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Retinoic acid induces discrete Wnt-signaling-dependent differentiation in F9 cells

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ABSTRACT

Retinoic acid (RA) induces F9 cells, the mouse teratocarcinoma cells, to differentiate into primitive endoderm and further into visceral and parietal endoderm depending on the culture conditions. To elucidate the instructive mechanisms involved in the differentiation steps we investigated the effects of Wnt-signaling members, Wnt3a and β-catenin, on the differentiation of F9 cells and β-catenin-deficient F9 cells (βT cells). RA up-regulated the expression of differentiation markers for primitive, visceral and parietal endoderm in F9 cells but not for visceral endoderm in βT cells. Wnt3a or leukemia inhibitory factor (LIF) inhibited the RA-induced differentiation in F9 cells. LIF but not Wnt3a could inhibit differentiation in βT cells. RA evoked ZO-1α+ signals at cell-to-cell contacts in F9 cells in a Wnt3a sensitive manner. The results suggest that Wnt3a inhibits differentiation into endoderm through a pathway involving β-catenin, and β-catenin might be necessary in the process leading from primitive to visceral endoderm in F9 cells.

Introduction

The cellular differentiation events that occur just prior to and following implantation of the blastocyst during mammalian embryogenesis are critical for establishment of the cell lineages required for normal development. In mice at 4.5 days after fertilization, cells on the surface of the inner cell mass in a blastocyst differentiate into the epithelial-like primitive endoderm, which is destined to generate the extraembryonic parietal and visceral endoderm, the former moving and covering the inner surface of the blastocoels and the latter, still with epithelial properties, enveloping the epiblast [1]. The instructive events involved in the determination of cell fate so early in development, however, remain unknown.

The early events in development have been studied using the mouse teratocarcinoma cell line F9 treated with retinoic acid (RA), a biologically active molecule related to vitamin A, as a model of in vitro endoderm differentiation [2]. F9 cells increase their expression of several genes such as tissue plasminogen activator (tPA), Hoxa1, Hoxb1, lamininB1, keratin18 (Ker18) and keratin8 (Ker8) genes in response to RA to differentiate into primitive endoderm. Rex1 mRNA levels are high in F9 stem cells and reduced during the differentiation induced by RA [3]. Thus Rex1 is a marker of undifferentiated F9 stem cells. Further differentiation of F9 cells occurs along two pathways depending on the culture conditions. After forming aggregates the cells can differentiate into visceral endoderm, as indicated by the expression of a unique marker, α-fetoprotein (αFP) [4]. Furthermore, the exposure of F9 cells to RA with dibutyryl cyclic AMP (db-cAMP) results in their differentiation into parietal endoderm indicated by the expression of another specific marker, thrombomodulin (Thmd) [5].

Primitive and visceral endoderm show epithelium-like properties during mammalian development. The simple epithelial formation has three types of junctions between adjacent cells, the desmosome, the adherens junction and the tight junction (TJ) to connect neighboring cells and to isolate the apical from the basolateral side. One component of the multimolecular TJ complex is ZO-1, which binds to the cytoplasmic domain of occludin, an integral membrane protein in the TJ complex [6]. ZO-1 has an alternatively spliced isoform (ZO-1α+), which has an extra 80 amino acid sequence called a domain and is expressed in conventional epithelial TJs [7]. In a monolayer culture, F9 cells are shown to differentiate into epithelial-like endoderm characterized by the expression and localization of ZO-1α+ in TJs [8].

Wnt-signaling is known to be involved in several cellular events including proliferation, development, differentiation and tumorigenesis. Wnt proteins act through their intrinsic receptors, frizzleds, to activate canonical β-catenin-dependent and β-catenin-independent pathways [9]. β-Catenin is a cytoplasmic protein that

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directly interacts with the C-terminal cytoplasmic domain of cadherin and associates with actin-based cytoskeletons. In the absence of Wnts, active glycogen synthase kinase 3 (GSK3) phosphorylates β-catenin which is then ubiquitinated and degraded in the proteosome system, keeping cytoplasmic levels of β-catenin low [10]. Canonical Wnt-signaling stabilizes β-catenin by inhibiting GSK3. The accumulated β-catenin then enters the nucleus to associate with T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family transcription factors and activate gene transcription [11]. β-Catenin is therefore part of the Wnt-signaling machinery and may be involved in the signaling related to cellular differentiation. Bidirectional effects of Wnts and/or β-catenin signaling in differentiation have been reported in various stem cells [12–16]. In F9 cells, activation of the Wnt/β-catenin/Tcf-Lef pathway through frizzled-1 is reported to accompany the formation of primitive endoderm in response to RA in F9 cells [17]. Moreover, Shibamoto et al. showed that Wnt-signaling inhibited the differentiation into epithelial-like visceral endoderm of F9 cells cultured in suspension with 50 nM RA [18].

Thus the effects of Wnt-signaling in the early events of mammalian development remain unclear in view of the following. (1) Are Wnt signals positively or negatively involved in the early differentiation into primitive, visceral or parietal endoderm? Then, how does the epithelium form? (2) What pathways are involved in the effects of Wnt-signaling on differentiation? (3) How is β-catenin signaling involved in the effects of Wnt proteins on the endoderm differentiation? Here we tested the involvement of Wnt and β-catenin in cellular differentiation by using purified Wnt protein and comparing the effects with those by leukemia inhibitory factor (LIF) in F9 cells and β-catenin-deficient F9 cells (βT) isolated by gene targeting [19]. LIF is the first soluble factor identified as having the ability to maintain the pluripotency of mouse embryonic stem cells [20].

Materials and methods

Cell culture. F9 and βT cells were grown on a gelatin-coated tissue culture dish in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (JRH Biosciences), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco) in 5% CO_2 at 37 °C. In differentiation experiments, cells were plated at a density of 6×10^3 cells/cm^2 in the presence of RA (all-trans-RA, Sigma–Aldrich).

Semi quantitative RT-PCR. Total RNA was prepared from the cells according to the acid guanidinium thiocyanate–phenol–chloroform method [21]. cDNA was obtained in a total volume of 20 μL using 4 μg of total RNA, 50 units of MuLV-reverse transcriptase (Applied Biosystems) and 50 pmol of random primers and subjected to quantitative PCR using a LightCycler (Roche). Briefly, the given amount of reverse-transcribed cDNA corresponding to 0.1 μg of original total RNA was used per reaction along with the LightCycler FastStart DNA Master SYBR Green I (Roche) with 3 mM Mg^{2+} and 1 μM of gene-specific primers. Primers for target genes tested were designed as Table 1. A gene-specific standard was generated by a constructing pT7Blue vector (Novagen) containing the RT-PCR product using each primer set, diluted repeatedly 10-fold and subjected to a real-time PCR to create a standard curve. The amounts of sample mRNA were determined as an absolute copy number from the standard curve according to the LightCycler software (Roche) and expressed as normalized values with 1000 copies of 18S ribosomal RNA.

Immunocytochemistry. The cells were plated on coverslips and treated with RA in the presence of Wnt3a. After culturing for 5 days, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min, blocked and permeabilized with 0.1% bovine serum albumin and 0.1% Triton X-100 in PBS for 10 min. Then staining was performed with a monoclonal rat anti-mouse ZO-1 antibody (1:400, Chemicon), which can detect the ZO-1α+ isoform [8], or a polyclonal rabbit anti-mouse β-catenin antibody (1:500, Pharmingen) for 1 h, followed by an Alexa Fluoro 488 or 546-conjugated anti-rat or rabbit IgG antibody (1:500, Molecular Probes). After three washes with PBS, cells were mounted with 15% glycerol in PBS on a glass slide. Images were acquired using MetaXpress software with a confocal microscope (LSM510, Carl-Zeiss MicroImaging).

Data analysis. Data for the quantification of mRNA were expressed as the mean ± SEM and mRNA levels were considered to be significantly different from those of the corresponding control using a one-way analysis of variance with pairwise comparison by the Bonferroni method when the p value was less than 0.05.

Table 1
Sequences of primers for the quantitative RT-PCR.

Targets	Sequences(in 5′ to 3′ orientation)	Amplicon length (bp)
REX1	F: atggactaagagctggg R: cttgaacaatgcctatgact	212
tPA	F: acgacatcgcattact R: gttcgtgacggttttat	251
Ker18	F: agatggagcagctcaatg R: gtttgcatggagttgctg	211
Ker8	F: gggatgcagaacatgag R: ggacacgacatcagaag	237
αFP	F: acctcagcagagctgatcga R: ttgcagcagtggctgatacca	180
ZO-1α$^+$	F: agtcccttacctttcgc R: gcaatggtggtccttca	197
Cldn6	F: gcctctactggtctgc R: caatgaggagggtgaca	268
Wnt3a	F: ccgtcacaacaatgagg R: actctcggtgtttctctac	194
DKK1	F: atgaggcacgctatgt R: acagtctgatgatcggag	232
Thmd	F: aacgaactctgcgagca R: cgaaacacggatccaga	247
18S rRNA	F: cgccgctagaggtgaaattct R: cgaacctccgactttcgttct	487

Results

Expression of marker genes

To determine the optimal term for differentiation, the time course of the effects of RA on gene expression was examined in F9 cells and β-catenin-deficient βT cells. Cells were cultured in the presence of 10^{-6} or 10^{-7} M RA for 6 days. Total RNA prepared everyday from the cells treated with 10^{-6} M RA was analyzed for differentiation markers, REX1, tPA, Ker18 and Ker8, adhesion-related genes of epithelial-like cells, Cldn6 and ZO-1α+ and Wnt-related genes, Wnt3a and DKK1, while total RNA from the cells treated with 10^{-7} M RA was analyzed for αFP (Fig. 1). REX1, a zinc finger protein which acts as a transcriptional regulatory protein was expressed in untreated stem cells. REX1 mRNA expression was reduced one-seventh relative to that in stem cells on the 3rd day of the RA treatment in both F9 and βT cells, which coincided with REX1's role as a marker [3,22]. The endoderm and epithelial markers tPA, Ker18, Ker8, Cldn6 and ZO-1α+ mRNA began to be expressed on the 2nd day and reached maximal levels on the 4th day in both F9 and βT cells, followed by a decrease on the 5th and 6th day in βT cells. αFP mRNA, a marker of visceral endoderm, was expressed in the F9 cells treated with 10^{-7} M RA on the 3rd day, increasing on the 5th day by 12-fold compared with that on the 3rd day, but only at a low level or not at all in F9 cells treated with 10^{-6} M RA. It was not expressed in βT cells treated with RA. Regarding Wnt-related genes, F9 and βT cells had a basal level of