Identification of an Enhancer That Controls Up-regulation of Fibronectin during Differentiation of Embryonic Stem Cells into Extraembryonic Endoderm*

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The extraembryonic endoderm is derived from inner cell mass cells of the blastocyst during early mouse embryogenesis. Formation of the extraembryonic endoderm, which later contributes to the yolk sac, appears to be a prerequisite for subsequent differentiation of the inner cell mass. While embryonic stem cells can be induced to differentiate into extraembryonic endoderm cells in vitro, the molecular mechanisms underlying this process are poorly understood. We used a promoter trap approach to search for genes that are expressed in embryonic stem cells and are highly up-regulated during differentiation to the extraembryonic endoderm fate. We showed that fibronectin fits this expression profile. Moreover we identified an enhancer in the 12th intron of the fibronectin locus that recapitulated the endogenous pattern of fibronectin expression. This enhancer carries Sox protein-binding sequences, and our analysis demonstrated that Sox7 and Sox17, which are highly expressed in the extraembryonic endoderm, were involved in enhancer activity.

Understanding cell fate commitment and differentiation at the molecular level is a major area of interest in the field of developmental biology. The first overt differentiation step in mouse development occurs at about 3.5 days postcoitum when the blastomeres segregate into two distinct cell lineages to form the blastocyst stage embryo (1). The trophectoderm, which contributes exclusively to extraembryonic tissues, forms the outer population of cells, while the inner cell mass (ICM),1 which is competent to differentiate into all embryonic tissue types, comprises the inner population of cells. Although extraembryonic tissues such as the placenta, amnion, and yolk sac are not part of the fetus proper, these tissues play pivotal roles in development, including nourishment and protection of the fetus within the uterus as well as embryonic axis formation by extraembryonic endoderm cells at early stages. While substantial portions of extraembryonic tissues are derived from the trophectoderm, the ICM also contributes significantly to extraembryonic tissues. For example, the extraembryonic or primitive endoderm, which gives rise to portions of the yolk sac, is derived from the ICM. This differentiation step can be mimicked in vitro by culturing ICM-derived embryonic stem (ES) cells in the absence of a cytokine such as leukemia inhibitory factor (1, 2), allowing systematic analysis of the gene regulation involved in this process. Recently Niwa et al. (3) demonstrated that although the major role of Oct-3/4 in ES cells is to maintain pluripotency, elevated Oct-3/4 levels provide a key signal for differentiation of ES cells into extraembryonic endoderm. However, the molecular mechanisms underlying this differentiation remain unknown at present.

To investigate the molecular basis of extraembryonic endoderm differentiation, we generated a promoter-trapped mouse ES clone in which expression of the pGTIRESH/geopA reporter gene (4) is highly up-regulated during differentiation of ES cells into extraembryonic endoderm. The reporter gene integrated into the fibronectin locus, which was previously reported to be expressed in the extraembryonic endoderm (5–8). Wartiovaara et al. (6) have shown that fibronectin is first detected between the cells of the inner cell mass in late blastocyst stage embryos with the onset of expression coinciding with the appearance of extraembryonic endoderm cells. However, since the trophectoderm also produces fibronectin, it is unclear which cell type is the major source of fibronectin in Reichert’s membrane. Our data clearly demonstrated that it is the extraembryonic endoderm, and not the trophectoderm, that represents the major source of fibronectin production. Moreover we identified a regulatory enhancer in intron 12 of the fibronectin gene that, when linked to a reporter gene, recapitulated endogenous expression during differentiation of ES cells to extraembryonic endoderm. We also showed that Sox7 and Sox17, whose expression is restricted to the extraembryonic endoderm in high mobility group; E14, embryonic day 14; EC, embryonic carcinoma; Fec, fibronectin enhancer involved in extraembryonic endoderm cells; PE, parietal endoderm; VE, visceral endoderm; RA, retinoic acid.

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1 The abbreviations used are: ICM, inner cell mass; ES, embryonic stem; BAC, bacterial artificial chromosome; tk, thymidine kinase; IRES, internal ribosomal entry site; EF-1, elongation factor 1; HMG, high mobility group; E14, embryonic day 14; EC, embryonic carcinoma; Fec, fibronectin enhancer involved in extraembryonic endoderm cells; PE, parietal endoderm; VE, visceral endoderm; RA, retinoic acid.
mouse early embryos (9–11), were able to transcriptionally activate this enhancer.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The pGTIRES\*ggpA reporter gene used for the promoter trap has been described by Mountford et al. (4). To construct genomic libraries carrying the fibronectin gene, a bacterial artificial chromosome (BAC) (GenBank\textsuperscript{TM} accession number AC091450) carrying the entire fibronectin gene (67 kb) as well as 5’ (86 kb) and 3’ (51 kb) flanking sequences was prepared and digested with Sau3A1 or HpaII. Subsequently Sau3A1-digested DNA was subcloned into the BamHI site of the tk-puro-Venus reporter plasmid in which the tk promoter (12) and CDNA encoding for a fusion protein (13) of puromycin-detoxifying enzyme and Venus fluorescent protein (14) were subcloned into the BamHI/SpeI site and SpeI/NotI sites of Bluescript II KS+, respectively. HpaII-digested DNA was inserted into the ClaI site of the vector. To construct the Feec1-tk-\(\beta\)-geo reporter gene, the splice acceptor domain of the Engrailed gene and the internal ribosomal entry site (IRES) sequence were removed from pGTIRES\*ggpA reporter plasmid (4), and a fibronectin enhancer involved in extraembryonic endoderm (Feec1) 1 DNA fragment obtained by PCR was subcloned as a 5′ flanking fragment of the tk promoter. To generate the puro-Venus reporter constructs bearing wild type and deletion mutants of Feec1, the Sail/BamHI PCR products shown in Fig. 5B were individually subcloned into the puro-Venus reporter plasmid together with the tk promoter. For construction of puro-Venus reporter genes bearing wild type, nucleotide substitution mutants of Feec1 shown in Fig. 7A, or canonical B1 repetitive sequence, we used our previously published method for amplifying oligonucleotides that carry the desired sequence (from 20 to 30 nucleotides) of the target DNA regions. For nucleotide substitution mutants, underlined nucleotides in Fig. 7A were converted to non-complementary ones, i.e. G, A, T, and C were changed to T, C, G, and A, respectively. Construction of the EF-1-puro-Venus reporter plasmid was described previously (13). For constructing Sox expression vectors, the entire coding regions of Sox2, Sox7, and Sox17 were recovered from fragments by PCR in which Asp718 sites were mutagenized at the initiating ATG codons. These were subcloned into an expression vector bearing a FLAG tag sequence, which was generated by introducing specific oligonucleotides into the pH\(\text{Apr}1\)-expression vector (16). To construct the Sox17 HMG-Engrailed expression vector, the amino-terminal portion of Sox17 (amino acids 1–164) bearing the HMG domain (17) and the Drosophila Engrailed repressor domain (amino acids 2–298) (18) were amplified by PCR and subcloned together into the pH\(\text{Apr}1\) expression vector. To construct the Feec1-tk-Luc reporter plasmid, the Feec1 regulatory region was amplified by PCR as an Asp718/XhoI fragment and was subcloned into the tk-Luc reporter plasmid.

**Culture and Transfection of ES Cells and Other Cells**—E14 ES cells were cultured as described previously (19). The pGTIRES\*ggpA reporter gene was introduced into ES cells by electroporation according to the method described by Thomas and Capecchi (20). After selection with G418, the drug-resistant clones were picked and expanded. These cells were stained for LacZ to examine the level and pattern of reporter gene expression. ZHTc4 and ZHTc6 ES cells were cultured as described by Niwa et al. (3) and were differentiated into trophoderm and extraembryonic endoderm, respectively. F9 embryonic carcinoma (EC) cells and COS cells were cultured as described previously (21). Introduction of plasmid DNAs into ZHTc4, ZHTc6, and F9 cells was done by lipofection using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen), while the conventional calcium phosphate method was used to introduce DNA into COS cells.

**In Vitro Differentiation of ES Cells and F9 EC Cells**—E14 ES cells were induced to differentiate in vitro essentially as described by Robertson (2). In brief, embryoid bodies were formed on bacteria grade plates in the absence of leukemia inhibitory factor for 4 days. These cells were further cultured to form cystic embryo bodies or replated on tissue culture grade plates at the absence of leukemia inhibitory factor for 4 days. F9 EC cells were induced to differentiate with 0.5 \(\mu\)M all-trans-retinoic acid alone or together with 1 mM dibutyryl cAMP as described by Hogan et al. (11).

**Determination of Integration Site of pGTIRES\*ggpA Reporter Gene**—Genomic DNA was prepared from ES cell clone 183, which has extraembryonic endoderm-specific reporter gene expression (see text for details). The DNA was cut with NcoI, which is present in the reporter gene (4), and BspHI, which is present in the chromosomal integration site of the reporter gene by Southern blot analysis (data not shown, see Fig. 3A for details). The genomic DNA was then circularized with T4 DNA ligase and used as template for PCR to identify the junction between the reporter gene and chromosomal DNA. The primers used for PCR were as follows: 5’-\(\text{GATGTAAGCTTGCCACAACCATG-3}'\) and 5’-\(\text{ACCAGCCACGATCATGCTTCT-3}'\). The PCR product was directly used as a sequencing template.

**RNA Preparation and RNase Mapping Analysis**—RNA was prepared from ZHTc4 and ZHTc6 ES cells using the RNeasy midikit from Qiagen. RNA was also prepared from undifferentiated as well as differentiated F9 cells and was used to examine gene expression levels by RNase protection as described previously (19). Probes to the following sequences were used: fibronectin, nucleotides 4081–4291; hepatocyte nuclear factor 3β, nucleotides 377–604; Rex-1, nucleotides 75–352; Mash2, nucleotides 190–408; and \(\beta\)-actin, nucleotides 903–1023.

**Reverse Transcription-PCR Analysis**—RNA was prepared as above from wild-type E14 ES cells and ES cell clone 183 (see text for details) and used to produce first strand complementary DNA by Powerscript reverse transcriptase from BD Biosciences. The cDNA was used for PCR analysis to detect the presence of wild-type fibronectin RNA and fibronectin fused to IRES-\(\beta\)-geo RNA derived from pGTIRES\*ggpA. The following primers were used: for wild-type fibronectin RNA: Primer A, 5’-\(\text{CTCCAGCGGTTGGAACACTTACCCATCC-3}'\); and Primer B, 5’-\(\text{GATCCAGTGACGAGATTCTTCACAGGAGG-3}'\); for fibronectin RNA fused to IRES-\(\beta\)-geo RNA: Primer C, 5’-\(\text{AACCGTCAGCTGAGTCTACTGCTTC-3}'\); and Primer D, 5’-\(\text{TTTCTTCAAGTGTACAGTTCGCCGCTTACCG-3}'\).

**Reporter Assay with puro-Venus Vector**—The puro-Venus reporter gene encodes a fusion protein of puromycin-detoxifying enzyme and Venus fluorescent protein (for details see Ref. 13). The reporter can be used for both transient and stable transfection assays with puromycin detoxifying activity used for the stable transformant analysis as shown in Figs. 5, 6B, and 7, while transient transfection analysis can be done by monitoring the level of Venus fluorescence protein as shown in Fig. 6D.

**Identification of a Fibronectin Regulatory Region That Functions in the Extraembryonic Endoderm**—Puro-Venus reporter plasmid libraries were constructed by inserting a small piece of genomic DNA from the fibronectin locus. These libraries were then introduced by lipofection into ZHTc6 cells that had been differentiated into extraembryonic endoderm, and puromycin-resistant colonies were obtained. After 1 week of expansion, genomic DNA was recovered from these cells, and PCR was performed with the reverse primer from the Bluescript II KS+ vector sequence and a primer recognizing a portion of the tk promoter sequence. PCR products derived from HpaII and Sau3AI libraries were digested with Sail/BamHI and Sail/Sau3AI, respectively, and subcloned into the tk-puro-Venus reporter gene. The constructed plasmids were again transfected into the differentiated ZHTc6 cells as above to examine the enhancer activities of the subcloned PCR fragments.

**Luciferase Reporter Assay**—COS cells in 10-cm dishes were transfected by the calcium phosphate method as described previously (21) with 2 \(\mu\)g of the Feec1-tk-Luc reporter plasmid and the indicated amounts of expression vectors. The total amount of DNA was adjusted to 12 \(\mu\)g with pUC18. At 48 h post-transfection, the transcription level was determined by the dual luciferase method according to the manufacturer’s instructions (Promega).

** Gel Shift Assay**—The Sox7 or Sox17 expression vector or empty vector was introduced into COS cells by the calcium phosphate method as described above. Preparation of whole cell extracts and subsequent gel shift analysis were done as described previously (21). The sequences used for the analysis were as follows: wild type, 5’-\(\text{TGGTGTCCAGACGCCAGGCTTAAACAAGGAGAACTCCCTCTCCAGAATCCACCC-3}'\); mutant, 5’-\(\text{TGGTGTCCAGACGCCAGGCTTAAACAAGGAGAACTCCCTCTCCAGAATCCACCC-3}'\). The underlined portion and bold portion indicate the wild-type and mutated Sox-binding sequence, respectively. The probe sequences represent part of the Feec1 regulatory sequence and contain two potential transcription factor binding sites (\(\gamma\) and \(\varepsilon\)) in addition to the Sox binding site (\(\delta\)). However, the \(\gamma\) and \(\varepsilon\) sites were mutated to simplify the analysis.

**RESULTS**

**Identification of a Gene Whose Expression Level Is Highly Up-regulated during Differentiation of ES Cells Into Extraembryonic Endoderm Cells**—To elucidate the molecular mechanism of extraembryonic endoderm differentiation from ES cells, we searched for genes whose expression is highly up-regulated during this process. To this end, we randomly integrated the pGTIRES\*ggpA reporter gene encoding a fusion protein of \(\beta\)-galactosidase and neomycin-detoxifying enzyme
To determine whether the endogenous expression of fibronectin is extraembryonic endoderm-specific, we examined expression in two specialized ES cell lines (ZHTc6 and ZHBTc4) (3) and their differentiated derivatives. ZHTc6 ES cells can be maintained in a pluripotent state in the presence of tetracycline, and withdrawal of tetracycline from the culture medium leads to extraembryonic endoderm differentiation. In contrast, ZHBTc4 ES cells are maintained in a pluripotent state in the absence of tetracycline, and addition of tetracycline results in dedifferentiation to trophectoderm fate. We prepared RNA from these cells cultured in the presence or absence of tetracycline and performed RNase protection assays. As shown in Fig. 4 when cultured in the absence of tetracycline, fibronectin was indeed highly expressed in ZHTc6 cells, which adopt extraembryonic endoderm fate. We also examined the expression levels of Rex-1 (22), hepatocyte nuclear factor 3β (23), and Mash2 (24) and confirmed that pluripotent/differentiated states of these ES cells are, as expected, regulated by tetracycline.

Identification of an Enhancer Involved in Fibronectin Gene Expression in Extraembryonic Endodermal Cells—To identify the regulatory element responsible for extraembryonic endoderm expression of fibronectin, we first digested BAC DNA bearing the entire fibronectin gene as well as 5' and 3' flanking regions into small fragments using the 4-base cutter Sau3AI or HpaII. The digested DNA was inserted upstream of the tk promoter in the tk-puro-Venus reporter plasmid to generate plasmid libraries (for details see “Experimental Procedures”). These plasmid libraries were then introduced by lipofection into ZHTc6 cells, which had been cultured in the absence of tetracycline, and puromycin-resistant colonies were obtained. The BAC DNA was recovered by PCR amplification of genomic DNA from 84 independent puromycin-resistant colonies. These fragments were again individually inserted upstream of the tk promoter of the reporter plasmid and introduced into ZHTc6 cells, and puromycin-resistant colonies were obtained. In most cases, an equivalent number of puromycin-resistant colonies was obtained with BAC-transfected and control tk-puro-Venus reporter plasmid-transfected cells, indicating that the original puromycin resistance observed from the primary screen was not due to the inserted BAC DNA but was instead dependent on the site of reporter gene integration. However, in one case, significantly more resistant colonies were obtained (Fig. 5A), suggesting that the BAC DNA inserted in the reporter plasmid was involved in producing the resistant colonies. Sequencing analysis revealed that the BAC DNA carries a portion of intron 12 (1568 bp) of the fibronectin gene, and preliminary characterization demonstrated that this DNA fragment exerted its activity rather specifically in extraembryonic ectodermal cells (data not shown, but see below for details). We next delineated the regulatory region using a series of deletion mutants shown in Fig. 5B. We characterized their enhancer activities by count-
ing the number of puromycin-resistant stable colonies, and these analyses allowed us to narrow down the regulatory region to 144 bp (the sequence is shown in Fig. 7A), and we termed this regulatory region Feec1.

The Feec1 Enhancer Drives Expression Specifically in Extraembryonic Endoderm Cells—Next we examined whether the Feec1 enhancer can drive expression specifically in extraembryonic endoderm cells. We constructed a tk-β-geo reporter plasmid carrying the Feec1 enhancer and introduced it into ES cells by electroporation along with pSV2Neo since Feec1 alone might not be strong enough to produce G418-resistant colonies when the ES cells are in the pluripotent state. We obtained four independent ES cell clones in which both Feec1-tk-β-geo and the pSV2Neo reporter gene integrated into the genome. These clones were then allowed to differentiate, and reporter gene expression was examined at day 8 following replating on tissue culture dishes. We found that in all cases, LacZ reporter expression was detected in cells derived from outer layer cells but not in pluripotential inner cells (two representative examples are shown in Fig. 6D), although the signal was not as strong as that shown in Fig. 2D.

Next we examined the activity of the Feec1 regulatory region in ZHTc6 and ZHBTc4 cells using the Feec1-puro-Venus reporter plasmid and found that, as expected, the Feec1 enhancer showed strong activity in ZHTc6 cells that had differentiated into endoderm-like cells (Fig. 6B). However, weaker activity was observed for Feec1 in ZHBTc4 ES cells that were maintained in pluripotent state.
Characterization of fibronectin expression in pluripotent ES cells and their differentiated derivatives. ZHTc6 and ZHTtc4 ES cells were cultured to maintain their pluripotent state or induced to differentiate according to Niwa et al. (3). Subsequently RNA was prepared, and levels of gene expression were examined by RNase protection as described under “Experimental Procedures.” For differentiation of ZHTc6 cells, cultures were maintained in the absence of tetracycline for 6 days, while ZHTtc4 cells were induced to differentiate by culturing in the presence of tetracycline for 4 days.

To obtain an independent line of evidence that the Feec1 functions as an extraembryonic endoderm-specific enhancer, we performed similar experiments using F9 EC cells. While extraembryonic endoderm cells are first comprised of primitive endoderm upon segregation from the embryonic ICM cells, the primitive endoderm differentiates further into parietal endoderm (PE) and visceral endoderm (VE) in vivo (1). F9 cells are able to mimic this process in culture when specific inducers are added (1, 25, 26). Indeed VE-like cells can be obtained by culturing F9 EC cells with retinoic acid (RA) and dibutyryl cAMP converts them to PE-like cells. We therefore used differentiated F9 EC cells to examine the specificity of the Feec1 regulatory region in PE- and VE-like cells. First we examined whether the endogenous expression levels of fibronectin were up-regulated in these differentiation-induced F9 EC cells. As shown in Fig. 6C, fibronectin mRNA levels were highly elevated in both VE- and PE-like F9 cells, although expression in VE-like cells appeared to be down-regulated at later stages of differentiation. Next we introduced the Feec1-tk-puro-Venus reporter plasmid into F9 cells and then induced differentiation into PE- or VE-like cells as above or else maintained them in an undifferentiated state. At 48 h post-transfection, we detected Venus fluorescence in both PE- and VE-like cells, although the Feec1 enhancer appeared to function much more efficiently in PE-like cells than in VE-like cells (Fig. 6D). We also noted that the Feec1 regulatory region had weak but noticeable activity even in undifferentiated F9 cells. Indeed the reporter plasmid with the Feec1 regulatory region had weak but noticeable activity even in undifferentiated F9 cells. It is noteworthy that this SRY sequence does not comprise part of the repetitive sequence, and repetitive sequences lacking the SRY motif did not show any noticeable activity (Fig. 7B), indicating that the extraembryonic endoderm-specific enhancer is not a general feature of repetitive sequences but is restricted to the Feec1 sequence bearing the SRY element.

It was previously shown that the SRY proteins Sox7 and Sox17 are abundantly expressed in the PE as well as the VE (9–11), Therefore, it seemed possible that these factors mediate Feec1 enhancer activity in extraembryonic endoderm cells. To test this possibility, we first examined whether the SRY element could serve as a potential binding site for Sox7, Sox17, and Sox2. Sox2, Sox7, and Sox17 expression vectors as well as an empty control vector were individually transfected into COS cells, and whole cell extracts were analyzed by a gel shift assay using a DNA probe recognizing this SRY motif (for details, see “Experimental Procedures”). We found that all of exogenously expressed Sox factors produced specific bands (Fig. 8A), although the signal obtained for Sox7 was relatively weak. No such specific bands were obtained with the SRY mutant probe (data not shown). Moreover we confirmed that specific signals observed were due to the binding of Sox proteins to the probe by using poly(dI·dC)/poly(dI·dC) as a nonspecific competitor (data not shown). These results indicate that the SRY sequence is indeed able to serve as a binding site for Sox2, Sox7, and Sox17.

Next we transiently transfected the Feec1 tk luciferase reporter plasmid along with Sox expression vectors into undifferentiated F9 EC cells in which the expression of Sox7 and Sox17 is hardly detectable. However, we could not detect an appreciable effect of these factors on the Feec1 enhancer (Fig. 8B). Since Sox2 is highly expressed in these cells (21, 28), it is possible that Sox2 abrogates the effect of Sox7 and Sox17 on the Feec1 regulatory region. We differentiated F9 cells in the presence of RA and dibutyryl cAMP, which results in very low levels of Sox2 expression. Consistent with the data shown in Fig. 6D, we found that Feec1 by itself showed strong transcriptional activation in these differentiated F9 cells (Fig. 8B) probably due to endogenous Sox7 and Sox17. However, introduction of the Sox7 or Sox17 expression vector to the differentiated F9 cells did not further activate but rather attenuated Feec1 activity. We presumed that these results were due to the squelching effect of these Sox factors. Therefore, we transiently transfected COS cells, which do not endogenously express Sox7 or Sox17, with the Sox factor expression vectors and the Feec1-tk-Luc reporter plasmid. Both Sox7 and Sox17 were able to increase the level of transcription significantly in these cells, while Sox2 induced much weaker transcriptional activation (Fig. 8C). The observed
**FIG. 5.** Identification and localization of the Feec1 regulatory region, which is highly active in the extraembryonic endoderm. A, identification of the Feec1 regulatory region as an enhancer that functions in differentiated ZHTc6 cells. Cloning of the Feec1 regulatory region from the tk-puro-Venus reporter plasmid libraries is described under “Experimental Procedures.” For the enhancer activity index, the number of the puromycin-resistant colonies obtained with the control tk-puro-Venus plasmid was arbitrarily set as one, and the -fold induction due to the presence of the regulatory region was calculated. B, localization of the Feec1 regulatory region. Deletion mutants of the Feec1 regulatory region were obtained by PCR and subcloned into the tk-puro-Venus reporter plasmid as described under “Experimental Procedures.” Enhancer activity indexes were calculated as in A.

**FIG. 6.** Functional characterization of the Feec1 regulatory region. A, the Feec1-tk-β-geo reporter gene has the same expression profile in ES cells as ES cell clone 183 during in vitro differentiation. E14 ES cells, in which Feec1-tk-β-geo reporter gene was stably integrated, were selected and subjected to in vitro differentiation. The same procedure used for the cells shown in Fig. 2D was used for these ES cells. B, ZHBTc4 and ZHTc6 ES cells, which were maintained in the pluripotent state and differentiated into extraembryonic endoderm cells, respectively, were transfected using the lipofection method with the indicated reporter plasmids, and puromycin-resistant colonies were obtained. The enhancer activity indexes were calculated as in Fig. 5. C, expression profile of the fibronectin gene during differentiation of F9 cells. Undifferentiated F9 cells were induced to differentiate into VE- and PE-like cells with RA alone and RA plus cAMP as described by Hogan et al. (1). D, Feec1 has stronger activity in PE cells than in VE cells. The Feec1-tk-puro-Venus reporter gene was introduced into undifferentiated F9 cells by lipofection. After 4 h, DNA was washed off, and F9 cells were maintained in the undifferentiated state or induced to differentiate with RA alone or RA plus cAMP. After 48 h of cultivation, puro-Venus protein was visualized under a fluorescence microscope.
activity of Sox7 and Sox17 was Feec1-dependent since no elevation in transcriptional level was detected in experiments using the reporter plasmid that lacks the Feec1. Thus, these results indicate that Sox7 and Sox17 can increase transcriptional activation through the Feec1 enhancer. These results also support the notion that Sox2, which is abundantly expressed in undifferentiated F9 cells, functions as a dominant negative factor when Sox7 or Sox17 are expressed in these cells by transient transfection.

To further examine the effect of Sox7 and Sox17 on fibronectin expression in extraembryonic endoderm cells, we performed loss-of-function type experiments by generating a fusion protein composed of the repressor domain of the Drosophila Engrailed protein and the HMG DNA binding domain of Sox17. Since this fusion protein acted as a strong negative regulator of Sox7- and Sox17-dependent reporter activation in the cotransfection system used in Fig. 8C (data not shown), we transiently transfected F9 cells, which had been treated with RA and dibutyryl cAMP, with the expression vector of fusion protein by lipofection. We did not observe any noticeable effect on endogenous fibronectin gene expression (data not shown), which was likely due to the low transfection efficiency of differentiated F9 cells. Therefore, we decided to use ZHTc6 cells cultured in the absence of tetracycline since the transfection efficiency of ZHTc6 cells is much higher than for differentiated F9 cells. Indeed, as shown in Fig. 8D, about 80% of the ZHTc6 cells became Venus-positive when the cells were transfected with Venus reporter plasmid, which drives Venus expression by the elongation factor (EF)-1 gene promoter. Since this high transfection efficiency could allow for examination of the effect of the fusion protein even in the presence of non-transfected cells, we prepared RNA from ZHTc6 cells transfected with the Sox17 HMG-Engrailed fusion protein expression vector or empty vector, and this RNA was used for RNase protection analysis. The expression level of fibronectin was indeed reduced when the Sox17-Engrailed fusion protein was expressed, whereas no noticeable effect was evident on the expression level of β-actin (Fig. 8E). These results indicate that the Sox17-Engrailed fusion protein represses expression of the fibronectin gene. Thus, these results support a model in which Sox7 and Sox17 play a significant role in gene regulation in the extraembryonic endoderm.

DISCUSSION

Fibronectin is an extracellular matrix protein that has been shown to play an important role in many developmental processes (29). Here we identified fibronectin as a gene whose expression level is highly up-regulated during differentiation of ES cells to extraembryonic endoderm. While induction of fibronectin expression during differentiation of human EC cells (NEC14) was demonstrated previously (30), these studies did not examine the cell type of differentiated cells, so it was not known whether these fibronectin-expressing cells represented extraembryonic endoderm. In addition to characterization of fibronectin expression profile, our analysis was extended to the identification of a regulatory enhancer, termed Feec1, which recapitulates endogenous fibronectin expression during the differentiation of ES cells to extraembryonic endoderm. Indeed Feec1 was able to activate the same expression pattern as clone 183 in which pGTIRESβgeoA integrated into the fibronectin locus in E14 ES cells. Furthermore experiments with ZHBTc4 and ZHTc6 ES cells suggested that the Feec1 enhancer functions more efficiently in extraembryonic endoderm than in pluripotent ES cells. Our data also established that the Feec1 enhancer functions in PE and VE cells, although activity in VE cells is much lower than in PE cells.

The Feec1 enhancer is located in the middle of a stretch of repetitive sequence in the mouse genome. However, the activity observed with the Feec1 enhancer is not a general feature of the repetitive sequence but specific to Feec1. Sequence analysis showed that a Sox-binding sequence is located in the Feec1 enhancer, and our mutagenesis analysis demonstrated that this site is absolutely required for the enhancer activity of the Feec1. Among the Sox genes, Sox7 and Sox17 are highly expressed in extraembryonic endoderm (9–11), whereas their expression is barely detectable in undifferentiated F9 cells (10) and pluripotent ES cells.2 In contrast, it is known that Sox2 is abundantly expressed in pluripotent ES cells (31). This suggested that Sox2 and Sox17 were involved in activation of the Feec1 enhancer in the extraembryonic endoderm. Consistent with this, cotransfection analysis with COS cells that do not endogenously express Sox2, Sox7, and Sox17 revealed that Sox7 and Sox17 increased transcriptional activation through

2 T. Shirai and A. Okuda, unpublished data.
Fig. 8. Sox7 and Sox17 are able to potentiate transcriptional activation through the Feec1 enhancer. A, the SRY site in Feec1 is able to serve as a specific binding site for Sox2, Sox7, and Sox17. COS cells were transfected with Sox2, Sox7, and Sox17 expression vectors or empty vector, and whole cell extracts were prepared as described previously (21). Gel shift analysis was done as described under “Experimental Procedures.” F represents free probe. B, exogenous expression of Sox7 and Sox17 did not lead to significant potentiation of Feec1 activity in F9 cells. Undifferentiated and differentiated F9 cells treated with RA plus dibutyryl cAMP for 48 h were transfected with the Feec1-tk-Luc reporter (2 μg) and varying concentrations of Sox expression vectors as indicated. In addition, an internal control luciferase gene (2 μg) of Rotylenchulus reniformis was also introduced. At 48 h post-transfection, transcriptional levels were determined by the dual luciferase system according to the manufacturer's protocol (Promega). Values obtained with the tk-Luc reporter plasmid bearing no canonical regulatory region were arbitrarily set as one, and -fold induction due to the presence of Feec1 was calculated. Data were obtained from three independent experiments with comparable results. C, Sox7 and Sox17 are able to increase the level of transcription from the Feec1 reporter plasmid in COS cells. Increasing concentrations of Sox expression vectors were transfected as in B into COS cells together with the Feec1-tk-Luc or tk-Luc reporter plasmid with no canonical regulatory region. Base-line activity obtained with Feec1-tk-Luc or tk-Luc was arbitrarily set to one, and -fold induction due to the presence of Sox1 was calculated. Data were obtained from three independent experiments with comparable results. D, the reporter plasmid was incorporated into about 80% of ZHTc6 cells by lipofection. The Venus reporter gene (2 μg), which is under the control of the constitutively active EF-1 gene promoter, or empty vector was transfected using Lipofectamine 2000 (Invitrogen) together with 6 μg of pUC18. At 48 h post-transfection, cells were dissociated to single cells, and Venus expression was quantitated on a FACS Vantage device (BD Biosciences). The green portion and white line correspond to cells transfected with the Venus expression vector and empty vector, respectively. The numbers in the vertical and horizontal axes represent cell counts and Venus fluorescence, respectively. E, the Sox17-Engrailed fusion protein functions as a negative regulator of endogenous fibronectin expression. ZHTc6 cells, which were cultured in the absence of tetracycline, were subjected to lipofection with an expression vector carrying a cDNA for the fusion protein of the Sox17 HMG box-Engrailed repressor domain or empty vector. At 48 h post-transfection, RNA was prepared, and expression levels of fibronectin and β-actin were analyzed by RNase protection. The right panel represents quantitative data calculated using the Fuji BAS-5000 image analyzer from Fujifilm with control cells arbitrarily set to 100%.
Feec1, while Sox2 showed weaker activity than these extraembryonic endoderm-specific Sox factors. Moreover expression of a fusion protein composed of the Sox17 HMG DNA binding domain and the transcriptional repression domain of the Dro sophila Engrailed protein in differentiated ZHTe6 cells led to a decline in the expression level of the endogenous fibronectin gene. However, our analysis also revealed that the Feec1 enhancer shows higher activity in PE-like cells than in VE-like cells even though both cells possess equivalent amounts of Sox7 and Sox17 proteins, casting doubt on the idea that the activity of the Feec1 enhancer is mainly regulated by these Sox proteins. However, Murakami et al. (10) have recently demonstrated that high levels of Sox2 expression are detected in VE-like cells as well as in undifferentiated F9 cells, though the gene is scarcely expressed in PE-like cells. Therefore, it is possible that Sox2 protein in VE-like cells functions as a competitive inhibitor of Sox7 and Sox17 for Feec1 binding, which could result in lower transcriptional activation of the Feec1enhancer in VE-like cells.

About 20 different Sox transcription factors have been identified so far (32, 33). These factors appear to share the need to associate with certain protein partners to activate transcription. Indeed naturally occurring Sox-dependent enhancers typically have an additional site close to the Sox binding site that allows for cooperation between the Sox factor and its partner to activate transcription (34). For example, Sox2 has been shown to function together with Oct-3/4 in ES cells (19, 21, 28), while in lens cells, it cooperates with Pax6 to boost transcription of δ-crystallin (35). Likewise Sox9 and steroidogenic factor 1 act synergistically to stimulate transcription of anti-Mullerian hormone (36). Therefore, it is possible that Sox7 and Sox17 also require partner proteins to activate transcription of target genes in extraembryonic endoderm cells, although candidates for such proteins have so far not been identified. Alternatively it is possible that unlike other Sox factors, Sox7 and Sox17 may not require partners for activating transcription in the extraembryonic endoderm. Consistent with this notion, Sox7 and Sox17 have stronger transcriptional activity in COS cells compared with Sox2, which must associate with partner proteins for maximum activity.

What role do Sox7 and Sox17 play in the extraembryonic endoderm? Gene targeting of Sox17 demonstrates that the protein is dispensable at the early stages of embryogenesis. Indeed embryonic abnormalities appear only after gastrulation (9). However, since Sox factors have been shown to be key players in regulation of embryogenesis and cell fate decision in many aspects of development (32–34) and Sox7 and Sox17 are major Sox factors expressed predominantly in extraembryonic endoderm (9–11), it is possible that disruption of both Sox7 and Sox17 would result in defects in the extraembryonic endoderm. However, the molecular mechanism by which Sox7 and Sox17 function in these cells is largely unknown at present possibly due in part to the fact that a number of downstream targets of these Sox factors remain to be identified. So far, only laminin α1 and fibroblast growth factor-3 genes have been shown to be downstream of Sox7 and Sox17 proteins (10, 11). We showed that fibronectin is under the control of these proteins. The identification and characterization of more Sox target genes will help to elucidate the mechanism of Sox function.

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