E-Cadherin Synergistically Induces Hepatospecific Phenotype and Maturation of Embryonic Stem Cells in Conjunction With Hepatotrophic Factors

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Abstract: Since effective cell sourcing is a major challenge for the therapeutic management of liver disease and liver failure, embryonic stem (ES) cells are being widely investigated as a promising source of hepatic-like cells with their proliferative and pluripotent capacities. Cell–cell interactions are crucial in embryonic development modulating adhesive and signaling functions; specifically, the cell–cell adhesion ligand, cadherin is instrumental in gastrulation and hepatic morphogenesis. Inspired by the role of cadherins in development, we investigated the role of expression of E-cadherin in cultured murine ES cells on the induction of hepatospecific phenotype and maturation. The cadherin-expressing embryonic stem (CE-ES) cells intrinsically formed pronounced cell aggregates and cuboidal morphology whereas cadherin-deficient cadherin-expressing embryonic stem (CD-ES) cells remained more spread out and corded in morphology. Through controlled stimulation with single or combined forms of hepatotrophic growth factors; hepatocyte growth factor (HGF), dexamethasone (DEX) and oncostatin M (OSM), we investigated the progressive maturation of CE-ES cells, in relation to the control, CD-ES cells. Upon growth factor treatment, the CE-ES cells adopted a more compacted morphology, which exhibited a significant hepatocyte-like cuboidal appearance in the presence of DEX-OSM-HGF. In contrast, the CD-ES cells exhibited a mixed morphology and appeared to be more elongated in the presence of DEX-OSM-HGF. Reverse-transcriptase polymerase chain reaction was used to delineate the most differentiating condition in terms of early (alpha-fetoprotein (AFP)), mid (albumin), and late-hepatic (glucose-6-phosphatase) markers in relation to growth factor presentation for both CE-ES and CD-ES cells. We report that following the most differentiating condition of DEX-OSM-HGF stimulation, CE-ES cells expressed increased levels of albumin and glucose-6-phosphatase, whereas the CD-ES cells showed low levels of AFP and marginal levels of albumin and glucose-6-phosphatase. These trends suggest that the membrane expression of E-cadherin in ES cells can elicit a marked response to growth factor stimulation and lead to the induction of later stages of hepatocytic maturation. Thus, cadherin-engineered ES cells could be used to harness the cross-talk between the hepatotrophic and cadherin-based signaling pathways for controlled acceleration of ES hepatodifferentiation.

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Keywords: embryonic stem cells; hepatic differentiation; maturation; E-cadherin

INTRODUCTION

Cell sourcing and organoid definition continue to challenge the promise of hepatocyte-based therapies for treatments of liver failure (Allen and Bhatia, 2002; Chapekar, 2000; Famulari et al., 2003; Griffith and Naughton, 2002; Haghighi et al., 2004; Kobayashi et al., 2003; Kulig and Vacanti, 2004; Langer and Vacanti, 1993; McKay, 2000; Selden and Hodgson, 2004; Yamamoto et al., 2003; Yarmush et al., 1992). Adult liver-derived stem cells are being widely investigated for their potential usage and clinical trials based on adult stem cells are now underway, however, sourcing and processing of adult stem cells is likely to be a challenge for the field (Dabeva and Shafritz, 2003). In contrast, embryonic stem (ES) cells isolated from the inner cell mass of blastocyst; (Chinezei et al., 2002; Evans and Kauffman, 1981; Hamazaki et al., 2001; Orkin, 2000) are easier to harvest and can potentially proliferate into lineage-specific cells, and thus may have significant potential to provide a promising alternative source of hepatic cells. For this potential to be realized for
regenerative medicine, the key molecular mechanisms that can control stem cell function in vivo require systematic elucidation (Nakano, 2003; Spradling et al., 2001; Watt, 2001). This study focuses on the concerted molecular regulation of hepatospecific maturation in ES cells by homotypic cell–cell interactions and hepatotrophic growth factors.

Cell–cell interactions are recognized to be of fundamental importance for embryonic development, tissue formation, and differentiation: (Betson et al., 2002; Omelchenko et al., 2001; Rosenberg et al., 1997; Steinberg and McNutt, 1999; Takeichi, 1991; Tunggal et al., 2005; Vleminkx and Kemler, 1999; Wang et al., 2004a). The calcium-dependent, transmembrane molecules, cadherins are centrally involved in establishing cell–cell adhesive structures during embryonic organoid formation (Angst et al., 2001). Cadherins directly regulate morphogenetic events, histogenesis and gastrulation in epithelial cells (Gonzalez-Reyes, 2003; Han et al., 2000; Kii et al., 2004; Larue et al., 1996; Rosenberg et al., 1997; Takeichi, 1991, 1995). They not only maintain the structural integrity of cells and tissues but also control a wide array of cellular behaviors including cell and tissue polarization and regulating cell rearrangements (Kawaguchi et al., 2001; Kii et al., 2004; Shin et al., 2000; Tepass, 1999; Tepass et al., 2000). The predominant cadherin of most epithelia, including liver cells, is Epithelial-cadherin (E-cadherin). Using coculture studies, we have shown that the expression of E-cadherin at hepatocyte-mesenchymal cell contact can promote hepatospecific differentiation (Brieva and Moghe, 2001). Recently, using E-cadherin chimeras, we reported the importance of E-cadherin engagement for modulation of growth versus differentiation balance in cultured hepatocytes (Brieva and Moghe, 2004a,b). Although E-cadherins may potentially have the ability to stimulate hepatic morphogenesis in ES cells (Larue et al., 1996), the role of E-cadherin in the induction of hepatospecific maturation in ES cells has not yet been directly investigated.

Largely inspired by the progression of hepatotrophic stimuli during development in vivo (Duncan, 2000, 2003; Lemaigre and Zaret, 2004; Zaret, 2001), there have been extensive studies of molecular growth factors that can interact with developing liver cells, fetal liver cells, and more recently, ES cells (Hu et al., 2003; Michalopoulos et al., 2003; Miyashita et al., 2002; Rossi et al., 2001; Yamada et al., 2002). A complex sequence of hepatotrophic factors has been implicated in the process of liver development as the foregut endoderm commits to the hepatic lineage (Lemaigre and Zaret, 2004; Zaret, 2001) primarily through the interactions between the endoderm and cardiac-mesoderm (Cascio and Zaret, 1991; Jung et al., 1999). This process results in the expression, as early as embryonic day 8–9 (E8–E9), of two major liver-specific markers alpha-fetoprotein (AFP) and albumin (ALB). At E10–E11, hematopoietic stem cells originating from the extrahepatic organ colonize the fetal liver region produce cytokines especially, oncostatin M (OSM) (Kamiya et al., 2001) while mesenchymal cells (non-parenchymal liver cells) produce the hepatocyte growth factor (HGF) (Kamiya et al., 1999).

Several investigators have examined the effect of hepatotrophic factors on the differentiation of ES cells into all three embryonic germ layers (meso, endo, ectoderm) (Kuai et al., 2003; Kubo et al., 2004; Nakano, 2003; Seo et al., 2005; Yoshimizu et al., 2001). The most significant of these include dexamethasone (DEX) and OSM, (Kinoshita and Miyajima, 2002; Matsui et al., 2002). DEX was shown to suppress AFP production and DNA synthesis, while upregulating ALB and glucose-6-phosphatase (G6P) (late hepatic marker) production. OSM, an interleukin (IL) 6 family cytokine, has been shown to upregulate G6P and tyrosine-amino-transferase (TAT) and glycogen accumulation. Another key hepatotrophic molecule, the HGF, is a paracrine mediator of morphogenetic epithelial-mesenchymal interactions, and one of the most established signals for liver development and regeneration (Huh et al., 2004). Recent studies have reported HGF to induce an early transition of albumin negative stem cells into albumin positive hepatic cells resembling hepatoblasts (Suzuki et al., 2003). Several other studies have shown HGF to be a powerful mitogen, playing an instrumental role in proliferation and differentiation into hepatic cells (He et al., 2003; Kamiya et al., 1999, 2001; Matsumoto and Nakamura, 1997; Schmidt et al., 1995; Wang et al., 2004b).

The current study was undertaken to examine the role of cadherin expression in ES cells as a possible regulatory parameter in guiding the process of hepatic differentiation. Murine embryonal stem cell line D3 (Doetschman et al., 1985) that were engineered to overexpress E-cadherin were grown on 0.1% gelatin-coated plates with LIF and 2-mercaptoethanol to maintain undifferentiated state. Gene transfer experiments were carried out as described previously (Larue et al., 1996) to generate Ecad+/Ecad+ cells, E-cadherin-expressing cells and Ecad-/Ecad- ES cells, E-cadherin-deficient cells. We compared morphogenesis and hepatotrophic maturation behavior of the E-cadherin expressing cells (CE-ES) to cadherin-deficient cells (CD-ES) cells. We report that cadherin expression can markedly promote the maturation responsiveness of ES cells to hepatotrophic growth factors. Only the CE-ES cells were able to express late stage maturation markers, indicating that cadherin expression may be a major molecular determinant of ES cell hepatospecific differentiation.

MATERIALS AND METHODS

Cell Culture Conditions

Two major murine ES cell lines (D3) were utilized (Larue et al., 1996): cadherin-expressing embryonic stem (CE-ES) cells and cadherin-deficient embryonic stem (CD-ES) cells. The CE-ES cell lines express high levels of E-cadherin, while, ES cells genetically modified through two rounds of homologous recombinations to genetically disrupt both copies of E-cadherin gene are CD-ES cells, which served as controls.
ES cells were cultured on 0.1% (w/v) gelatin (Sigma, St. Louis, MO) coated tissue culture polystyrene plates (TCPS). Undifferentiated cells were maintained on knock-out Dulbecco’s modified Eagle’s medium (KDMEM) with high glucose (Invitrogen Corp., Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen Corp.), 100 U/mL penicillin/streptomycin (Biowhittaker, Walkersville, MD), 2 mM glutamine (Biowhittaker), 1,000 U/mL leukemia inhibiting factor (LIF) ESGRO (Chemicon, Temecula, CA) and 2-beta-mercaptoethanol (Invitrogen Corp.). Cells were split every 2–3 days with 0.25% Trypsin/0.02% EDTA solution (Sigma) and media were exchanged every other day (Larue et al., 1996).

**Initiation of ES Cell Differentiation**

The Hanging Drop method was utilized to initiate embryoid body (EB) formation (Hamazaki et al., 2001; Metzger et al., 1994). The EBs were allowed to form over 18 days, which elicited the outgrowth of cells of the hepatocytic lineage from the EB. Basal Media consisted of DMEM (Cellgro, Herndon, VA), 20% FBS (Invitrogen Corp.), 4 mM L-glutamine (Biowhittaker), and 100 U/mL Penicillin/streptomycin (Biowhittaker). Cells were diluted to 9.9 × 10^4 cells/mL (one drop is approximately 30 μL, which should contain 3,000 cells). Drops were placed on the inside of the lid of 100 mm polystyrene petri-dish and spaced at least 1 cm apart. To ensure gas exchange, 5 mL of basal media were placed on the bottom petri-dish lid and the cells were incubated at 37°C and 5% CO2 for 2 days. After day 2, the EBs were washed with fresh media, transferred to a new 100 mm petri-dish and incubated for another 2 days. At day 4, single EBs were transferred to TCPS plates and allowed to attach by incubating for 7 days. Media was exchanged and cultures incubated for six more days. At day 18, the differentiated cells migrating out of the EB were harvested (Hu et al., 2004) by trypsinization and plated on collagen gels (1 mg/mL) with varying hepatotrophic growth factor treatments (in C+H medium without glucagon): no hepatotrophic growth factors, DEX only (10^-7 M), DEX-OSM (10^-7 M, 10 ng/mL), and DEX-OSM-HGF (10^-7 M, 10 ng/mL, 20 ng/mL) (Sigma) (Kamiya et al., 1999, 2001; Kinoshita and Miyajima, 2002), following which the morphogenesis and hepatogenesis were examined. Figure 1 provides an illustration of the initiation process.

**E-Cadherin Staining and Flow Cytometry Analysis**

Single cell suspensions of undifferentiated and differentiated ES cells were used for flow cytometric analysis. Cell identification was confirmed using fluorescent-activated cell sorter (FACS) (Becton Dickinson, Franklin Lakes, NJ) analysis by staining with fluorescent antibodies against mouse E-cadherin. Cells were fixed lightly with 0.1% formaldehyde (15 min, on ice) and washed again twice. Cell identification was confirmed by staining with fluorescent antibodies against mouse E-cadherin-FITC clone 36 (BD Biosciences, San Jose, CA), dilution 1:100. Isotype controls utilized a FITC-conjugated mouse IgG2A Isotype (BD Biosciences), whereas non-specific controls involved secondary antibodies alone.

ES Cells cultured on collagen gels were fixed with 4% paraformaldehyde (15 min/room temperature) and blocked with 3% bovine serum albumin (BSA), 1% normal goat serum (NGS) for 1 h/room temperature. After washing steps, cells were stained with fluorescent antibodies against mouse E-cadherin-FITC clone 36 (BD Biosciences), diluted 1:100 (1 h/4°C), washed with Dulbecco's phosphate buffer solution (DPBS) and viewed under oil immersion Zeiss LSM laser scanning microscope at 40×. Imaging was done in Leica.

**Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was extracted by using an RNA aqueous kit (Roche, Indianapolis, IN). cDNA was synthesized from 5μL.

![Figure 1. Schematic illustrating ES cell culturing and the steps initiating differentiation. At Eb-D0, the hang-drop method (Dang et al., 2002) was utilized to instigate differentiation through LIF removal and embryoid body (Eb) formation. Ebs recapitulate embryogenesis with facilitating extensive 3-D cell-cell interactions. At Eb-D4, Ebs are plated onto tissue culture polystyrene (TCPS) plates to enable cell growth for 18 days. At Eb-D12, ES cells are emanating and migrating out of the core EB. At Eb-D18, a sub-population of cells stemming out of Eb are harvested and cultured to produce hepatic cells.](image-url)
total RNA by using Superscript II first-strand synthesis with oligo dT (Promega, Madison, WI) (48 °C, 45 min first strand synthesis; 94 °C, 2 min, RT inactivation/denaturation). PCR was performed using SYBR green PCR master mix (Qiagen, Valencia, CA). Primers were synthesized for the following mouse genes as per Hamazaki et al. (2001). Oligonucleotide sequences are given in brackets in the order of antisense, sense primers followed by annealing temperature, cycles used for PCR and length of amplified fragment: (i) α-fetoprotein (AFP) (5′TCGTATTCCAACAGGAGG, 5′ AGGGCTTTGTGCTTACCAG; 55°C, 25 cycles, 173 bp); (ii) albumin (ALB) (5′GCTACGGCACAGTGCTTG, 5′ CAGGATTGCAGACAGATAGTC; 55°C, 25 cycles, 260 bp); (iii) glucose-6-phosphatase (G6P) (5′ CAGGACTGTTCTCATCCTT, 5′ GTTGCTGTAAGTAGTCCGT, 55°C, 30 cycles, 206 bp); (iv) Endogenous housekeeping gene, β-actin (5′ TTTCTCTTTGGTAGATGGAAT, 5′ GAGCAATCATCTTGATCTTC, 55°C, 20 cycles, 200 bp).

RESULTS

ES Cell Characterization: Cell Morphology and E-Cadherin Visualization

Cells were grown on collagen gels to assess the effect of endogenous cadherin on ES cell phenotype. As shown in Figure 2, ES cells readily attached and proliferated on collagen gels. CE-ES cells attained a more aggregated, spheroidal phenotype whereas CD-ES cells appear very spread and elongated. CD-ES cells grew with little cell aggregation as opposed to CE-ES cells which showed prominent cell aggregation with few isolated cells. In visualizing E-cadherin, distinct E-cadherin engagement/staining was seen at cell–cell borders in CE-ES cells; E-cadherin is localized distinctly at the cell–cell contacts (data not shown). This suggests that cell aggregation is enhanced through homotypic cell–cell contacts mediated via E-cadherin.

Characterization of E-Cadherin Expression in ES Cells

The level of E-cadherin expression in CE-ES cells and CD-ES cells was assessed by flow cytometry of the E-cadherin receptors. E-cadherin expression of undifferentiated cells was first conducted to characterize the cells, and then, to evaluate the stability of E-cadherin expression during the cell differentiation process. Cadherin expression was assessed in undifferentiated state and at day 0 (18 day when cells harvested) and day 8 (post day 18 of EB). As shown in Figure 3, the CE-ES cells exhibit stable expression of E-cadherin without downregulation at different stages of development during ES cell culture. Furthermore, E-cadherin staining was conducted to determine if the different hepatotrophic (no GFs, DEX/OSM, DEX/OSM/HGF) treatments adversely affected E-cadherin expression and/or localization. There seemed to be no significant change in cadherin localization, which was still distinctly prominent at cell–cell junctions. This further proved E-cadherin expression at cell–cell contacts was stable under the different treatments.

Effect of Hepatotrophic Factors CE-ES Cell Hepatic Differentiation

Different hepatotrophic factors (DEX, OSM, HGF) were assessed for their ability to activate differentiation of CE-ES (control: CD-ES) cells into hepatic-like cells. Our data shown in Figure 4 indicates that cell culturing in the absence of any hepatotrophic growth factors yielded several aggregated CE-ES cells and more spread and elongated CD-ES cells. With the addition of DEX, CE-ES became more cuboidal whereas, the combination of DEX-OSM and DEX-OSM-HGF, elicited even further rounded, cuboidal cells. With this treatment, distinct, prominent nuclei were observed in CE-ES cells. In CD-ES cells, only modest levels of rounding of cell phenotype were observed.
Expression of hepatic specific markers in differentiating ES cells cultured on collagen gel was assessed to elucidate hepatic maturation. Specifically, mRNA expression of AFP (early hepatic marker), ALB (mid-hepatic marker), G6P (late hepatic markers), and beta-actin was examined by RT-PCR and in each condition, this was normalized to the beta-actin mRNA expression. As shown in Figure 5, the normalized AFP expression was observed in both cell types when cultured with no growth factors indicating that immature fetal cells are present. However, greater levels of the late hepatic marker, G6P are present in CE-ES cells. Following the incorporation of DEX-OSM, DEX-OSM-HGF, the normalized expression of AFP and ALB in CD-ES cells diminished, and, G6P expression was not detectable. In contrast, the CE-ES cells expressed low levels of AFP and ALB, but significantly higher levels of G6P, which were further increased ($P < 0.05$) in the presence of all three growth factors, DEX/OSM/HGF. Thus, the expression of E-cadherin clearly correlated with increased expression of G6P, a mature hepatospecific marker, in the hepatotrophically stimulated ES cells.

**DISCUSSION**

The goal of this study was to investigate the role of endogenous epithelial cadherin expression on hepatospecific maturation in murine ES cells following stimulation with hepatotrophic growth factors. Our key findings are: (a) the presence of E-cadherin correlated with striking differences in ES cell morphogenesis both in the presence and absence of hepatotrophic factors; (b), E-cadherin expressing ES cells showed more pronounced morphogenesis and maturation toward late-stage hepatospecific phenotype versus CD-ES cells following DEX-OSM-HGF stimulation.

In our cultures, ES cells were allowed to aggregate into structures called embryoid bodies (Ebs) in which all three germ layers (endoderm, mesoderm, and ectoderm) develop and interact with each other. These Ebs recapitulate many of the processes that take place during embryogenesis and development (Dang et al., 2002; Doetschman et al., 1985; Leahy et al., 1999). Subcultures of ES cells emanating out from the Ebs were harvested to propagate (Hu et al., 2004) and establish hepatic-like cells. Notably, there were significant differences in the morphogenic behavior of cadherin-expressing (CE-ES) cells relative to the cadherin-deficient (CD-ES) control cells. In adult hepatic cells, compact
cuboidal hepatic morphology conventionally represents more differentiated cells (Semler et al., 2000; Semler and Moghe, 2001; Singhvi et al., 1994). Similarly, in our ES cultures, CE-ES cell morphology on collagen gels attained a more spheroidal, aggregated phenotype, suggesting that the presence of E-cadherin overexpression facilitated a more native cytoskeletal organization and phenotype indicative of native, hepatic-like parenchyma. In contrast, CD-ES cells were more spread and elongated yielding a more fibrotic phenotype. The corresponding E-cadherin visualization in CE-ES cells revealed distinct staining at cell–cell borders with E-cadherin engagement, while as expected CD-ES cells showed no E-cadherin staining. E-cadherin molecules are instrumental in modulating cell morphogenesis and tissue architecture by facilitating cell adhesion, regulating the cytoskeleton and maintaining cell polarization in epithelia. Thereby, the presence of the cadherin perhaps enables the CE-ES cells to acquire/maintain more hepatic phenotypes by establishing correct cell polarity (Knudsen et al., 1998; Kovacs et al., 2002; Larue et al., 1996; Stamatoglou and Hughes, 1994). In addition to supporting cell–cell adhesion, E-cadherin activates cell signaling pathways (Tunggal et al., 2005), which affords another major avenue to guide ES cell hepatic differentiation/maturation.

The cadherin-engineered ES cells used in our study had a significantly higher cadherin expression than the CD-ES cells, and notably, this expression was maintained even upon the onset of differentiation (embryoid body induction). Since E-cadherin expression is typically thought to be downregulated in order to facilitate migratory phenotype (Pagan
et al., 1999), E-cadherin expression may decrease within the elongated cells growing out of the embryoid body, indicative of the so-called epithelial-mesenchymal transition (EMT) process (Pagan et al., 1999). It is plausible that the enhanced expression of E-cadherin in our system may have slowed down the EMT process. Further, by primarily harvesting an early subpopulation (Hu et al., 2004) of less motile ES cells emanating from the EB by day 18, we may have selected for the less motile cells along the hepatic-like lineage that maintain their cadherin expression. Additionally, many studies have reported that the expression of E-cadherin decreased with HGF exposure (Martin et al., 2001; Miura et al., 2001). However, these reports involve highly motile adult endothelial cells or tumorigenic cells for which HGF is known to be a potent mitogen and motogen, regulating cell proliferation and motility by disrupting cell–cell adhesion junctions (causing cell scattering) and phosphorylating catenins, which in turn disrupted E-cadherin function (Hiscox and Jiang, 1999; Martin et al., 2001). Our flow cytometry studies indicate that E-cadherin expression is not significantly downregulated upon exposure to HGF (data not shown). Furthermore, E-cadherin staining confirmed qualitatively that E-cadherin localization at cell–cell contacts was stably conserved following stimulation with hepatotrophic factors (DEX/OSM/. DEX/OSM/HGF). Thus, we concluded that the CE-ES cells continued to stably express E-cadherin at the cell–cell contacts under all key conditions compared.

The cadherin-engineered ES cells exhibited pronounced morphogenic responses to hepatotrophic stimulation. In the absence of any growth factors, CE-ES cells showed aggregated phenotype compared to CD-ES cells, which were more elongated. Upon the addition of DEX, more pronounced cell aggregation was observed in both cell types. However, the DEX-OSM and DEX-OSM-HGF treatments produced more hepatic-like phenotypes; CE-ES cells became more cuboidal with even distinct, prominent nuclei being observed. The effect of ES cell phenotype is in agreement with DEX and DEX-OSM treatment on fetal liver cells (E14.5) as reported by (Kamiya et al., 1999, 2001; Matsui et al., 2002). In CD-ES cells, there was some rounding of cell phenotype with the addition of DEX-OSM which has not been documented before, but the response to hepatotrophic factor was far less pronounced than that observed in CE-ES cells.

Previously, we have demonstrated that the E-cadherin based adhesion of hepatocytes augmented mature hepatocyte function (increased liver-specific secretion markers) in both co-culture systems (Brieva and Moghe, 2001) and acellular presentation (Semler et al., 2005). One of the major findings of the study is that cadherin-engineered ES cells exhibit altered hepatospecific maturation responsiveness to hepatotrophic stimulation. In the absence of any hepatotrophic growth factors, AFP was expressed at basal levels in both cell types indicating that immature fetal cells were being developed. This suggested the correct lineage (endoderm) was being followed and cells were being differentiated along the early phases of the hepatic lineage (Zaret, 2001). However, without the incorporation of additional growth factors, only immature hepatic cells were being produced; the mid-late hepatic markers (ALB, G6P) were not highly expressed. To further probe the development process, specific hepatotrophic factors were added to instigate hepatic maturation in conjunction (Kamiya et al., 1999, 2001; Matsui et al., 2002) with the presence of endogenous E-cadherin. By adding DEX, DEX-OSM, DEX-OSM-HGF, AFP expression decreased as the expression levels of the late hepatic marker (G6P) increased in CE-ES cells. In CD-ES cells, AFP expression continued to be expressed, however, the mid-late markers (ALB, G6P) were not expressed at levels comparable to CE-ES cells. The pattern of the onset of mid-late hepatic markers was not observed in CD-ES cells. Thus, CE-ES cells appear to be more responsive to the addition of hepatotrophic factors. Although G6P was highly expressed in CE-ES cells following DEX-OSM-HGF stimulation, the behavior of ALB expression in CE-ES cells, which appears to be maintained at modest levels, is somewhat intriguing. The albumin gene is considered to be an intermediate maturation marker and encodes a key hepatic protein expressed in mature hepatocytes. The major reason why growth factor
responsiveness of albumin and G6P expression do not seem to correlate in the CE-ES cells may be likely due to the fact that the expression of G6P and albumin may not be as stringently regulated as that in adult hepatocytes. Secondarily, our results reflect the mRNA levels of these genes, not the gene products, which may be differentially processed in ES cells. Thus, further studies will be necessary to comprehensively elucidate the complete profile of gene expression and protein synthesis in ES cells.

On a molecular level, there is a significant potential to harness the cross-talk between the DEX/OSM/HGF and cadherin-based signaling pathways (Nelson and Nusse, 2004) for strategic acceleration of ES hepatodifferentiation. The HGF/c-met signaling pathway supports development, proliferation, scattering, and branching morphogenesis (Birchmeier and Gherardi, 1998; Michalopoulos and DeFrances, 1997; Zhang and Vande Woude, 2003). HGF activates c-Jun pathways in hepatocytes (Auer et al., 1998; Gomez-Lechon et al., 1996), which are critical for hepaticogenic development (Behrens et al., 2002). Additionally, the Wnt family proteins participate in morphogenesis and cell proliferation during embryogenesis/differentiation (Ohnaka et al., 2004). The HGF/c-met and cadherin-based pathways can clearly converge; the presence of HGF can facilitate cell growth/proliferation activities by increasing the amount of cytosolic β-catenin available for nuclear translocation induced by Wnt signaling. On the other hand, cadherin-mediated contacts also activate and regulate β-catenin levels that can partake in Wnt signaling (Wheelock and Johnson, 2003); cell differentiation is instigated at the expense of cell growth. The regulatory roles of DEX and OSM are likely to be somewhat different from HGF. Specifically, DEX suppresses the canonical Wnt signaling, by also decreasing the availability of cytosolic β-catenin and inhibiting nuclear translocation of β-catenin; consequently also reducing the transcriptional activity of T-cell factor (TCF)/lymphoid enhancer factor (LEF) (Ohnaka et al., 2005). Moreover, DEX suppresses growth (by transcription factors: IL-6, CXC-chemokine receptor, amphiregulin, COX-2) and induces hepatocyte maturation (induces expression of both HNF4 and C/EBPα which are essential transcription factors for hepatocyte differentiation) (Michalopoulos et al., 2003).

Lastly, OSM may exert a feedback via downstream mediator, K-Ras, on sub-localization of E-cadherin/β-catenin at cell–cell contact sites and stabilizing cell polarity (Matsui et al., 2002; Kamiya et al., 2000; van der Woude et al., 2002), although within our system, E-cadherin expression levels were not overtly altered in the presence of OSM.

The key findings of this study may have several implications for the molecular engineering strategies available to accelerate hepatospecific maturation of stem cells. Insights gained from this study would be useful to design optimal molecular strategies to prime ES cells via E-cadherin expression and growth factor stimulation. Such approaches may aid in the isolation of the sub-populations of ES cells that express high levels of late maturation markers: the isolation of a homogeneous hepatic-like cell source from a mixed population of ES cells is a major challenge for the future. Insights on the regulation by cadherin-based signaling of hepatospecific maturation events could also reveal appropriate molecular targets that could potentially be engineered through gene silencing. If the underlying challenges can be resolved, such efforts could aid in the engineering of hepatic ES cells for cell sourcing in tissue engineering of the liver.

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