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Embryoid Body–Mediated Differentiation of Mouse Embryonic Stem Cells Along a Hepatocyte Lineage: Insights from Gene Expression Profiles

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Abstract

Pluripotent embryonic stem (ES) cells represent a promising renewable cell source for the generation of functional differentiated cells. Previous studies incorporating embryoid body (EB)-mediated stem cell differentiation have, either spontaneously or after growth factor and extracellular matrix protein supplementation, yielded populations of hepatocyte lineage cells expressing mature hepatocyte markers such as albumin (ALB). In an effort to promote ES cell commitment to the hepatocyte lineage, we have evaluated the effects of four culture conditions on albumin and gene expression in differentiating ES cells. Quantitative *in situ* immunofluorescence and cDNA microarray analyses were used to describe not only lineage specificity but also to provide insights into the effects of disparate culture environments on the mechanisms of differentiation. The results of these studies suggest that spontaneous and collagen-mediated differentiation induce cells with the highest levels of ALB expression but mature liver specific genes were only expressed in the spontaneous condition. Further analysis of gene expression profiles indicated that two distinct mechanisms may govern spontaneous and collagen-mediated differentiation.

INTRODUCTION

PLURIPOTENT EMBRYONIC STEM CELLS (ES) represent a promising renewable cell source to generate a variety of differentiated cell types including cardiomyocytes,¹ neural precursors,² and hepatocyte lineage cells,³ and may ultimately be incorporated into implantable engineered tissue constructs, or *ex vivo* cell-based therapeutic devices.^{4–7} *In vitro* aggregation of murine ES cells initiates the formation of embryoid bodies (EBs), which can facilitate spontaneous, unguided differentiation analogous to that seen in developing mouse embryos. During mouse embryogenesis, the primitive ectoderm gives rise to three distinct embryonic germ layers: definitive endoderm, mesoderm, and ectoderm.^{8,9} The primitive endoderm gives rise to both the visceral and parietal endoderm and while the definitive endoderm ultimately differentiates into mature hepatic tissue, the visceral endoderm is an extraembryonic tissue, which expresses many genes also commonly expressed in differentiating liver cells.¹⁰

Analyses characterizing the extent of EB differentiation have been limited to one or several lineage-specific protein or gene expression patterns. For example, after extracellular matrix (ECM) and exogenous growth factor supplementation, several groups of investigators have demonstrated EB-mediated differentiation of a population of albumin-positive cells,^{3,11–13}

or cells that express genes for albumin (ALB), alpha-feto protein (AFP), and transthyretin protein (TRT).^{14–18} Spontaneous differentiation of human EBs into hepatocyte lineage cells, in the absence of both ECM and exogenous growth factor supplementation, has also been identified using gene microarray analysis of several known mature liver specific proteins.^{20,21} Despite the growing number of studies reporting ES/hepatocyte differentiation, isolation of pure populations of neither committed hepatocyte precursors nor fully differentiated hepatocytes have been reported. Furthermore, it is unclear whether both spontaneous and directed EB-mediated differentiations are controlled by the same regulatory pathways or whether they yield identical hepatocyte lineage cells.

In the present study, we have evaluated the effects of four culture configurations on albumin and gene expression in order to gain further insight into the regulation of hepatocyte lineage commitment. We have assessed the dynamic expression of albumin within individual EBs. In addition, using complete mouse genome cDNA microarray analyses, we have assessed not only mature hepatocyte gene expression but have also explored the mechanism of hepatocyte lineage differentiation from ES cells. These studies identified unique hepatocyte-specific differentiation profiles among the culture conditions that may assist in unraveling the mechanisms that control EB-mediated hepatocyte differentiation and/or albumin expression.

MATERIALS AND METHODS

ES cell culture

All cell cultures were incubated at 37°C in a humidified 5% CO₂. The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated dishes (Biocoat, BD-Biosciences, Bedford, MA) in Knockout Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) containing 15% knockout serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), 10 µg/mL gentamicin (Gibco), 1000 U/mL ESGRO (Chemicon, Temecula, CA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Media was changed every 2 days. In order to induce differentiation, cells were suspended in Iscove's modified Dulbecco's medium containing 20% fetal bovine serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco), 10 µg/mL gentamicin (Gibco). Embryoid bodies were formed and cultured for 2 days using the hanging drop method (1 × 10³ ES cells per 30-µL drop). Hanging drops were transferred to suspension culture in 100-mm Petri dishes and cultured for an additional 3 days. The EBs were then plated, one EB per well, in six-well plates coated with or without collagen type I (40–60 µg/mL, Discovery Labware, Bedford, MA). When growth factors were supplemented, 100 ng/mL acidic fibroblast growth factor (aFGF) (days 8–11) and 20 ng/mL hepatocyte growth factor (HGF) (Sigma, St. Louis, MO) (days 11–17) were added to the culture medium. Growth factors were selected to induce early hepatocyte commitment and not late-stage differentiation. The Hepa 1-6 cell line (ATCC, Manassas, VA) was maintained in Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), and 4 mM L-glutamine (Gibco).

EB size

EB size was evaluated as a function of cell number/EB. Individual EBs were trypsinized and cells were counted. Twelve EBs were counted for each experimental day, and the experiments were repeated three times. The same procedure was followed for all culture conditions.

In situ indirect immunofluorescence

On evaluation days 11, 14, and 17, EBs were washed in phosphate-buffered saline (PBS) (Gibco) and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature. The EBs were washed twice for 10 min in cold PBS and then twice for 10 min in cold saponin/PBS (SAP) membrane permeabilization buffer containing 1% bovine serum albumin (Sigma-Aldrich), 0.5% saponin (Sigma-Aldrich), and 0.1% sodium azide (Sigma-Aldrich). The EBs were subsequently incubated for 30 min at 4°C in a SAP solution containing rabbit anti-mouse albumin antibody (150 µg/mL) (MP Biomedicals, Irvine, CA), or normal rabbit serum (NRS) (150 µg/mL) (MP Biomedicals) as an isotype control of the background intensity generated by nonspecific binding, washed twice for 10 min in cold SAP buffer, and then treated for 30 min at 4°C with the secondary antibody, fluorescein isothiocyanate-conjugated donkey anti-rabbit, diluted 1:500 (Jackson Immuno Labs, Westgrove, PA). EBs were then washed once with cold SAP buffer and once with cold PBS. Fluorescent images were acquired with an Olympus IX70 microscope and Olympus digital camera using an excitation filter of 515 nm. Fluorescent albumin expressing cells were only observed in the outer EB region and not in the dense intermediate region or the central core. Image fluorescence was quantified with Olympus Microsuite. To generate intensity values for each of the samples, 10 cells located within this outer monolayer EB region were chosen at random from the phase contrast image. The average intensity value for 10 randomly chosen cells was measured in the NRS (control) case, and this average background intensity was then subtracted from each value of the 10 randomly chosen cells in the anti-albumin (experimental) case. This was done in triplicate experiments yielding a total of 30 cells per condition.

Each data point represents the mean, and the error bars represent the standard error of the mean. Statistical significance was determined using the student t-test for un-paired data. Differences were considered significant when the probability was less than, or equal to, 0.05. Each experiment was repeated a minimum of three times, with a minimum of three replicates per experiment.

RNA preparation for microarray analysis

RNA was prepared from ES- and EB-derived cells. Cells were homogenized with a tissue grinder in TRIzol (Invitrogen). Chloroform was added to the TRIzol homogenate to separate phases, then the aqueous phase was removed, mixed with an equal volume of 70% ethanol, and loaded onto an RNeasy column (Qiagen, Valencia, CA). The column was washed and RNA was eluted according to manufacturer's recommendations. RNA was subjected to spectroscopic analysis of quantity and purity, with A_{260}/A_{280} ratios, at pH 8.0, between 1.9 and 2.1 for all samples. All RNA samples were subjected to capillary electrophoresis on an Agilent 2100 Bioanalyzer (Palo Alto, CA), with all samples demonstrating sharp 18S and 28S ribosomal RNA bands.

Microarray design, printing, and processing

The 21,997 probes on the custom microarrays contain a collection of oligonucleotides specific for mouse cDNA clusters purchased from Compugen/Sigma-Gnesis Oligos. The probes, 65–70 nt in length, are standardized for melting temperature and homology is minimized. Microarrays were printed at the Rutgers University Keck Center on poly-L-lysine-coated glass slides using an OmniGrid microarrayer (GeneMachines, San Carlos, CA) and quill-type printing pins. Poly-L-lysine slides were prepared and scanned at 532 nm and 635 nm in an Axon GenePix 4000B Scanner (Axon Instruments, Union City, CA) to evaluate surface quality. Slides were stored in a bench-top desiccator for at least 3 weeks prior to use. Oligonucleotides were resuspended to 40 µM in $3 \times$ SSC and rehydrated with shaking at room temperature. Printing was performed at 24°C with a relative humidity of

50%. After printing, arrays were stored overnight in a Parafilm (Fisher Scientific)-sealed plastic slide box in a desiccator at room temperature and postprocessed by standard procedures. Slides were stored at room temperature in a sealed plastic slide box in a desiccator and used for up to 3 months after printing.

Hybridization

Fluorescent probes were prepared using the Genisphere 3DNA dendrimer system (Genisphere, Hatfield, PA). Two micrograms of total cellular RNA was reverse-transcribed from a “capture-sequence”-containing oligod(T)₁₈ primer using SuperScript II (Invitrogen). After alkaline hydrolysis and neutralization, the cDNA target was hybridized with probes on the array at 58°C for 12 h using a Ventana Discovery workstation (Ventana Medical Systems, Tuscon, AZ) and washed to high stringency. Dye- and capture sequence-specific fluorescent dendrimers (Genisphere) were then hybridized at 58°C for 2 h. The arrays were washed after removal from the Discovery workstation and scanned on an Axon GenePix 4000B.

Microarray data analysis

Intensity values were obtained from the 16-bit tif. images generated from the scanner using TIGR Spotfinder (TIGR, Rockville, Maryland). A quality control check for spot size (using the Otsu algorithm) and intensity was applied at this point. Spots smaller than 2 pixels in diameter, larger than 25 pixels in diameter, or with fewer than 50% of the pixels being higher than 2 standard deviations of the background were flagged for later filtering. Local background values were also subtracted from spot intensity values using TIGR Spotfinder (TIGR, Rockville, Maryland). Intensity values were then normalized per channel per chip using the Lowess function contained in TIGR Midas (TIGR, Rockville, MD). The normalized data set was passed through a series of two filters to obtain a list of annotated genes that demonstrated differential expression in intensity between the experimental and control case in each of the respective experimental scenarios. In filter 1, genes are discarded in each experimental condition if any replicate within either the cy3 or the cy5 data set did not pass the aforementioned TIGR Spotfinder quality control check. The genes that passed this criterion were subjected to a second filter where analysis of variance (ANOVA) was performed to test each gene independently for a statistical difference in expression between the experimental condition and its respective control. The output of the analysis is the probability (*p* value) that a difference in expression can be observed by chance, i.e., probability of getting a false positive. Although the occurrence of false positives can be controlled by choosing a higher significance level (0.01 or 0.05), it also concomitantly increases the false negatives. Therefore, in this study we have chosen to work with ANOVA *p* value cutoff of 0.1. However, the large majority of genes reported here had *p* values below 0.05. To calculate the *p* value, we created an algorithm using the VBA package in Excel (Microsoft, Redmond, WA), which utilizes a two-sample, one-way ANOVA, analogous to a global two-tailed t-test. Three biological replicates were performed for each differentiation condition.

Gene network construction

Signaling pathways were modeled using Pathway Assist software (Ariadne Genomics, Rockville, MD). The Pathway Assist package utilizes a natural language algorithm (MedScan) to extract information on gene interactions from the ResNet database. In our analysis, we utilized the shortest path approach to link differentially expressed genes, with filters inclusive for protein nodes as well as regulatory controls.

RESULTS

Dynamic studies of hepatocyte function after ES differentiation

Cultures were established to study the differentiation of individual EBs in the absence of outer wall mechanical constraint or interacting EB interference. Hepatocyte lineage commitment was initially assessed by examining the dynamics of albumin expression after LIF removal, within the four culture environments, spontaneous, thin-layer collagen supplementation (C), FGF and HGF supplementation (GF), and a combination of thin-layer collagen and growth factor supplementation (C+GF). One EB was plated into the center of a well and evaluated on days 11, 14, and 17 postplating. Cell number continued to increase throughout the 17-day culture period, but the cells did not reach the outer well boundary (Fig 1A). Cell numbers, approximately 1.2×10^5 cells per EB, were similar in all differentiation conditions. At the end of the culture period, the EB was characterized by a variety of cell morphologies contained outside the dense center core. Cells were rounded and densely packed within the intermediate region located adjacent to and surrounding the center core. The outer region was populated by elongated cells (Fig. 1B). These regions generally did not become pronounced until day 11 post-differentiation induction.

Experiments were designed to evaluate the differentiation of individual cells within the EB population and outside the EB central core by assessing *in situ* intracellular albumin expression. EB cultures were initiated as outlined above, and albumin levels were qualitatively assessed on day 17 post-differentiation induction using indirect immunofluorescence with either primary anti-albumin antibody or an immunoglobulin control serum and subsequently fluorescently labeled secondary antibody. Images were captured using digital microscopy in order to determine the location of albumin expressing cells within the EB. As depicted in Fig. 2, albumin expression was apparent within the EB outer region and not in the core or dense intermediate region, in both the spontaneous and C conditions. C+GF cultured cells expressed intermediate albumin levels. Albumin was not detected within either undifferentiated ES or GF cultured cell populations.

A dynamic profile of intracellular albumin expression was then established by quantifying the relative fluorescence intensity values in the digitized images (Fig. 3A). Intracellular albumin was expressed on day 11 in all differentiation conditions, but expression was not maintained in the GF condition. In fact, albumin levels were negligible in GF-cultured cells by day 17, where only 10% of the cells outside the EB core were albumin positive (Fig. 3B). In contrast, both C and spontaneous cultures expressed the highest albumin levels in 80% of the evaluated cells at day 17, whereas C+GF cultured cells expressed intermediate levels, with only 33% albumin-expressing cells outside the EB core.

Microarray analysis

In order to better evaluate maturational commitment to the hepatocyte lineage within the four culture environments, cDNA microarrays were used to generate gene expression profiles 17 days post-differentiation induction. The entire EB population was analyzed after EB incubation with trypsin and cell recovery. It is important to note that at later stages of differentiation, the inner central core was resistant to trypsin treatment, and therefore these cells were not included in the analysis. Differential gene expression was measured using a 22k complete mouse cDNA microarray and gene expression within differentiated cells quantified relative to undifferentiated ES cells. Data analysis revealed that 3079 genes were differentially expressed in the spontaneous condition, 2079 genes were differentially expressed in the GF condition, 1137 genes were differentially expressed in the C condition, and 2053 genes were differentially expressed in the C+GF condition relative to control ES cells (Table 1).

Hepatocyte lineage gene expression

Hepatocyte lineage differentiation was assessed by examining differential expression of liver-specific genes. Data analysis indicated that spontaneously cultured cells upregulated seven cytochrome P450 enzymes expressed in mature liver cells and downregulated fetal liver cytochrome P450 3A13. In addition, these cells expressed cytokeratin 18, a hepatocyte-specific cytoskeletal intermediate filament, cadherin 17, a liver and intestine cadherin, and transthyretin. Alcohol dehydrogenase 1 and aldehyde dehydrogenase 3A2 were upregulated in both the spontaneous and GF conditions (Table 2). Interestingly, of the 174 genes expressed in both the spontaneous and C conditions, which express albumin at almost equal levels on day 17, 138 or ~80% of the genes upregulated in one condition were downregulated in the other (data not shown). In fact, although albumin was expressed in day 17 C cultured cells, mature hepatocyte genes were not. The disparity between the differentially expressed genes in these two conditions suggested that different differentiation mechanisms may result in similar albumin expression levels.

Regulation of hepatocyte lineage commitment

In an effort to probe the mechanism of *in vitro* EB differentiation, we examined the expression of genes known to regulate liver development during normal *in vivo* embryogenesis, including growth factors and their receptors, ECM proteins, cell–matrix adhesion proteins, and cell–cell adhesion proteins.^{22,23} FGF2, HGF, and FGF receptor 1 were only expressed in the spontaneous condition, as shown in Table 3. FGF2 receptor was upregulated in the spontaneous condition as well as the GF condition. Exposure to collagen significantly decreased the number of differentially expressed growth factors and growth factor receptors.

Expression profiles of the fibronectin, laminin, and procollagen families of ECM proteins were also investigated. Fibronectin 1 was upregulated in both the spontaneous and GF conditions, but the expression was significantly greater in the spontaneous condition. Laminin expression was upregulated or downregulated in the spontaneous, GF, and C+GF conditions, but expression was unchanged in the C condition. Ten different members of the procollagen gene family were differentially expressed in the spontaneous condition, but only two were upregulated. A variety of cell adhesion genes, notably, integrins and cadherins, were also differentially expressed (Table 3). Thus, spontaneously differentiating cells, which expressed the most mature hepatocyte genes, also expressed the largest number of genes known to direct the differentiation of mature hepatocytes during embryogenesis.^{22–24}

Acute phase gene expression

In order to gain further insight into the differentiated state of albumin-producing EB cells, acute phase genes, known to be expressed in both regenerating liver and in primitive endodermally derived cells, were examined. Interleukin 1 alpha (IL1 α) was upregulated in both C and C+GF conditions, and interleukin 6 and tumor necrosis factor were downregulated in the spontaneous condition. In addition, cAMP responsive element (CREMP), activator protein 1 (AP-1), and STAT 1 genes were upregulated in the C+GF condition. Extracellular matrix remodeling genes, metalloproteinase 2 and 9, were also upregulated in the C+GF condition. These results are summarized in Table 4. The data suggest that both C and C+GF cell populations, which express albumin and acute phase but not mature hepatocyte genes, represent a maturational stage that is dramatically different from spontaneously differentiating cell populations.

Regulation of albumin expression

Our experimental results indicated that two conditions, spontaneous and C, induced high levels of albumin expression. However, although the spontaneous condition expressed hepatocyte specific genes, the C condition expressed genes commonly expressed during the acute phase response. In order to gain further insight into albumin regulation in these conditions, Pathway Assist software was used in conjunction with differentially expressed genes identified by our microarray analysis. This program generates regulatory pathways for input genes, upregulated in these conditions, from gene and protein interactions defined in current literature. As shown in Fig. 4A, albumin expression in the spontaneous condition is regulated by HGF and FGF. Interestingly, although epidermal growth factor and insulin were not differentially expressed in our system, these genes were identified as key mediators in this pathway. In contrast, a distinct albumin regulatory pathway was identified in the C condition (Fig. 4B). Albumin expression in the C condition was found to be potentially regulated by IL1 and IL2, and albumin in turn could regulate matrix metalloproteinase 9.

DISCUSSION

The development of implantable engineered liver tissue constructs and *ex vivo* hepatocyte-based therapeutic devices are limited by an inadequate hepatocyte cell source. Pluripotent embryonic stem cells may solve this cell source limitation problem, but their utility is contingent upon correct functional characterization and isolation. In the present study, we compared the effectiveness of guided and spontaneous differentiation from ES cell populations to generate a population of committed hepatocyte precursors. The present studies indicate that hepatocyte lineage commitment, characterized by a combination of hepatocyte specific gene expression and albumin production, was induced in the absence of both growth factors and ECM supplementation. In addition, although collagen-supplemented cultures produced albumin, their gene profiles and associated regulatory pathways are distinct from those of spontaneously induced cultures.

Although others have reported spontaneous differentiation of hepatocyte lineage cells,^{20,21} the mechanism of differentiation induction has not been reported. *In vivo*, FGF is known to direct embryonic liver development. In fact, during *in vivo* mouse embryogenesis, induction of hepatic gene expression from the definitive endoderm is initiated after FGF signaling.²⁴ Cells committed to the hepatocyte lineage subsequently respond to HGF and other late-stage growth factors to complete their functional differentiation.^{26,27} In addition, ES cells differentiated in the presence of cardiac mesoderm, an FGF-producing tissue, induces differentiation of neighboring cells into hepatocytes *in vitro*.^{23,25} The results of our microarray analyses indicate that FGF2, HGF, and FGF1 receptors were all upregulated in spontaneously differentiating EBs, but not in the other differentiation conditions tested. This suggests that an autoregulatory growth factor mechanism, similar to that of FGF-stimulated embryonic liver development, controls spontaneous EB differentiation and likely parallels normal embryonic development. FGF10, FGF17, and FGF receptor 2 regulation was also observed in other culture conditions. The data also suggest that the addition of this growth factor cocktail induces the upregulation of FGF10 and that collagen supplementation suppresses the expression of FGF receptor 2.

Interestingly, when both aFGF and HGF were supplemented to the EB cultures, either in the presence or absence of collagen, albumin expression actually diminished and/or mature hepatocyte genes were not expressed. In fact, in the absence of ECM supplementation, the addition of aFGF initially increased albumin expression on day 11, but expression was not sustained after aFGF removal and subsequent HGF addition. These data suggest that optimum concentrations of growth factors and/or their receptors may be autoregulated in the spontaneous EB culture and that the growth factor cocktail added in these experiments voids

the autoregulatory EB response. It is important to note that in our system, EBs cultured on collagen-coated surfaces in the presence of these growth factors, generated cells with significantly less albumin than either spontaneous or collagen-mediated differentiation and did not express mature hepatocyte genes. However, in our studies we evaluated only early (aFGF) and midstage (HGF) growth factors in order to evaluate commitment to the hepatocyte lineage. In contrast, others such as Hamazaki *et al.* added late-stage growth factors in addition to the ones used here, to induce end-stage differentiation. Further studies will evaluate the effect of late-stage growth factors such as insulin, transferrin, and selenous acid (ITS) and oncostatin M (OSM) on either functional augmentation or terminal differentiation of cells isolated from either the spontaneous or supplemented cultures.

Many investigators have measured ALB, AFP, and TRT expression in order to identify hepatocyte lineage cells. However, although these markers are widely used to assess function of mature hepatocytes isolated from adult livers, they may also be expressed in nonhepatic lineage embryonic cells.^{14–19} In addition, other markers often used in evaluating hepatocyte differentiation, such as glucose-6-phosphatase, have also been found, using reverse transcription-polymerase chain reaction (RT-PCR), in cells of a variety of other tissues including pancreatic islets.²⁸ In general, although RT-PCR is a powerful tool, only a limited number of genes are generally evaluated, and many of these genes are expressed in a variety of embryonic and adult tissues. Because EB differentiation produces a very heterogeneous population, we have elected to evaluate gene expression within the entire mouse genome. Although this approach may not be as quantitative as PCR, it provides a comparative platform of gene expression among parallel experimental cultures. The results of these analyses indicate that although high levels of albumin were expressed in both the spontaneous and C conditions, and intermediate albumin levels were expressed in C+GF condition, mature hepatocyte genes were only expressed in spontaneously differentiating cells. This suggests that albumin expression can be regulated by disparate mechanisms in these culture conditions and that distinct albumin-expressing cell lineages may be differentiated in unique culture configurations.

In addition, EB cultures can yield not only hepatocyte lineage cells but may also induce albumin-expressing visceral endodermal lineage cells of the primitive endoderm.¹⁰ This is also suggested in our studies by the expression of many acute phase response genes in EB-derived cells and is supported by the Pathway Assist analysis suggesting distinct albumin regulatory networks in the different culture conditions. Although primitive endodermal cells may initially be generated in the spontaneous culture condition as well, these cells may be replaced by the differentiating hepatocyte lineage cells in this culture configuration. A dynamic gene array study will assist in elucidating both the differentiation profile and the pathways that may be activated during hepatocyte differentiation of ES cells.

Because neither the C+GF nor the C condition expressed a significant number of mature hepatocyte genes, we explored alternative mechanisms to explain the appearance of embryonic albumin-producing cells. IL1 α , in conjunction with collagen, has been shown to induce myofibroblastic activation, through matrix remodeling proteins such as matrix metalloproteinase 9 (MMP-9), during the liver acute phase response to injury.²⁹ In addition, expression of extracellular MMPs is developmentally controlled during the differentiation and spreading of the parietal endoderm.³⁰ In both C and C+GF conditions, gene expression of IL1 α was significantly increased. Although metalloproteinase was not upregulated in the C condition, the C+GF condition demonstrated not only increased MMP-9 gene expression but also a variety of other early acute phase genes listed in Table 4. These data suggest that the mechanism activated upon exposure to collagen may initiate upregulation of acute phase genes within primitive endodermal cells and ultimately an increased number of primitive endodermal cells within the EB. The effect was more pronounced in the C+GF condition,

which also demonstrated decreased albumin expression and may therefore be regulated by the addition of early-stage growth factors. Therefore, although collagen may initiate the acute phase response during EB differentiation, the effect may be more pronounced in the presence of growth factors.

Unlike the other culture conditions established in our studies, GF did not express albumin, acute-phase genes, or mature hepatocyte genes. Although we cannot definitively assign these cells to a specific embryonic lineage, they may represent a more primitive embryonic stage. It is also important to note that although identifying up-regulated genes may assist in characterizing the cell lineage of differentiating cells, these data may not reflect actual protein synthetic levels. Future studies will assess the protein expression profiles of the genes identified in these studies. In addition, although we have identified hepatocyte lineage cells in spontaneously differentiating cultures, the incorporation of these cells into tissue constructs is contingent upon optimizing differentiated function and cell purification. The intrinsic heterogeneity of the differentiating EB makes characterization of a specific cell type difficult, and further cell purification may also help yield a more accurate analysis. This is especially important since we have identified other lineage specific cells including nerve, lymphoid and muscle in our EB cultures (data not shown). In fact, even though the dense inner region did not contain albumin-expressing cells, it contains cells more reminiscent of mature hepatocyte morphology.

Nevertheless, our data indicate that spontaneous differentiation induces high ALB expression and liver-specific gene upregulation. In addition, we have identified disparate pathways that may regulate albumin expression during EB differentiation. Experimental manipulation of these pathways may assist in engineering more efficient hepatocyte differentiation from ES cells.

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FIG. 1.

EB size and morphology characterization. **(A)** EB cell number was assessed by counting total cells per EB dissociated after trypsinization. Asterisk (*) indicates time points at which the central condensed core was not dissociated by trypsin. **(B)** All images were acquired on day 17 post-differentiation induction. (a) 4× magnification, phase contrast image of the dense EB core and sparser migrating outer cells. (b) 10× magnification, phase contrast image of core border with monolayer cells that have migrated out of the core. (c) 40× magnification, phase contrast image of cells of an elongated morphology, located on the outer edge of the growing EB. (d) 40× magnification, phase contrast image of densely packed cells located in between the core and outer region of elongated cells. These cells are reminiscent of the typical cobblestone morphology indicative of hepatic cells.

FIG. 2.

10× images of phase contrast and *in situ* albumin staining on day 17 of differentiation. Differentiated EBs in the four culture conditions were fixed in place with paraformaldehyde and stained with an anti-albumin antibody followed by fluorescently labeled anti-immunoglobulin antibody. The images (phase contrast, left and fluorescent, right) were captured with a computer-interfaced Zeiss inverted microscope. (A) Spontaneous, (B) C, (C) GF, (D) C+GF, (E) ES control. (Color images are available at <www.liebertpub.com/ten>.)

FIG. 3.

Intracellular albumin expression. **(A)** Time course of albumin expression levels in the four conditions. Undifferentiated ES cells were used as a negative control (average intensity (0)). Each data point represents the mean of a sample size of 30 cells from the monolayer, where the mean average intensity value across the entire cell is used in the average of the 30 cells and error bars represent standard error of the mean of 30 cells. Asterisk indicates statistically significant differences ($p < 0.05$) from all other conditions on that day. **(B)** Time course of the percentage of outer region EB cells expressing albumin in the four conditions. Each data point represents the percentage of cells with an intensity reading above 0. The average of three experiments is presented. All values were statistically significant as compared to the ES control.

FIG. 4.

Pathway Assist Generated Pathways. The pathways were generated utilizing the shortest path approach to link differentially expressed genes. **(A)** Input genes were regulated in the spontaneous condition only. Asterisk indicates genes identified and inserted by Pathway Assist. **(B)** Input genes were regulated in the C+GF and C conditions. Asterisk indicates genes identified and inserted by Pathway Assist. (Color images are available at www.liebertpub.com/ten.)

Table 1

Effects of the Four Culture Conditions on Differential Gene Expression

Conditions	Upregulated	Downregulated	Total
Spontaneous	1410	1663	3073
+GF	996	1082	2078
C	626	511	1137
C+GF	1261	792	2053

Microarray analysis of differentially expressed genes 17 days after the start of differentiation. Expression levels of differentiated cells were compared to expression profiles of ES cells. Here, all genes that were upregulated or downregulated as compared to ES cells ($p < 0.1$) are shown for the four conditions.

Table 2

Representative Genes Differentially Expressed in at Least One of the Four Differentiation Conditions

Gene	Spontaneous	GF	C	C+GF
Cytochrome P450s				
CYP 1B1	0.891	N/S	N/S	N/S
CYP 19A1	0.414	N/S	N/S	N/S
CYP 2E1	0.385	N/S	N/S	1.405
CYP 2J6	1.098	N/S	N/S	N/S
CYP 2S1	-0.567	N/S	N/S	N/S
CYP 3A13	-0.346	N/S	N/S	N/S
CYP 3A24	1.981	1.59	N/S	N/S
CYP 46A1	-0.18	N/S	N/S	N/S
Cytokeratin 18	0.607	N/S	N/S	N/S
Alcohol dehydrogenase 1	0.919	1.919	N/S	N/S
Aldehyde dehydrogenase family 3A2	1.679	0.985	N/S	N/S
Cadherin 17	0.755	N/S	N/S	N/S
Tranthyretin	0.551	N/S	N/S	0.769

All numbers represent the log ratio (base 2) of the (cy3/cy5 mean), $\log_2$ (mean cy3/cy5) of that specific gene. N/S is representative of all nonsignificant differences ($p > 0.1$) in the $\log_2$ (mean cy3/cy5) ratio. All positive numbers indicate upregulation and negative numbers indicate downregulation. Undifferentiated mouse ES cells were used as the control cell type.

Table 3

Representative Differentially Expressed Growth Factor, Growth Factor Receptor, and ECM Protein Genes

Gene	Spontaneous	GF	C	C+GF
Growth factors				
Hepatocyte growth factor	0.595	N/S	N/S	N/S
Fibroblast growth factor 2	0.273	N/S	N/S	N/S
Fibroblast growth factor 10	N/S	0.99	N/S	0.81
Fibroblast growth factor 17	0.913	N/S	-0.79	-1.11
Fibroblast growth factor receptor 1	0.864	N/S	N/S	N/S
Fibroblast growth factor receptor 2	1.367	1.456	N/S	N/S
Matrix proteins				
Fibronectin 1	0.573	1.107	N/S	N/S
Laminin gamma 3	-1.059	-1.03	N/S	N/S
Laminin, alpha 4	N/S	0.358	N/S	N/S
Laminin, beta 3	0.955	1.092		-0.809
Procollagen, type II, alpha 1	N/S	N/S	-0.66	N/S
Procollagen, type IV, alpha 3	-0.447	N/S	N/S	1.184
Procollagen, type IV, alpha 6	-2.057	-2.044	N/S	N/S
Procollagen, type V, alpha 2	0.837	N/S	N/S	N/S
Procollagen, type V, alpha 2	0.688	N/S	N/S	N/S
Procollagen, type X, alpha 1	-1.281	N/S	N/S	N/S
Procollagen, type XI, alpha 2	N/S	N/S	-0.787	0.727
Adhesion proteins				
Integrin alpha 2	N/S	N/S	N/S	0.76
Integrin alpha 3	-1.11	-1.307	N/S	0.976
Integrin beta 4	0.909	1.005	N/S	N/S
Integrin beta 5	-0.738	N/S	N/S	N/S
Integrin beta 6	0.64	N/S	-0.78	N/S
Cadherin 6	0.78	0.78	N/S	N/S
Cadherin 9	N/S	N/S	N/S	0.874
Cadherin 17	0.755	N/S	N/S	N/S
Cadherin 22	N/S	-0.213	N/S	N/S

All numbers represent the log ratio (base 2) of the (cy3/cy5 mean), $\log_2$ (mean cy3/cy5) of that specific gene. N/S is representative of all nonsignificant differences ($p > 0.1$) in the $\log_2$ (mean cy3/cy5) ratio. All positive numbers indicate upregulation and negative numbers indicate downregulation. Undifferentiated mouse ES cells were used as the control cell type.

Table 4**Acute Phase Response Genes**

Gene	Spontaneous	GF	C	C+GF
Interleukin 1 alpha	N/S	N/S	1.02	0.92
Interleukin 6	-0.470	N/S	N/S	N/S
Tumor necrosis factor	-0.980	-0.922	N/S	N/S
CAMP responsive element modulator	N/S	N/S	N/S	0.694
AP-1, gamma 1 subunit	N/S	N/S	N/S	1.281
STAT 1	N/S	N/S	N/S	1.732
Protein inhibitor of activated STAT 3	N/S	N/S	0.937	0.877
Matrix metalloproteinase 2	N/S	N/S	N/S	5.132
Matrix metalloproteinase 9	N/S	N/S	N/S	0.868

All numbers represent the log ratio (base 2) of the (cy3/cy5 mean), $\log_2$ (mean cy3/cy5) of that specific gene. N/S is representative of all nonsignificant differences ($p > 0.1$) in the $\log_2$ (mean cy3/cy5) ratio. All positive numbers indicate upregulation and negative numbers indicate downregulation. Undifferentiated mouse ES cells were used as the control cell type.