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Transdifferentiation of mouse visceral yolk sac cells into parietal yolk sac cells *in vitro*

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ABSTRACT

The mouse embryonic yolk sac is an extraembryonic membrane that consists of a visceral yolk sac (VYS) and parietal yolk sac (PYS), and functions in hematopoietic-circulation in the fetal stage. The present study was undertaken to examine the normal development of both murine VYS and PYS tissues using various molecular markers, and to establish a novel VYS cell culture system *in vitro* for analyzing differentiation potentials of VYS cells. RT-PCR and immunohistochemical analyses of gene expression in VYS and PYS tissues during development revealed several useful markers for their identification: HNF1β, HNF4α, Cdh1 (E-cadherin), Krt8 and Krt18 for VYS epithelial cells, and Stra6, Snail1, Thbd and vimentin for PYS cells. PYS cells exhibited mesenchymal characteristics in gene expression and morphology. When VYS cells at 11.5 days of gestation were cultured *in vitro* for 7 days, the number of HNF1β-, HNF4α-, E-cadherin- and cytokeratin-positive VYS epithelial cells was significantly reduced and, instead, Stra6-and vimentin-positive PYS-like cells increased with culture. RT-PCR analyses also demonstrated that gene expression of VYS markers decreased, whereas that of PYS markers increased in the primary culture of VYS cells. These data indicate that VYS epithelial cells rapidly transdifferentiate into PYS cells having mesenchymal characteristics *in vitro*, which may provide a culture system suitable for studying molecular mechanisms of VYS transdifferentiation into PYS cells and also epithelial-mesenchymal transition.

1. Introduction

The mouse embryonic yolk sac is comprised of a visceral yolk sac (VYS) and a parietal yolk sac (PYS), which are extraembryonic membranes derived from the primitive endoderm [9,15]. The VYS functions as an exchange system for nutrients, waste and gas, and a hematopoietic-circulation system [4]. It develops as an epithelial layer over the epiblast and extraembryonic ectoderm, which expresses E-cadherin as a cell adhesion molecule [12]. VYS epithelial cells secrete various plasma proteins, including α-fetoprotein, albumin, and Apoe and Apoa1 of apolipoproteins, which are also produced by hepatoblasts and hepatocytes [3,14,22]. VYS epithelial cells express HNF1β and HNF4α, liver-enriched transcription factors [8,18]. In contrast, the PYS functions to protect the early embryo and fetus, and its cellular component is dispersed on the trophoblast giant cell layer or Reichert's membrane, which is a thick basement membrane. These cells abundantly produce extracellular matrix components such as laminin and type IV collagen [2,10]. PYS cells have minimal cell-to-cell contact on Reichert's membrane and coexpress cytokeratins and vimentin as epithelial and mesenchymal markers, respectively [6,13]. Snail1, which may act on embryonic mesoderm formation and be a mesenchymal marker, is also expressed in PYS cells [16]. Furthermore, PYS cells express transmembrane proteins Stra6 and Thbd, which are critical for cellular vitamin A uptake and homeostasis [5], and for the anticoagulant activity of blood [23], respectively. PYS regresses and is degraded after formation of the chorioallantoic placenta [7].

Irrespective of their remarkable differences in morphology and functions, the VYS and PYS share the same origin from the primitive endoderm in embryonic development [9,15]. It has been shown that, in an organ culture system, visceral endoderm (VE), which is the precursor of VYS epithelial cells, has the potential for conversion or transdifferentiation into parietal endoderm (PE) cells, which form the parietal yolk sac in later developmental stages [11,17]. The extraembryonic mesoderm lining the VE may inhibit the transdifferentiation, but its molecular mechanisms have not been

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Table 1
Primary antibodies used for immunocytochemistry.

Antibody	Source	Dilution
Goat anti-mouse vimentin antibody	Sigma–Aldrich, St. Louis, MO, USA	1:200
Guinea pig anti-mouse CK8/18 antibody	Progen Biotechnik GmbH, Heidelberg, Germany	1:200
Rabbit anti-calf keratin antiserum	Dako, Carpinteria, CA, USA	1:200
Rabbit anti-mouse HNF4α antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:200
Rabbit anti-mouse HNF1β antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:200
Rabbit anti-mouse Stra6 antibody	Abcam, Cambridge, UK	1:200
Rabbit monoclonal anti-vimentin antibody	Abcam, Cambridge, UK	1:200
Rat monoclonal anti-mouse CK19 antibody	Developmental Studies Hybridoma Bank, Iowa, USA	1:200
Rat monoclonal anti-mouse E-cadherin antibody	Takara Bio Inc., Kusatsu, Japan	1:200

elucidated. A cell line with bipotential for differentiation into VE and PE cells, called XEN (extraembryonic endoderm) cells, has been established, and demonstrated to exhibit its VE or PE phenotype depending on culture conditions, especially the presence or absence of BMP signaling [19]. Although studies suggest that VE or VYS epithelial cells have the potential for conversion or trans-differentiation into PE or PYS cells *in vivo* and *in vitro*, it remains to be studied in detail how VYS and PYS morphologically develop and exhibit their respective gene expression during development, as well as demonstrated whether VYS cells in later stages have the potential or not. Since VE tissue in the early stages is very small and difficult to manipulate [17], it is necessary to establish an *in vitro* culture system for the transdifferentiation in which VYS or VE cells can easily be prepared in large numbers.

The present study was undertaken to analyze the normal development of VYS and PYS tissues using various markers, and to establish a novel VYS cell culture system *in vitro* for analyzing the differentiation potentials of VYS cells.

2. Materials and methods

2.1. Animals

C3H/HeSlc strain mice (Japan SLC, Hamamatsu, Japan) were used. Animals were mated during the night, and noon of the day when a vaginal plug was found was considered 0.5 days of gestation (E0.5). Extraembryonic tissues at E9.5-15.5 were used for this study. All animal experiments were carried out in compliance with the "Guide for Care and Use of Laboratory Animals" of Shizuoka University.

2.2. Primary culture

E11.5 VYS tissues were removed aseptically, minced into 1 × 2 mm squares in phosphate-buffered saline (PBS), and then digested by 0.5% collagenase I (Wako Pure Chemical Industries, Osaka, Japan) in Dulbecco's modified minimum essential medium (DMEM; Life Technologies, Carlsbad, CA, USA)-10% fetal bovine serum (FBS) at 37 °C for 1 h. The digested tissues were further dissociated into single cells by gentle pipetting. The resultant cell suspensions were centrifuged at 400 × g for 5 min, and washed twice in DMEM-10% FBS. The cell suspensions of 70 μL were cultured for 7 days on the glass areas of Teflon-coated slides (5×10^4 cells/cm^2) (AR Brown Co., Tokyo, Japan) at 37 °C in a water-saturated atmosphere containing 5% CO_2. The medium was changed on day 3. Cultured cells were fixed in cold acetone (−20 °C) for periodic acid-Schiff (PAS) staining and immunohistochemistry or in 0.1 M MOPS-2 mM EGTA-1 mM magnesium sulfate-3.7% formaldehyde (pH7.4) (MEMFA) for *in situ* hybridization.

2.3. Immunohistochemistry

Developing VYS and PYS tissues were fixed in a chilled mixture of 95% ethanol and glacial acetic acid (99:1 v/v) overnight, and embedded in paraffin. Hydrated paraffin sections and cultured cells were incubated overnight at 4 °C with the primary antibodies listed in Table 1. After thorough washing with PBS, sections were incubated with horseradish peroxidase- or fluorescent dye-conjugated secondary antibodies of appropriate animal species (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature [1].

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

Total RNA was extracted from developing VYS and PYS tissues and cell culture samples using ISOGEN II (Nippon Gene Co., Tokyo, Japan). Complementary DNA (cDNA) was synthesized from total RNA, and the PCR reaction was conducted according to Akai et al. [1]. The primers listed in Table 2 were used at 0.5 μM.

Table 2
Primer sequences used for RT-PCR.

Gene	Sequence (from 5′ to 3′)		Size (bp)
Actb	(fwd)	GGCTGTATTCCCCTCCATCG	154
	(rev)	CCAGTTGGTAACAATGCCATGT	
Alb	(fwd)	CTTGTGCTTCACCAGCTCAG	179
	(rev)	GTAGTGGATCCCTGGTGGAA	
Apoa1	(fwd)	GTGGCTCTGGTCTTCCTGAC	218
	(rev)	ACGGTTGAACCCAGAGTGTC	
Apoe	(fwd)	AGCAAATACGCCTGCAGG	230
	(rev)	GAGAGGTGCTTGAGACAGGG	
Cdh1	(fwd)	CCGGTGTCCCTATTGACAGT	391
	(rev)	GCCAAGGAGCTGACAAACC	
Hnf4a	(fwd)	CTGGCAGATGATCGAACAGA	160
	(rev)	ATTGGTGCCCATGTGTTCTT	
Hnf1b	(fwd)	CGGCGACGACTATGACACTC	692
	(rev)	GCTTCTGCCTGAACGCCTCT	
Krt8	(fwd)	TCCGGCAGATCCATGAAGAG	505
	(rev)	CGGTAGGTGGTGATCTCGATG	
Krt18	(fwd)	AGAAGGAGACCATGCAAGACC	382
	(rev)	AGTGCCTCGATTTCTGTCTCC	
Krt19	(fwd)	AAACCTCAATGATCGTCTCGCC	197
	(rev)	TCTTGGAGTTGTCAATGGTGGC	
Lama1	(fwd)	GGATGGGAATGGGAGCTGAG	153
	(rev)	CTGAGCCGAGCCTTTGACCT	
Snail1	(fwd)	AGCCCAACTATAGCGAGCTG	476
	(rev)	TCGGATGTGCATCTTCAGAG	
Stra6	(fwd)	AGCCAAGTCAGACTCCAAGAG	111
	(rev)	CAGAGAGCACACTAACTTCTTTCA	
Thbd	(fwd)	GCGAGCATTTTTGTGTCAGCA	604
	(rev)	TCCTCTTCCTTGGTGTCTTCC	
Vim	(fwd)	AAGGAAGAGATGGCTCGTCA	199
	(rev)	TTGAGTGGGTGTCAACCAGA	