee DA. 1998. FGF is an essential regulator of the fifth cell division in preimplantation mouse embryos. *Dev Biol* 198:105–115.
- Charlier M, Hue D, Boissard M, Martal J, Gaye P. 1991. Cloning and structural analysis of two distinct families of ovine interferon-alpha genes encoding functional class II and trophoblast (*oTP*) alpha-interferons. *Mol Cell Endocrinol* 76:161–171.
- Charpigny G, Reinaud P, Huet JC, Guillomot M, Charlier M, Pernellet JC, Martal J. 1988. High homology between a trophoblastic protein (trophoblastin) isolated from ovine embryo and alpha-interferons. *FEBS Lett* 228:12–16.
- Chawengsaksophak K, James R, Hammond VE, Kontgen F, Beck F. 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* 386:84–87.
- Demmers KJ, Derecka K, Flint A. 2001. Trophoblast interferon and pregnancy. *Reproduction* 121:41–49.
- Ealy AD, Larson SF, Liu L, Alexenko AP, Winkelman GL, Kubisch HM, Bixby JA, Roberts RM. 2001. Polymorphic forms of expressed bovine interferon- τ genes: Relative transcript abundance during early placental development, promoter sequences of genes and biological activity of protein products. *Endocrinology* 142:2906–2915.
- Ezashi T, Roberts RM. 2004. Regulation of interferon- τ (IFN τ) gene promoters by growth factors that target the Ets-2 composite enhancer: A possible model for maternal control of IFN- τ production by the conceptus during early pregnancy. *Endocrinology* 145:4452–4460.
- Ezashi T, Ealy AD, Ostrowski MC, Roberts RM. 1998. Control of interferon- τ gene expression by Ets-2. *Proc Natl Acad Sci USA* 95:7882–7887.
- Ezashi T, Ghosh D, Roberts RM. 2001. Repression of Ets-2 induced transactivation of the interferon- τ promoter by Oct-4. *Mol Cell Biol* 21:7883–7891.
- Farin CE, Imakawa K, Roberts RM. 1989. *In situ* localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. *Mol Endocrinol* 3:1099–1107.
- Farin CE, Cross JC, Tindle NA, Murphy CN, Farin PW, Roberts RM. 1991. Induction of trophoblastic interferon expression in ovine blastocysts after treatment with double-stranded RNA. *Interferon Res* 11:151–157.
- Godkin JD, Bazer FW, Moffatt J, Sessions F, Roberts RM. 1982. Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts at day 13–21. *J Reprod Fertil* 65:141–150.
- Guillomot M, Michel C, Gaye P, Charlier M, Trojan J, Martal J. 1990. Cellular localization of an embryonic interferon, ovine trophoblastin and its mRNA in sheep embryos during early pregnancy. *Biol Cell* 68:205–211.
- Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM. 1987. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophoblast. *Nature* 330:377–379.
- Imakawa K, Hansen TR, Malathy PV, Anthony RV, Polites HG, Marotti KR, Roberts RM. 1989. Molecular cloning and characterization of complementary deoxyribonucleic acids corresponding to bovine trophoblast protein-1: A comparison with ovine trophoblast protein-1 and bovine interferon- α II. *Mol Endocrinol* 3:127–139.
- Imakawa K, Helmer SD, Nephew KP, Meka CS, Christenson RK. 1993. A novel role for GM-CSF: Enhancement of pregnancy specific interferon production, ovine trophoblast protein-1. *Endocrinology* 132:1869–1871.

- Imakawa K, Tamura K, Lee RS, Ji Y, Kogo H, Sakai S, Christenson RK. 2002. Temporal expression of type I interferon receptor in the peri-implantation ovine extra-embryonic membranes: Demonstration that human IFN α can bind to this receptor. *Endocrine J* 49:195–205.
- Ko Y, Lee CY, Ott TL, Davis MA, Simmen RC, Bazer FW, Simmen FA. 1991. Insulin-like growth factors in sheep uterine fluids: Concentrations and relationship to ovine trophoblast protein-1 production during early pregnancy. *Biol Reprod* 45:135–142.
- Leaman DW, Roberts RM. 1992. Genes for the trophoblast interferons in sheep, goat, and musk ox and distribution of related genes among mammals. *J Interferon Res* 12:1–11.
- Matsuda F, Xu N, Kijima S, Tachi C, Christenson RK, Sakai S, Imakawa K. 2004. Analysis of transcriptional control elements in the 5'-upstream region of ovine interferon- τ gene using feeder-independent caprine trophoblast cell line, HTS-1. *Placenta* 25:166–175.
- Nephew KP, Whaley AE, Christenson RK, Imakawa K. 1993. Differential expression of distinct mRNAs for ovine trophoblast protein-1 and related sheep type I interferons. *Biol Reprod* 48:768–778.
- Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95:379–391.
- Nojima H, Nagaoka K, Christenson RK, Shiota K, Imakawa K. 2004. Increase in DNA methylation downregulates conceptus interferon- τ gene expression. *Mol Reprod Dev* 67:396–405.
- Palmieri SL, Peter W, Hess H, Scholer HR. 1994. Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev Biol* 166:259–267.
- Pestka S, Langer JA, Zoon KC, Samuel CE. 1987. Interferons and their actions. *Annual Rev Biochem* 56:727–777.
- Ralston A, Rossant J. 2005. Genetic regulation of stem cell origins in the mouse embryo. *Clin Genet* 68:106–112.
- Rappolee DA, Basilico C, Patel Y, Werb Z. 1994. Expression and function of FGF-4 in peri-implantation development in mouse embryos. *Development* 120:2259–2269.
- Rings EH, Boudreau F, Taylor JK, Moffett J, Suh ER, Traber PG. 2001. Phosphorylation of the serine 60 residue within the Cdx2 activation domain mediates its transactivation capacity. *Gastroenterology* 121:1437–1450.
- Roberts RM, Cross JC, Leaman DW. 1992. Interferons as hormones of pregnancy. *Endocr Rev* 13:432–452.
- Ryan AM, Womack JE. 1993. Type I interferon genes in cattle: Restriction fragment length polymorphism, gene numbers and physical organization on bovine chromosome 8. *Anim Genet* 24:9–16.
- Scholer HR, Balling R, Hatzopoulos AK, Suzuki N, Gruss P. 1989. Octomer binding proteins confer transcriptional activity in early mouse embryogenesis. *EMBO J* 8:2551–2557.
- Stewart HJ, McCann SH, Barker PJ, Lee KE, Lamming GE, Flint AP. 1987. Interferon sequence homology and receptor binding activity of ovine trophoblast antiluteolytic protein. *J Endocrinol* 115:R13–R15.
- Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J. 2005. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* 132:2093–2102.
- Suh E, Chen L, Taylor J, Traber PG. 1994. A homeodomain protein related to caudal regulates intestine-specific gene transcription. *Mol Cell Biol* 14:7340–7351.
- Vallet JL, Bazer FW, Fliss MF, Thatcher WW. 1988. Effect of ovine secretory proteins and purified ovine trophoblast protein-1 on interoestrous interval and plasma concentrations of prostaglandins F-2 α and E and of 13,14-dihydro-15-keto prostaglandin F-2 α in cyclic ewes. *J Reprod Fertil* 84:493–504.
- Wakiya K, Begue A, Stehelin D, Shibuya M. 1996. A cAMP response element and an Ets motif are involved in the transcriptional regulation of flt-1 tyrosine kinase (vascular endothelial growth factor receptor 1) gene. *J Biol Chem* 271:30823–30828.
- Xu N, Takahashi Y, Matsuda F, Sakai S, Christenson RK, Imakawa K. 2003. Coactivator CBP in the regulation of conceptus IFN τ gene transcription. *Mol Reprod Dev* 65:23–29.
- Yamaguchi H, Ikeda Y, Moreno JI, Katsumura M, Miyazawa T, Takahashi E, Imakawa K, Sakai S, Christenson RK. 1999. Identification of a functional transcriptional factor AP1 site in the sheep interferon- τ gene that mediates a response to PMA in JEG3 cells. *Biochem J* 340:767–773.
- Yamaguchi H, Nagaoka K, Imakawa K, Sakai S, Christenson RK. 2001. Enhancer regions of ovine interferon- τ gene that confer PMA response or cell type specific transcription. *Mol Cell Endocrinol* 173:147–155.