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Differentiated Human Embryonic Stem Cells Enhance the *In vitro* and *In vivo* Developmental Potential of Mouse Preimplantation Embryos

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ABSTRACT : In differentiating human embryonic stem (d-hES) cells there are a number of types of cells which may secrete various nutrients and helpful materials for pre-implantation embryonic development. This study examined whether the d-hES could function as a feeder cell *in vitro* to support mouse embryonic development. By RT-PCR analysis, the d-hES cells revealed high expression of three germ-layered differentiation markers while having markedly reduced expression of stem cell markers. Also, in d-hES cells, LIF expression in embryo implantation-related material was confirmed at a similar level to undifferentiated ES cells. When mouse 2PN embryos were cultured in control M16 medium, co-culture control CR1aa medium or co-cultured with d-hES cells, their blastocyst development rate at embryonic day 4 (83.9%) were significantly better in the d-hES cell group than in the CR1aa group (66.0%), while not better than in the M16 group (90.7%)($p<0.05$). However, at embryonic days 5 and 6, embryo hatching and hatched-out rates of the d-hES cell group (53.6 and 48.2%, respectively) were superior to those of the M16 group (40.7 and 40.7%, respectively). At embryonic day 4, blastocysts of the d-hES cell group were transferred into pseudo-pregnant recipients, and pregnancy rate (75.0%) was very high compared to the other groups (M16, 57.1%; CR1aa, 37.5%). In addition, embryo implantation (55.9%) and live fetus rate (38.2%) of the d-hES cell group were also better than those of the other groups (M16, 36.7 and 18.3%, respectively; CR1aa, 23.2 and 8.7%, respectively). These results demonstrated that d-hES cells can be used as a feeder cell for enhancing *in vitro* and *in vivo* developmental potential of mouse pre-implantation embryos. (**Key Words :** Mouse Embryo, d-hES Feeder Cell, Co-culture, Developmental Potential)

INTRODUCTION

Embryonic stem (ES) cells are derived from the inner cell mass cells of early mammalian blastocyst. These cells are pluripotent and thus they retain long-term proliferative potential in an undifferentiated state, and can differentiate into derivatives of all three embryonic germ layers on *in vitro* differentiation culture conditions or an *in vivo* environment (Thomson et al., 1998; Reubinoff et al., 2000). ES cells become a powerful tool for *in vitro* investigation of developmental processes at both cellular and organism levels, and offer remarkable potential for clinical application as an unlimited source of cells for transplantation and tissue generation therapies. ES cells are differentiated into specified cells by embryoid body (EB) formation and addition of trophic factors, co-culture with feeder cells or others (Assady et al., 2001; Kaufman et al., 2001; Kehat et al., 2001). However, all of those methods resulted in appearance of various lineage specific cells after differentiation and it is problematic for the application of stem cells. Nevertheless, these cells can be efficiently used according to their characteristics because the cells may secrete valuable metabolites, signal transcripts, growth factors, cytokines and etc.

Successful pre-implantation embryo development *in vitro* requires a culture environment similar to the *in vivo* environment (Fukuda et al., 1990; Bavister et al., 1992). In general, co-culture with somatic cell is useful for overcoming the *in vitro* developmental block of cultured mammalian embryos. Also, it has been already known that co-culture cells may secrete nutrients and embryotrophic factors, or cell-specific glycoproteins (Hwu et al., 1998). And there were reports that embryo co-culture with cells such as cumulus cell, granulosa cell, oviductal cell, endometrial cell or vero cells was effective (Mercader et al., 2003; Malekshah and Moghaddam, 2005; Noh et al., 2006).

In this study, we prepared differentiating cells derived

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from hES cell (d-hES) for use as a feeder cells of embryo co-culture. We expect that co-culture with d-hES feeder cells may offer the best environment for embryo development *in vitro*, because it is also young-aged cells derived from embryo. The purpose of this study was to examine whether the d-hES feeder cells have the ability to support the *in vitro* and *in vivo* development of mouse embryos.

MATERIALS AND METHODS

hES cell culture and preparation of d-hES feeder cells

MB06 hES cells (35-45 passages), one of hES cell lines established at Maria Biotech (MB01-09), were maintained in an undifferentiated state by culturing on a mitomycin C-treated (10 μg/ml, Sigma, St. Louis, MO) STO cell feeder layer (CRL-1503, ATCC, USA). hES cell culture methods were previously described by Park et al. (2004). The hES cell culture medium consisted of Knockout-Dulbecco's modified Eagle's medium (KO-DMEM, Gibco, Grand Island, New York) supplemented with 20% serum replacement (SR, Gibco), 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma), 1% non-essential amino acids (Sigma), and 4 ng/ml bFGF (Koma Biotech. Inc). Cells were sub-cultured approximately every 6 to7 days.

To make d-hES cells, recovered hES clumps after 0.05% collagenase type IV (Sigma) treatment and mechanical dissection were suspension cultured for EB formation using bacteriological dishes (#1007, Becton Dickinson, Bedford, MA) in hES cell culture medium devoid of bFGF. Medium was changed every 2 days and day 4 EBs were treated with 1 uM retinoic acid (RA, Sigma) for further 4 days under the suspension culture environment. A number of day 8 cystic EBs were plated onto a 0.1% gelatin-coated dish in differentiation medium (DMEM/F12 containing 10% FBS) and d-hES cells were sub-cultured every 4 to 5 days into 1:3. d-hES cells used for feeder cells were prepared from four to five sub-culture and were mitotically inhibited with 10 μg/ml mitomycin-C treatment for 1.0 h. In co-culture, mouse 2PN embryos were cultured in 50 μl sized d-hES cell micro-drops (1×10^5 cells/ml) overlaid with mineral oil (Sigma).

Mouse embryo culture

In vivo fertilized 2 pronuclei (PN) embryos were recovered from four to six week-old F1 female mice (C57BL/6×CBA) that had been superovulated by intraperitoneal (ip) injection with 5 IU PMSG (Sigma) followed by ip injection of 5 IU of hCG (Sigma) 48 h later, then immediately mated with fertilizable F1 male mice of the same strain. Cumulus cell-enclosed embryos were collected 18 to 20 h after hCG administration, and cumulus cells were removed with 0.01% hyaluronidase solution (Sigma). Two PN-containing healthy zygotes (designated as embryonic day 1) were equally allocated, and cultured in control M16 medium supplemented with 0.4% BSA (M16 group), co-culture control medium CR1aa supplemented with 10% FBS (CR1aa group), or co-cultured on d-hES feeder cells in CR1aa medium (d-hES group) for 3 days (designated as embryonic day 4) or 5 days (designated as embryonic day 6). All treatment groups were cultured in 50 μl drop, and their development rates were compared.

Embryo transfer and pregnancy check

Embryonic day 4 blastocysts cultured *in vitro* in the two medium control groups (M16, CR1aa) and the co-culture group (d-hES) were transferred surgically onto the uterine horn (8-9 embryos/horn) of ICR recipient female mice on day 3 of pseudo-pregnancy. The day on which a copulation plug was found was designated day 1 of pseudo-pregnancy. Implantation rates were scored by total number of fetuses, including resorption sites, at day 15 of gestation, and live births.

Reverse transcription (RT)-PCR analysis

Total RNA was isolated using TRI reagent, according to the manufacturer's protocol (Sigma-Aldrich Company, Dorset, UK, http://sigma-aldrich.com). Complementary DNA was synthesized from about 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY). PCR was done with AccuPrime DNA Taq polymerase (Invitrogen). Synthesized cDNA was amplified using 30 cycles of PCR with an annealing temperature of 52-60°C. The PCR products were size fractionated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Final analysis was obtained in an image analyzer (Biorad). Primer sequences for representative stem marker genes were as follows; human Oct-4 (hOct-4, 450 bp, up; 5'-act cga gca att tgc caa gc-3'; down 5'- cgt tgt gca tag tcg ctg ct-3'), hNanog (498 bp, up; 5'-caa agg caa aca acc cac tt-3'; down 5'- ctg ttc cag gcc tga ttg tt-3') and hTert (508 bp, up; 5'-gta ctt tgt caa ggt gga tgt gac-3'; down 5'-agg tgt cac caa caa gaa atc at-3') were used as stem cell markers. hkeratin (783 bp, up; 5'-agg aaa tca tct cag gag gaa ggg c-3'; down 5'- aaa gca cag atc ttc ggg agc tac c-3') and hMap2 (640 bp; 5'-agg aaa agg agt cag aga ag-3'; down 5'-ggt aag gtc att gcc tct ga-3') were used as ectoderm markers. hEnolase (503 bp, up; 5'-tga ctt caa gtc gcc tga tga tcc c-3'; down 5'- tgc gtc cag caa aga ttg cc ttg tc-3') and hRenin (607 bp, up; 5'-agt cgt ctt tga cac tgg ttc gtc c-3'; down 5'-ggt aga acc tga gat gta gga tgc-3') were used as mesoderm markers. hAmylase (493 bp, up; 5'-gct ggg ctc agt att ccc caa ata c-3'; down 5'- gac gac aat ctc tga cct gag tag c-3') and hAlbumin (355 bp, up; 5'-cct ttg gca caa tga agt ggg taa cc-3'; down 5'- cag cag tca gcc att tca cca tag g-3') were used as endoderm markers. hLIF (501 bp, up; 5'-ctc tgg gta

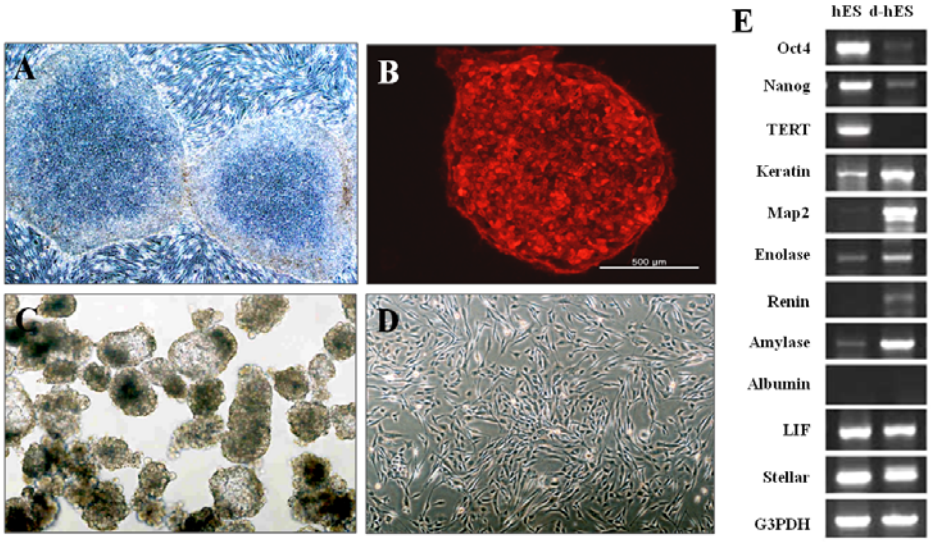

Figure 1. Morphological changes of hES cells throughout *in vitro* differentiation (A-D) and RT-PCR analysis of hES and d-hES cells (E). (A) Undifferentiated hES cell colonies. (B) SSEA-4 immuno-stained hES cell colony. (C) Day 8 EBs produced by suspension culture. (D) d-hES cells that have acquired a fibroblast morphology after EB plating and a few (3-5) rounds of subculturing. (E) Comparison of gene expression of hES and d-hES cells. Expression of three germ layered differentiation markers was very clear in d-hES cells, contrary to expression of undifferentiated hES cell markers. Also, expression of LIF was not reduced in d-hES cells. Scale bar in A, C and D = 100 μm.

aag gtc tgt aag aag g-3'; down 5'- aca cgc taa agc aag tca cag tag-3') and hStellar (168 bp, up; 5'-gtt act ggg cgg agt tcg ta-3'; down 5'- tga agt ggc ttg gtg tct tg-3') were used as human markers. Also, as a control for mRNA quality, hG3PDH (504 bp, up; 5'- ctc atg acc aca gtc cat gc-3'; down 5'- ggt ggt cca ggg gtc tta ct-3') was used.

Stem cell marker immunostaining

To detect the expression of the stem cell marker stage-specific embryonic antigen (SSEA)-4 (Developmental Studies Hybridoma Bank, Iowa City, IA), hES cell colonies were fixed with 4% paraformaldehyde solution at 4°C for 20 min. and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were incubated with primary antibody overnight at 4°C. Antibody localization was determined by staining with secondary anti-mouse antibody labeled with tetramethyl rhodamine isothiocyanate (TRITC, 1:200, Jackson Immunoresearch Lab Inc. Baltimore, PA) at RT for 1 h.

Statistical analysis

Differences in the developmental rates between treatment and control groups were assessed using the Chi-square test ($p<0.05$).

RESULTS

In this study, MB06 hES cells that had been sub-cultured 35-45 times (Figure 1A) were used to generate d-hES cells. Undifferentiated hES cells were very strongly positive for the stem cell marker SSEA-4, as assessed by indirect immunocytochemistry (Figure 1B). To generate d-hES cells, firstly, we generated a number of EBs, as shown in Figure 1C. And then day 8 EBs were plated onto a 0.1% gelatin coated plate and attached cells were continuously cultured in differentiation medium. Throughout a few rounds of subculture, the d-hES cell morphology was changed into fibroblast shaped and the cell size was large as somatic cell (Figure 1D). The preparing time of d-hES cells from hES cells was about 3 weeks. After the 3 rounds of subculture, a sufficient number of d-hES cells were obtained for feeder cell preparation.

To compare the gene expression between hES cells and d-hES cells, RT-PCR was done using primers specific for the stem cell markers Oct-4, nanog, and Tert; the ectoderm markers keratin and Map2; the mesoderm markers enolase and renin; the endoderm markers amylase and albumin; LIF; the human marker Stellar; and the housekeeping gene G3PDH. As shown in Figure 1E, compared to hES cells, d-hES cells revealed high expression in all three germ layered markers, with exception of albumin. In the meanwhile, there was markedly reduced expression of stem cell markers. Also, our d-hES cells presented that unchanged LIF expression similar to undifferentiated hES cells.

In this study, we employed two types of control medium for mouse embryo culture: M16 containing 0.4% BSA and CR1aa supplemented 10% FBS. To examine the effect of co-culture with d-hES cells, we selected CR1aa medium, which is generally not appropriate for mouse embryo culture. When we designated mouse 2PN embryonic day as

Table 1. *In vitro* development of mouse embryos co-cultured on the d-hES feeder cells (r = 3)

Treatment group	No (%)[1] of developed embryos						
	D1 (2PN)	D2 (≥2-cell)	D3 (Morula)*	D4 (≥Bla.)‡	D5 (≥HgB)**	D6 (≥HdB)**	D6 Outgrowth*
M16	108	108	96 (88.9)[a]	98 (90.7)[a]	44 (40.7)[b]	44 (40.7)[a]	
CR1aa	106	106	44 (41.5)[b]	70 (66.0)[b]	30 (28.3)[c]	26 (24.5)[b]	14 (13.2)[b]
d-hES cell[2]	112	112	92 (82.1)[a]	94 (83.9)[a]	60 (53.6)[a]	54 (48.2)[a]	72 (64.3)[a]

[1] Means in the column without common superscripts are significantly different (* $p<0.01$, ** $p<0.05$).
[2] Coculture on d-hES feeder cell.
HgB = Hatching blastocyst, HdB = Hatched blastocyst.

day 1, as shown in Table 1, the *in vitro* embryonic development rate until day 4 was very high in the M16 group (90.7%) compared to the others (CR1aa group, 66.0%; d-hES cell group, 83.9%). After that time, embryo hatching (day 5) and hatched-out rates (day 6) of the d-hES cell group (53.6 and 48.2%, respectively) were significantly different with those of CR1aa group (28.3 and 24.5%), which was better than those of the M16 group (40.7 and 40.7%), respectively ($p<0.05$). In particular, at embryonic day 6, when we examined the embryo morphology, the d-hES cell group displayed high percent of active attachment and outgrowing potential from hatching or hatched-out cells (64.3%, Table 1, and Figure 2C), characteristics similar to what occurs during implantation of embryos *in vivo*.

Also, *in vitro* cultured day 4 blastocysts (8-9 embryos/group) were transferred to the uterine horn of day 3 synchronized pseudopregnant recipients, as shown in Table 2, the pregnancy rate in the d-hES group (75.0%) was

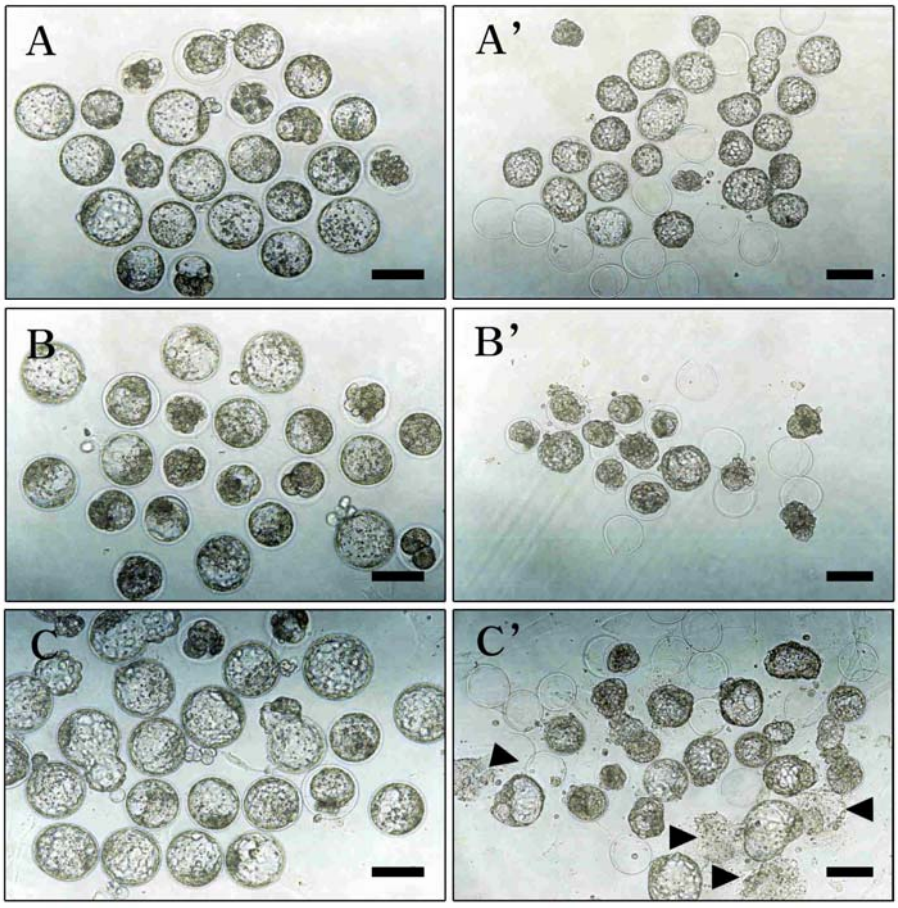

Figure 2. *In vitro* development of mouse embryos cultured in two different media, or co-cultured on d-hES feeder cells. Day 4 (A-C) and day 6 (A'-C') embryos cultured in M16 medium (A and A'), CR1aa medium (B and B'), and with d-hES cells (C and C'). Embryos co-cultured with d-hES cells displayed better *in vitro* developmental capacity and outgrowth patterns (arrowheads), similar to implantation morphology, compared to the other groups. Scale bar = 100 μm.

Table 2. *In vivo* development of mouse blastocysts co-cultured on the d-hES feeder cells (r = 2)

Treatment group	No. (%) of pregnant recipients	No. (%)*of blastocyst transferred		No. (%)* of day 15 of gestation		
		Total	Pregnancy	Resorption sites	Live fetuses	Total implantation
M16	4/7 (57.1)	60	34 (56.7%)[ab]	11 (18.3)	11 (18.3)[a]	22 (36.7)[a]
CR1aa	3/8 (37.5)	69	28 (40.6%)[a]	10 (14.5)	6 (8.7)[a]	16 (23.2)[a]
d-hES cell	6/8 (75.0)	68	46 (67.6%)[b]	12 (17.6)	26 (38.2)[b]	38 (55.9)[b]

* $(p<0.05)^{a-b}$.

higher than that in any other groups (M16 group, 57.1%; CR1aa group, 37.5%). This result demonstrated that d-hES feeder cells provide enhanced developmental capacity of mouse embryos. Although we couldn't estimate whether there were statistically significant differences among the groups, due to the limited number of recipients, the rate of embryo implantation (55.9%) and live fetus production (38.2%) of the d-hES group were also superior to those of the other groups (M16, 36.7 and 18.3%, respectively; and CR1aa, 23.2 and 8.7%, respectively).

DISCUSSION

This study demonstrated that the d-hES feeder cells can provide an enhanced co-culture environment for the development of mouse preimplantation embryos *in vitro*. Evidence of enhanced developmental potential of mouse embryos was seen in rates of blastocyst production, pregnancy, implantation, and live fetus production. These results may due to the presence of d-hES cells supplying a variety of nutrients (metabolites, glycoprotein, signal transcripts, growth factors cytokines…) and important other components (LIF, hCG…) for embryonic development.

ES cells are derived from the ICM cells of the early mammalian blastocyst and thus these cells are very young in age. Depending on the culture conditions, ES cells can either continue to grow in a pattern of prolonged self-renewal, or differentiate into derivatives of all three embryonic germ layers (Thomson et al., 1998; Reubinoff et al., 2000). Under differentiation conditions, pluripotent stem cells express a wide range of receptors for growth factors, and many types of human cells may be enriched *in vitro* by specific factors (Schuldiner et al., 2000).

Among the differentiation inducers, RA was identified as a morphogenic and teratogenic compound, and as a signaling molecule that influences gene expression in a complex manner via a family of RA receptors (Rohwedel et al., 1999). Through a process of EB formation, RA treatment, and through the 3 to 5 rounds of sub-culturing, we prepared d-hES cells that had characteristics of various cell types, as shown in RT-PCR analysis. Ectoderm (keratin and Map2), mesoderm (enolase and rennin), and endoderm (amylase) marker expression was very clear in d-hES cells, while expression of the stem cell markers Oct-4, Nanog and TERT was visibly decreased. Also, it is interesting that LIF (a differentiation inhibition factor) expression was continued in d-hES cells like as undifferentiated hES cells. Accordingly, we expect that this co-culture system might provide functional bio-material useful for embryo growth.

On the other hand, successful pre-implantation embryo development requires culture environment similar to *in vivo*. Accordingly, an effective culture medium added essential protein supplements with co-culture system are nedded for the best condition (Bavister et al., 1992). In general, somatic cell co-culture is considered the method of choice for overcoming the *in vitro* developmental block of cultured mammalian embryos. As feeder cells for embryo co-culture, cumulus cells (Malekshah and Moghaddam, 2005), oviductal cells (Khatir et al., 2004), endometrial cells (Mercader et al., 2003), vero cells (Noh et al., 2006), and other somatic cells (Hotoya et al., 2006; Srinivasan et al., 2006) have been used. All of these cells are adult somatic cells unlike our young aged d-hES cells. For this reason, our d-hES cells may supply more valuable proteins and enhance the developmental potential of preimplantation mouse embryos better than any of the other somatic cells described above, although we didn't directly compare the effects.

Among the secreted factors in d-hES cells, LIF is a cytokine belonging to the IL-6 family which was initially identified by its ability to induce differentiation of MI leukemia cells (Tomida et al., 1984). In embryogenesis, LIF is normally expressed in the trophectoderm of the developing embryo, with its receptor LIFR expressed throughout the inner cell mass. As embryonic stem cells are derived from the inner cell mass at the blastocyst stage, removing them from the inner cell mass also removes their source of LIF. Therefore LIF is essentially needed for mouse ES cell culture and removal of LIF pushes them toward differentiation (Smith et al., 1988). However, LIF is

not required for culture of human embryonic stem cells. On the other hand, it has been reported that LIF plays a major role in the uterus, and in its absence, embryos fail to implant. Also, profound disturbances in normal luminal epithelial and stromal cell differentiation occur during early pregnancy in LIF-null mice, such as failure to develop apical pinopods, no decidualization in the stroma, and low expression of decidualizing-related proteins (desmin, tenascin, Cox-2, BMP-2 and -7, Hoxa-10) (Fouladi-Nashta et al., 2005). In addition, high levels of LIF protein, LIF receptor, and gp130 are found in the day 4 uterus of the mouse, consistent with LIF being important for blastocyst implantation (Yang et al., 1995). In view of these results, LIF expression in d-hES cells likely plays a role in supporting the *in vivo* development of mouse embryos, such as implantation and live fetus production.

Although we selected a poor medium for mouse embryo co-culture, in the presence of CR1aa plus 10% FBS, d-hES cells overcame the sub-optimal environment for embryonic development. Also, from later developmental stages beyond embryonic day 5, the d-hES treatment group displayed better developmental capacity, including *in vivo* development, compared to the control M16 group. Thus, we guess that if the more appropriate medium was added in the present d-hES cell co-culture system, it will support higher *in vitro* and *in vivo* embryonic development than what we observed in the current study. At present, we are analyzing the bioactive material derived from d-hES cell and our future studies will carry out to define the specific component helpful for embryonic development and other applications.

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