Internalization of REIC/Dkk-3 protein by induced pluripotent stem cell-derived embryoid bodies and extra-embryonic tissues

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Abstract. REIC/Dkk-3 was first identified as a down-regulated gene in a number of human immortalized cells and human tumor-derived cell lines. Overexpression of the REIC/Dkk-3 gene using an adenovirus vector (Ad-REIC) has showed a potent selective therapeutic effect on various human cancers through induction of ER stress. Furthermore, we recently showed that Ad-REIC has an indirect host-mediated anti-tumor activity by induction of IL-7. However, the physiological function of REIC/Dkk-3 is still unclear. As a first step to study the possible receptor(s) for secreted REIC/Dkk-3, we analyzed the internalization of Cy3-labeled recombinant REIC/Dkk-3 protein. Among the cell lines screened, mouse induced pluripotent stem (iPS) cells showed a unique pattern of internalization. The internalization was observed in peripheral cells of spherical colonies formed spontaneously, but not in undifferentiated iPS cells. When we analyzed embryoid bodies (EBs) derived from iPS cells, REIC/Dkk-3 protein was internalized specifically by differentiated cells located at the periphery of EBs. Interestingly, Dkk-1 was internalized by undifferentiated cells at the center of the EBs. When developmental tissue was analyzed, internalization of REIC/Dkk-3 protein was strictly limited to extra-embryonic tissue, such as the trophectoderm layer of 4.5 days post-coitus (dpc) blastocysts and the chorionic membrane at 16.5 dpc. The mechanism of the internalization was confirmed to be endocytosis. These findings will contribute to knowledge on the interaction of REIC/Dkk-3 with a possible receptor(s).

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Introduction

REIC/Dkk-3 was first identified as a down-regulated gene in a number of human immortalized cells (1). The expression of REIC/Dkk-3 was reduced in many human cancer cells and tissues, including prostate cancer (2), renal clear cell carcinoma (3), testicular (4), non-small cell lung (5) and cervical cancer (6), malignant glioma (7), and malignant mesothelioma (8). Overexpression of REIC/Dkk-3 gene using an adenovirus vector (Ad-REIC) induced apoptosis in various human cancers. The induction of apoptosis was demonstrated to be selective, i.e., efficient killing of cancer cells with only a marginal effect on normal counterparts (2). In addition, Ad-REIC reversed resistance of cancer cells to anti-cancer drugs via down-regulation of P-glycoprotein (9). Furthermore, we recently showed that Ad-REIC has an indirect host-mediated anti-tumor activity *in vivo* by the induction of IL-7 (10). These anti-tumor activities of Ad-REIC *in vitro* and *in vivo* are triggered by ER stress caused by overexpression of REIC/Dkk-3 (2,10). However, the physiological function of REIC/Dkk-3 remains largely unclear.

Among the four Dkk family members, Dkk-1, -2, and -4 are known to interfere with Wnt signaling through the specific receptor LRP6 (11,12). After binding, Dkk-1 was internalized with its receptor LRP6 by endocytosis (13-15). Receptor-mediated endocytosis of ligand proteins is a common phenomenon for attenuation of the signal and recycling of the receptors (16,17). REIC/Dkk-3 is a highly-glycosylated secretory protein (18) and is considered to physiologically act on cells via a yet unidentified receptor. As a first step to obtain insight into a putative receptor(s) for REIC/Dkk-3, we analyzed the internalization of REIC/Dkk-3 protein in induced pluripotent stem cells and developing mouse embryos after Cy3 labeling.

Materials and methods

Preparation of recombinant REIC/Dkk-3 protein. OUMS-24 fibroblasts were infected with an adenovirus carrying full-length human REIC/Dkk-3 gene (2) and the secreted REIC/Dkk-3 protein was purified as previously described (10).

Briefly, the recombinant protein was purified from the conditioned medium of Ad-REIC-infected cells by two-step column chromatography using TSK-gel DEAE-Toyopearl 650S (Tosoh, Tokyo, Japan) and Resource-Q (GE Healthcare, Buckinghamshire, England). Purified REIC/Dkk-3 protein was analyzed and fractionated by high-performance liquid chromatography (HPLC) using a LaChrom Elite system (Hitachi, Tokyo, Japan). To confirm glycosylation of the recombinant REIC/Dkk-3 protein, the fractionated protein preparations were treated with peptide-N-glycosidase F (1,700 U/ml; New England Biolabs, Ipswich, MA) at 37˚C for 1 h (18).

Labeling of proteins with Cy3. Recombinant human REIC/Dkk-3 protein and recombinant Dkk-1 protein (R&D Systems, Minneapolis, MN) were labeled using Cy3 Mono-functional Reactive Dye (GE Healthcare) under the conditions described by the manufacturer.

Cell culture. The normal human fibroblast cell line OUMS-24 (19) was propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). A mouse induced pluripotent stem (iPS) cell line, iPS-MEF-Ng-20D-17 (20), was maintained in high-glucose DMEM supplemented with 15% FBS (Invitrogen), 0.1% leukemia inhibitory factor (LIF; Wako Pure Chemical Industries, Osaka, Japan) and 100 mM 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan) on mouse embryonic fibroblast (MEF) feeder cells. The iPS cell line carries a green fluorescent protein (GFP) cDNA under the control of the *nanog* promoter. MEFs were propagated from 12.5 days post-coitus (dpc) C57BL/6J embryos (CLEA, Tokyo, Japan) and were treated with 10 mg/ml mitomycin C (Sigma, St. Louis, MO) for 2 h.

Embryoid body formation. Undifferentiated iPS cells were passed through a 40 μm cell strainer (BD Biosciences, Bedford, MA) to prepare a single cell suspension. The cells were inoculated either into a petri dish for suspension culture (Sumitomo Bakelite, Tokyo, Japan) at 5,000 cells/ml or into round-bottom 96-well plates (Sumitomo Bakelite) at 500 cells/well (21). LIF was omitted from the medium.

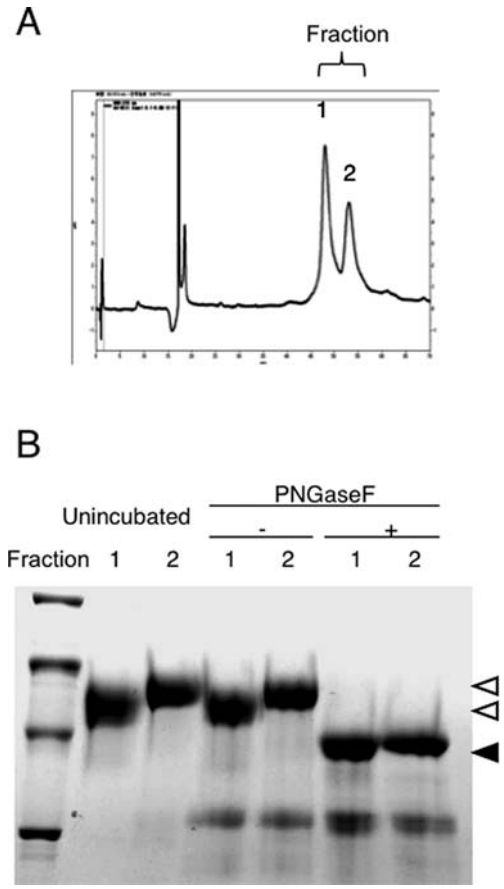

Figure 1. Preparation of recombinant human REIC/Dkk-3 protein from mammalian cells. (A) Recombinant REIC/Dkk-3 protein produced by OUMS-24 was fractionated using HPLC. (B) Glycosylation of the recombinant protein was confirmed by PNGaseF treatment. Open and closed arrowheads indicate glycosylated and deglycosylated REIC/Dkk-3 proteins, respectively.

Protein internalization assay. Cells were incubated with 2 μg/ml Cy3-labeled proteins at 37˚C for 1 h, followed by rinsing with PBS twice. Fluorescent signal was observed using an inverted fluorescence microscope (IX71; Olympus, Tokyo, Japan) or a confocal laser scanning microscope (FV10i; Olympus).

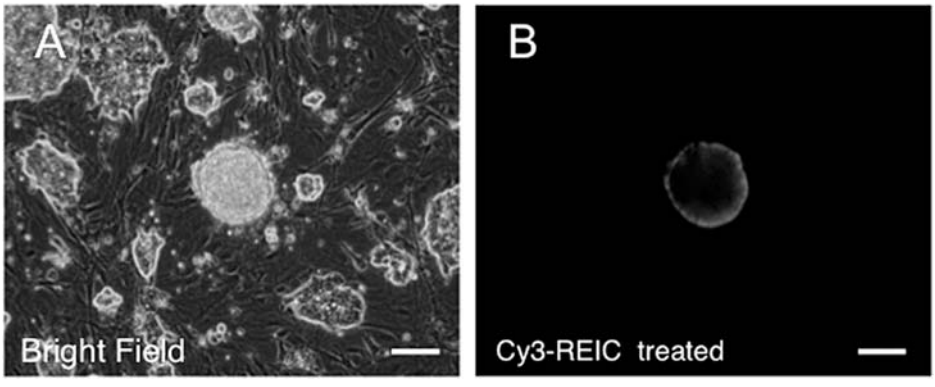

Figure 2. Internalization of Cy3-REIC by mouse iPS cells. (A) Mouse iPS cells with a few spontaneously differentiated cell colonies. (B) Cy3-REIC was internalized by a spheroidal colony. Bars, 200 μm.

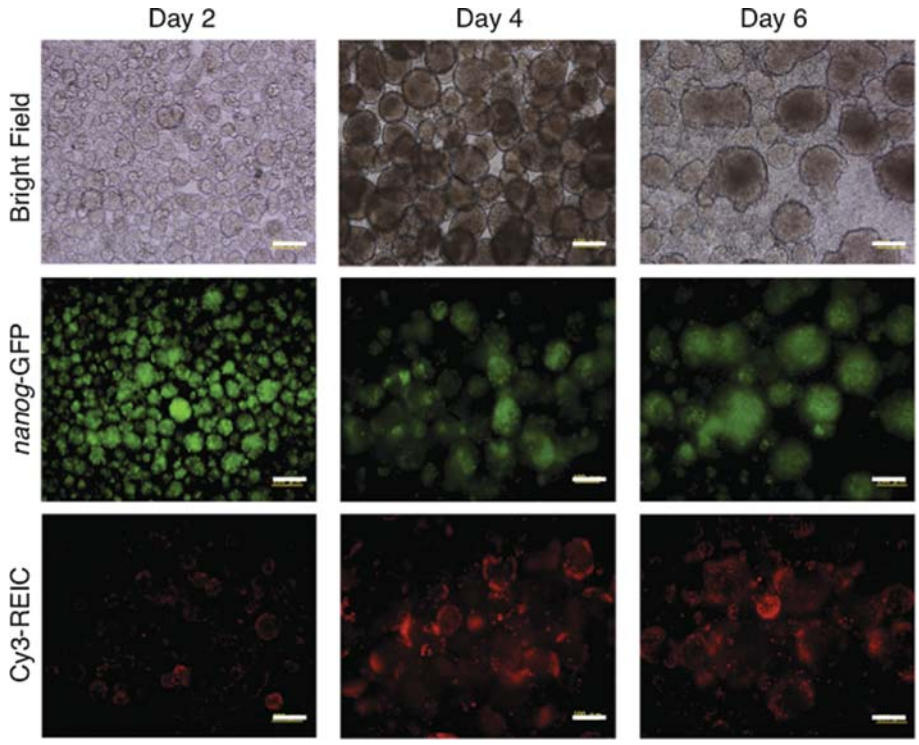

Figure 3. Internalization of Cy3-REIC by EBs derived from mouse iPS cells. (Top) EBs formed by mouse iPS cells in a suspension culture. (Middle) Expression of *nanog*. GFP cDNA conjugated to the *nanog* promoter *(nanog*-GFP*)* was constitutively integrated into the iPS cell genome. (Bottom) Internalization of Cy3-REIC became more remarkable with progression of EB formation. Bars, 200 μm.

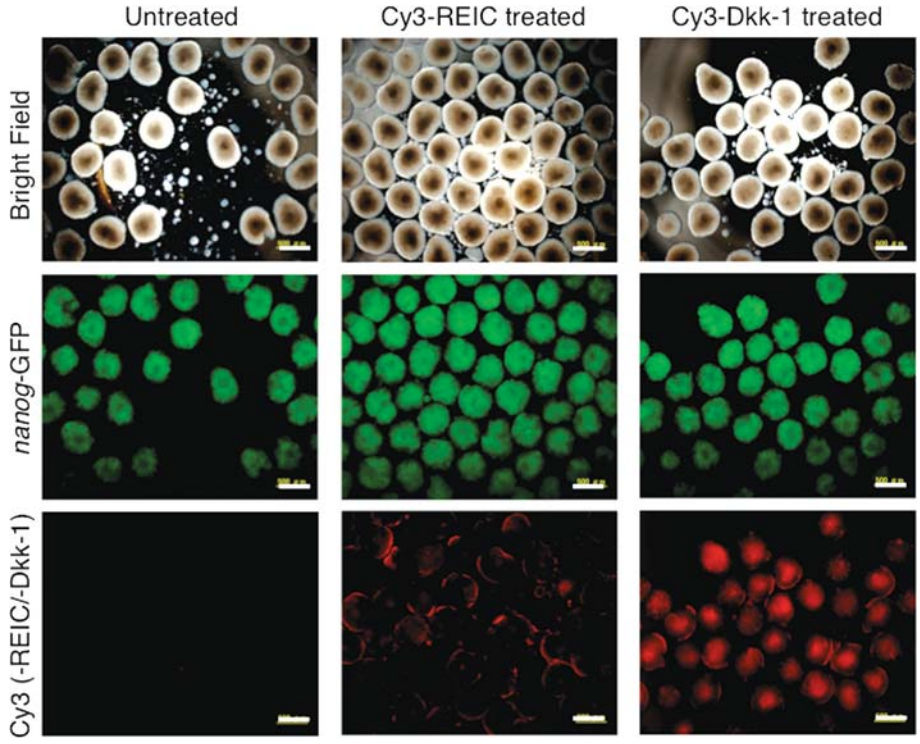

Figure 4. Differential internalization pattern of Cy3-labeled Dkk family proteins. Internalization of Cy3-REIC was observed in the outer layer of EBs, but that of Cy3-Dkk-1 was observed in the inner cells of EBs. Bars, 500 μm.

Internalization of Cy3-labeled REIC/Dkk-3 was assayed using 4.5 dpc blastocysts of ICR mice (Charles River, Yokohama, Japan) and 16.5 dpc embryonic and extra-embryonic tissues of BALB/c mice (Charles River). All animal-related procedures in this study were performed according to the institutional ethical code for the care and use of experimental

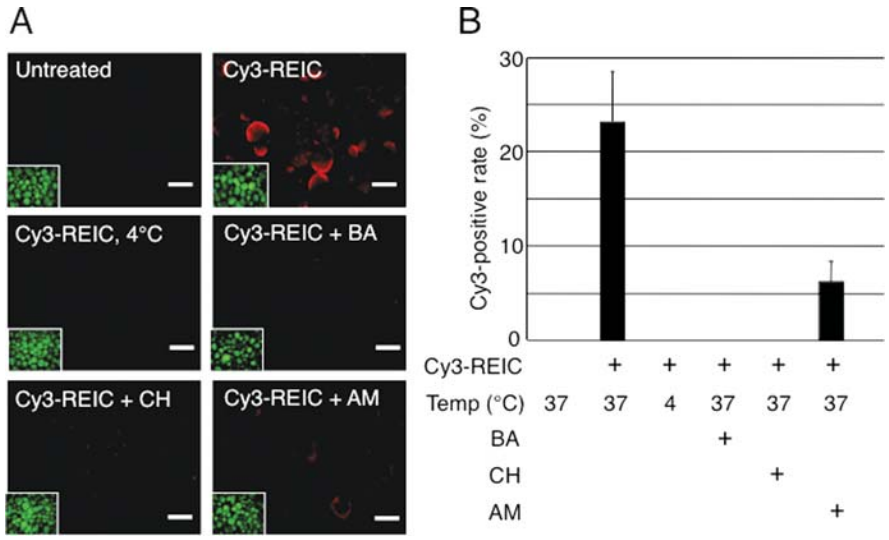

Figure 5. Suppression of Cy3-REIC internalization by inhibiting endocytosis. (A) Internalization of Cy3-REIC was assayed in iPS cells either cultured at 4˚C or with inhibitors of endocytosis, including bafilomycin A (BA; 20 mM), chloroquine (CH; 200 mM), and ammonium chloride (AM; 1 mM). Insets, *nanog*-GFP expression showing total EBs. Bars, 200 μm. (B) Rate of Cy3 signal internalization by EBs. Mean and standard deviation are shown.

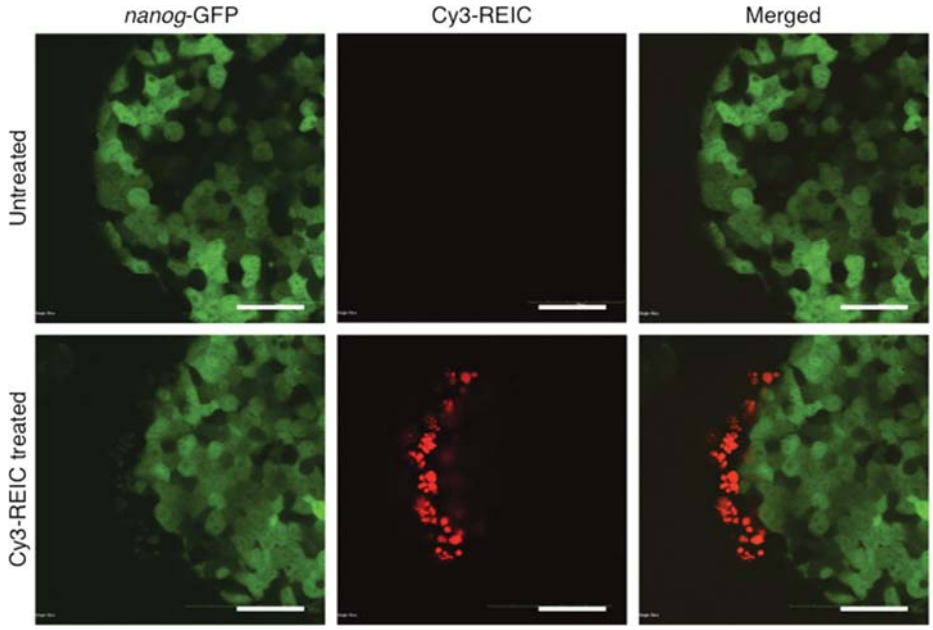

Figure 6. Internalization of Cy3-REIC by the outer cell layer of iPS cell-derived EBs. (A) Fluorescent images of GFP indicating the expression of *nanog* (Green) and internalized Cy3-REIC (Red). Bars, 50 μm.

animals. The developing tissues were incubated with Cy3-labeled REIC/Dkk-3 and observed under the same conditions as those described above. A stereo fluorescence microscope (SZX12; Olympus) was also used.

FACS analysis. After incubation with Cy3-labeled proteins for 1 h, embryoid bodies (EBs) were treated with 0.2% trypsin/0.02% EDTA/PBS solution at 37˚C for 20 min. Dissociated cells were passed through a 40 μm cell strainer. Individual cells were analyzed using a FACScalibur cytometer (BD Biosciences).

Inhibition of endocytosis. In order to inhibit endocytosis, EBs were cultured at a low temperature (4˚C) or treated with 20 μM bafilomycin A (Sigma), 300 μM chloroquine (Sigma), or 1 mM ammonium chloride (Nacalai Tesque) 1 h prior to Cy3-REIC treatment (22). The culture conditions for inhibiting endocytosis were maintained throughout Cy3-REIC treatment. The rate of Cy3 signal internalization by EBs was calculated from 10 individual fields for each group.

Results and Discussion

REIC/Dkk-3 protein was internalized by differentiated iPS cells. We first screened for cells that actively internalize REIC/Dkk-3 protein. Such cells or tissues should be good sources for identification of a specific receptor(s) for REIC/Dkk-3. Since REIC/Dkk-3 is known to be highly glycosylated

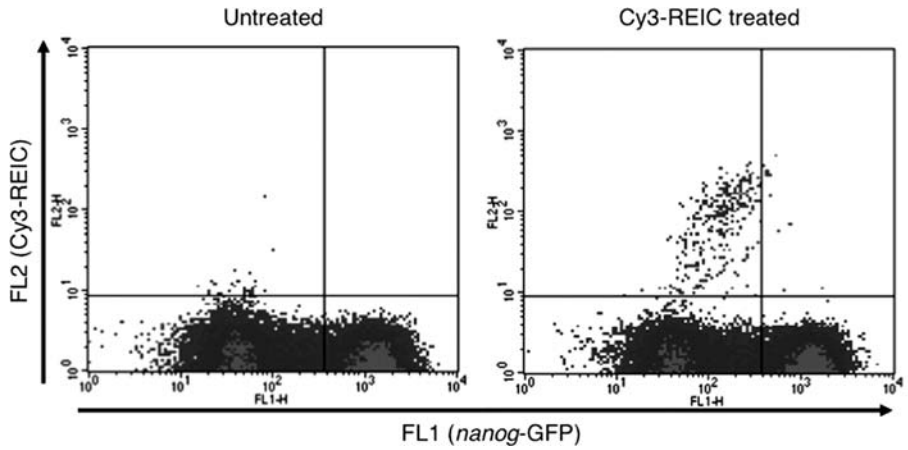

Figure 7. FACS analysis of Cy3-REIC-treated and untreated EB-forming cells. Cy3-positive cells were negative for GFP.

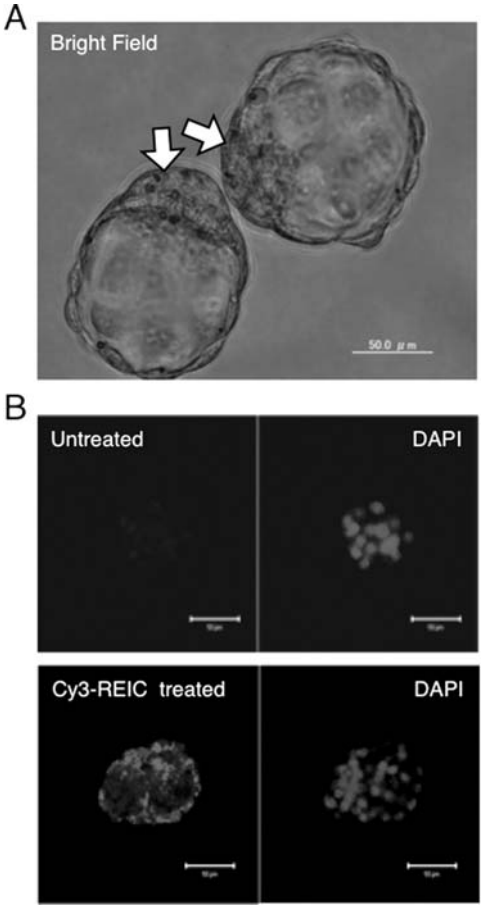

Figure 8. Internalization of Cy3-REIC by mouse blastocysts. (A) Mouse 4.5 dpc blastocysts consist of the inner cell mass (ICM) and the trophectoderm. Arrows indicate the ICM region. Bars, 50 μm. (B) Confocal images revealed that internalized Cy3-REIC was present specifically in the outer layer of EBs and not in the ICM. Bars, 50 μm.

(18), recombinant REIC/Dkk-3 was produced in OUMS-24 fibroblasts, not in *E. coli*, to prepare native protein. After purification of REIC/Dkk-3 by column chromatography, we obtained two fractions (Fig. 1A), probably due to different levels of glycosylation. This was confirmed by PNGaseF treatment, which gave rise to proteins with similar molecular size (Fig. 1B). REIC/Dkk-3 in the two fractions (Fig. 1A, fractions 1 and 2) was labeled with Cy3 fluorescent dye (Cy3-REIC).

Among the cell lines we screened, mouse iPS cells showed an interesting pattern of internalization of Cy3-REIC (Fig. 2). Internalization of Cy3-REIC was not observed in undifferentiated iPS cells and feeder MEFs. On the other hand, iPS cells that spontaneously formed spherical colonies strongly internalized Cy3-REIC (Fig. 2B). Mouse embryonic stem (ES) cells showed a similar pattern of Cy3-REIC internalization (data not shown). Since the spherical colonies resembled EBs, we analyzed EBs formed by iPS cells. As expected, EBs derived from iPS cells showed an increasing extent of Cy3-REIC internalization with time, while the expression of a stem cell marker, *nanog*, decreased (Fig. 3).

Since REIC/Dkk-3 and Dkk-1 belong to the same Dkk protein family, we compared Cy3-REIC with Dkk-1. As shown in Fig. 4, Cy3-REIC was internalized mainly by peripheral cells of EBs, while Cy3-Dkk-1 was internalized by central cells. The distinct pattern of protein internalization indicates that the internalization of Cy3-REIC as well as Cy3-Dkk-1 is an active and selective event. Dkk-1 has been reported to direct neural cell differentiation of ES cells (23). Though belonging to the same Dkk protein family, REIC/Dkk-3 is unique compared to the other 3 family members concerning DNA sequence, expression profile, and biological functions (24,25).

Internalization of REIC/Dkk-3 protein is due to endocytosis. Endocytosis controls entry of large molecules into the cell and has a crucial role in development, immune response, neurotransmission, intracellular communication, signal transduction, and homeostasis (26). Upon binding of a ligand to the corresponding receptor protein, the ligand-receptor complex is generally internalized by endocytosis for attenuating signal transduction and for recycling of the receptor (17). Sakane *et al* revealed that Dkk-1 was internalized with its receptor LRP6 by endocytosis and that the internalized LRP6 was recycled (15). The endocytosis of Dkk-1/LRP6 complex resulted in modulation of Wnt/ß-catenin signaling (14). Based on those findings, we examined whether the internalization of Cy3-REIC is due to endocytosis. If it is

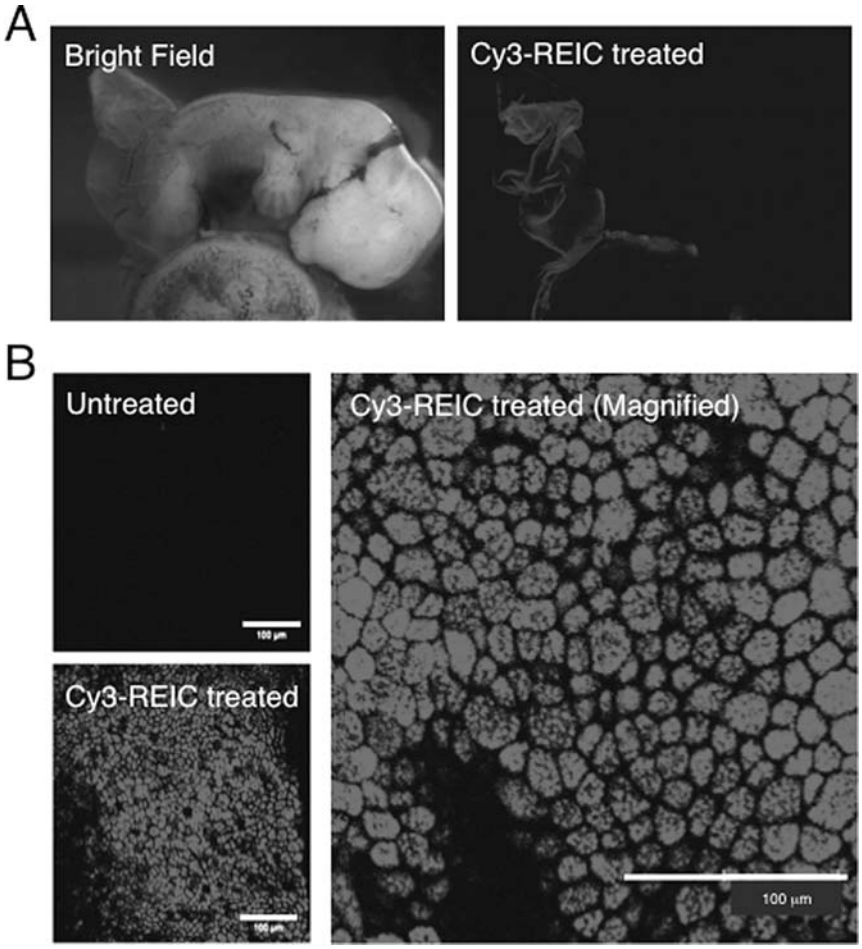

Figure 9. Internalization of Cy3-REIC by mouse extra-embryonic tissue. (A) Mouse embryo with extra-embryonic tissues at 16.5 dpc observed by a stereoscopic fluorescent microscope after Cy3-REIC treatment. (B) Presence of Cy3-REIC in chorionic cells observed by a confocal microscope. Bars, 100 μm.

caused by endocytosis, the data, though not sufficient, strongly support the existence of a REIC/Dkk-3 specific receptor(s).

The EBs derived from iPS cells were exposed to a low temperature or treated with inhibitors for endocytosis (22). The internalization of Cy3-REIC by the EBs was strongly suppressed by exposure to the low temperature and by all of the inhibitors tested (Fig. 5). It is well known that endocytosis of a ligand-receptor complex occurs within a few minutes (27,28). Internalization of Cy3-REIC was a rapid event, being observed within 5 min after addition of Cy3-REIC (data not shown). These results indicate an endocytotic mechanism for the internalization of REIC/Dkk-3 protein.

REIC/Dkk-3 protein was internalized by mouse extra-embryonic tissues. EB formation from pluripotent stem cells is an *in vitro* model for early development (29,30). Confocal microscopy of iPS-derived EBs exposed to Cy3-REIC clearly revealed that Cy3-REIC was internalized specifically by the outer cells of EBs that did not express *nanog* (Fig. 6). Cy3-positive cells prepared by the EBs were consistently negative for *nanog*-GFP as shown by FACS analysis (Fig. 7). The peripheral layer of EBs derived from ES cells was reported to differentiate into an extra-embryonic lineage (31). Therefore, we analyzed extra-embryonic cells in normal developmental tissues. When blastocysts of 4.5 dpc were incubated with Cy3-REIC, internalization was observed in the outer layer of blastocysts but not in the inner cell mass (Fig. 8). The cells at the outer layer of blastocysts, called the trophectoderm, develop into extra-embryonic tissues, such as the chorionic membrane and the placenta. We next analyzed mouse developmental tissues at a later stage of development. Stereoscopic observation of 16.5 dpc embryo and extra-embryonic tissues exposed to Cy3-REIC revealed that Cy3-REIC was internalized only by the chorionic membrane (Fig. 9). These results indicate that during mouse development Cy3-REIC is most actively internalized by cells of extra-embryonic lineage .

It is not yet fully understood which signaling pathways regulate differentiation of stem cells into extra-embryonic tissues in early development. Fibroblast growth factors, bone morphogenetic proteins (BMPs), and activin were reported to be involved in the development of the extra-embryonic lineage from pluripotent stem cells (32,33). Our preliminary data show that REIC/Dkk-3 protein induces phosphorylation of Smads, downstream targets of BMPs and other growth factors, in HEK293 cells (unpublished data). However, REIC/Dkk-3 itself was not expressed in 4.5 dpc blastocysts. Interestingly, decidual cells of the mouse uterus strongly expressed REIC/Dkk-3. Taking into account results showing that Dkk-1 was secreted by decidual cells and had an effect on trophoblast invasion (34,35), it is possible that REIC/Dkk-3

is involved in implantation of early embryos. Further analysis is needed to fully understand the function of REIC/Dkk-3 in the development of extra-embryonic lineage.

In conclusion, REIC/Dkk-3 protein was internalized by EBs derived from mouse iPS cells and mouse extra-embryonic tissue by an endocytotic mechanism. The function of REIC/Dkk-3 and the effect of its internalization are still unclear, but these findings will contribute to knowledge on the interaction of REIC/Dkk-3 with a possible receptor(s).

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