Development of definitive endoderm from embryonic stem cells in culture

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Summary

The cellular and molecular events regulating the induction and tissue-specific differentiation of endoderm are central to our understanding of the development and function of many organ systems. To define and characterize key components in this process, we have investigated the potential of embryonic stem (ES) cells to generate endoderm following their differentiation to embryoid bodies (EBs) in culture. We found that endoderm can be induced in EBs, either by limited exposure to serum or by culturing in the presence of activin A (activin) under serum-free conditions. By using an ES cell line with the green fluorescent protein (GFP) cDNA targeted to the brachyury locus, we demonstrate that endoderm develops from a brachyury[+] population that also displays mesoderm potential. Transplantation of cells generated from activin-induced brachyury^{+} cells to the kidney capsule of recipient mice resulted in the development of endoderm-derived structures. These findings demonstrate that ES cells can generate endoderm in culture and, as such, establish this differentiation system as a unique murine model for studying the development and specification of this germ layer.

Key words: Stem cells, Endoderm

Introduction

The development of definitive endoderm and its subsequent patterning and differentiation leads to the formation of many of the major organs including the liver, pancreas, lungs, thyroid and intestines (Wells and Melton, 1999). In the mouse, endoderm, together with mesoderm and ectoderm, is formed from the embryonic ectoderm of the epiblast through the process of gastrulation beginning at approximately day 6.5 of gestation (Tam and Behringer, 1997). During gastrulation, cells from specific regions of the epiblast are recruited to a structure known as the primitive streak where they undergo an epithelial-to-mesenchymal transition giving rise to mesoderm and definitive endoderm. This close proximity of mesoderm and endoderm formation within the primitive streak of the mouse embryo supports a concept established in other model systems that these lineages are generated from a bipotential population known as mesendoderm (Kimelman and Griffin, 2000; Rodaway and Patient, 2001).

The close developmental association between mesoderm and endoderm, possibly through a common progenitor, suggests that the same mechanisms regulate the early induction events leading to the establishment of these lineages. Although many factors and cellular interactions have been implicated in the regulation of different stages of endoderm and mesoderm formation, a consensus of findings from different studies point to an essential role for members of the TGFβ family of

molecules at the earliest stages in this process (Smith, 1993; Hogan, 1996; Schier and Shen, 2000; Stainier, 2002). Two of the most notable factors in this regard are activin and nodal. Activin was identified as a potential regulator of these early developmental decisions based on its capacity to induce mesoderm and endoderm in *Xenopus* animal caps in vitro (Smith et al., 1990; Jones et al., 1993; Gamer and Wright, 1995; Ninomiya et al., 1999) and from the findings that interference with its receptor function inhibited the development of these germ cell layers in the embryo (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994). Nodal and related factors are expressed prior to and during the onset of gastrulation and have been shown to play pivotal roles at the earliest stages of mesoderm and endoderm development in the mouse, *Xenopus* and zebrafish embryo (Zhou et al., 1993; Conlon et al., 1994; Jones et al., 1995; Feldman et al., 1998; Osada and Wright, 1999; Gritsman et al., 2000; Lowe et al., 2001).

Signaling events in the early embryo initiate the activation of a cascade of transcription factors that function at different stages in the induction and specification of definitive endoderm. With respect to the earliest induction steps, studies in *Xenopus* and zebrafish have clearly demonstrated a role for factors such as the homeodomain protein Mixer/Mix.3, (Henry and Melton, 1998) the Sry-related HMG-box transcription factor Sox 17 (Hudson et al., 1997) and the zinc-finger

transcription factor Gata5 (Reiter et al., 1999; Reiter et al., 2001; Weber et al., 2000). Targeting experiments in the mouse have similarly shown that Sox17 (Kanai-Azuma et al., 2002) and the mix-like gene, Mixl-1 (Hart et al., 2002) are essential for endoderm development, indicating that the mechanisms regulating early induction events are evolutionarily conserved. Hepatocyte nuclear factors (HNFs), a group of proteins originally identified as regulators of liver gene expression, also play important roles in endoderm development (Darlington, 1999). In the mouse, Foxa2 (previously known as HNF3β), a factor expressed in the anterior region of the primitive streak, in endoderm and the early liver (Monaghan et al., 1993; Sasaki and Hogan, 1993), is essential for the development of prospective foregut and midgut endoderm (Ang et al., 1993; Weinstein et al., 1994). Beyond the induction stage, numerous other transcription factors are required for endoderm patterning and organ development. Nkx2.1 is required for thyroid development and lung morphogenesis (Lazzaro et al., 1991), Hhex for liver and thyroid development (Martinez Barbera et al., 2000), and Ipf1 (Pdx1) for the formation of the ventral and dorsal pancreas (Jonsson et al., 1994).

Much of our current knowledge of endoderm induction is based on findings from studies using model systems such as *Xenopus* and zebrafish that provide easy access to early embryonic stages of development at a time when lineage commitment decisions are taking place. By contrast, the mouse embryo is much less amenable to such experimental approaches because of its difficult accessibility and limiting amounts of tissue. The in vitro differentiation of embryonic stem (ES) cells provides an attractive alternative model system to the mouse embryo for addressing questions relating to early lineage commitment (Keller, 1995; Smith, 2001). Under appropriate culture conditions, ES cells will differentiate into embryoid bodies (EBs) that can contain derivatives of all three germ cell layers. To date, the majority of such studies have focused on the development of mesoderm and ectoderm derivatives and as a consequence, conditions have been established for the efficient and reproducible differentiation of the hematopoietic, vascular, muscle and neural lineages (Keller et al., 1993; Nakano et al., 1994; Rohwedel et al., 1994; Bain et al., 1995; Okabe et al., 1996; Vittet et al., 1996; Nishikawa et al., 1998; Czyz and Wobus, 2001). More recently, several studies have provided evidence for endoderm development in ES differentiation cultures and demonstrated the generation of insulin-expressing cells and cells with hepatocyte characteristics (Abe et al., 1996; Hamazaki et al., 2001; Lumelsky et al., 2001; Hori et al., 2002; Jones et al., 2002; Yamada et al., 2002a; Yamada et al., 2002b; Blyszczuk et al., 2003). None, however, has established conditions for the efficient induction of endoderm, nor has any defined the origin of the specific cell populations in the study.

In this report, we have investigated the events that regulate endoderm development in ES differentiation cultures and demonstrate that exposure of EBs to factor(s) present in serum for restricted periods of time is essential for the establishment of this lineage. In addition to serum factors, we show that activin A can induce mesoderm and endoderm formation in EBs in serum-free cultures and that lineage development is dependent on the concentration of factor used. Using an ES cell line with the GFP cDNA targeted to the brachyury locus (Fehling et al., 2003), we provide evidence that the endoderm

lineage develops from a brachyury$^+$ population with mesoderm potential.

Materials and methods

Growth and differentiation of ES cells

The development, characterization and maintenance of the GFP-Bry ES cell line has been recently reported (Fehling et al., 2003). Prior to the initiation of differentiation, feeder-dependent ES cells were passaged twice on gelatin-coated dishes to deplete the population of feeder cells. To generate EBs, ES cells were dissociated with trypsin and cultured at various concentrations (10^3 to 8×10^4 cells/ml) in 60 mm petri-grade dishes in differentiation media that consisted of Iscoves' modified Dulbecco's medium (IMDM) supplemented with penicillin/streptomycin, 2 mM glutamine (Gibco/BRL, Grand Island, NY), 0.5 mM ascorbic acid (Sigma), 4.5×10^{-4} M MTG, 15% FCS (Summit, Ft Collins, CO), 5% protein free hybridoma medium (PFHM-II; Gibco/BRL) and 200 µg/ml transferrin (Boehringer Mannheim, Indianapolis, IN). Cultures were maintained in a humidified chamber in a 5% CO_2-air mixture at 37°C.

For endoderm differentiation, serum-stimulated EBs were harvested at day 2.5 of differentiation, allowed to settle by gravity and then replated in 60 mm dishes in IMDM supplemented with 15% serum replacement (SR; Gibco/BRL), penicillin/streptomycin, 2 mM glutamine, 0.5 mM ascorbic acid and 4.5×10^{-4} M MTG (hereafter referred to as serum replacement medium). Activin induction was carried out using a two-step culture protocol. In the first step, EB differentiation was initiated in Stem Pro 34 medium (Gibco) supplemented with 2 mM glutamine, 0.5 mM ascorbic acid, 4.5×10^{-4} M MTG and Kit ligand (1% conditioned medium) at a concentration of 2×10^3 ES cells/ml. For the second step, EBs were harvested at 48 hours of differentiation, allowed to settle by gravity in a 50 ml tube and transferred to new dishes and cultured in IMDM supplemented with 15% SR, 2 mM glutamine, 0.5 mM ascorbic acid, 4.5×10^{-4} M MTG and different concentrations of human activin A (R&D Systems).

Hepatocyte differentiation

For the hepatocyte differentiation, day 2.5 serum-induced EBs were transferred to serum replacement media for an additional 3.5 to 7.5 days. At days 6 or 10 of differentiation, EBs were harvested and replated intact on six-well tissue culture dishes coated with matrigel (Becton Dickenson, San Jose, CA) (~20 EBs per well) in IMDM with 15% FCS and 10^{-7} M dexamethasone (Dex) (Sigma) (referred to as serum hepatocyte cultures). Cells were harvested for expression analysis at day 14 (total time) of culture. For the analysis of brachyury subpopulations, GFP-Bry$^+$ and GFP-Bry$^-$ cells were sorted at day 2.5, and reaggregated (3×10^5 cells/ml) for 1 day in ultra low attachment 24-well plates (Costar) in serum replacement media. The reaggregated EBs were transferred to 60 mm petri-grade dishes with serum replacement media. At a total of 6 or 10 days of differentiation, EBs were replated on matrigel coated 6 well dishes in serum replacement media supplemented with 5 ng/ml bFGF (R&D Systems, Minneapolis, MN) (serum-free hepatocyte conditions). Cells from the replated cultures were harvested at day 14 (total time for differentiation) for RNA isolation and immunostaining. For activin-induced cultures, cells from the pre-sorted, the GFP-Bry$^+$ and GFP-Bry$^-$ populations from day five EBs were reaggregated in serum replacement media and then cultured in the same media in the absence of activin for 8 days. At day 13, the reaggregated EBs were replated in serum hepatocyte conditions for four days and then harvested for RT-PCR analysis.

Hematopoietic progenitor assays

For hematopoietic colony assays, EBs were trypsinized to single cell suspensions and plated (5×10^4–1×10^5 cells/ml) in 1% methylcellulose

Table 1. Primers used and PCR conditions

Gene	Forward	Reverse	Product size (bp)	Taq polymerase used	Annealing temperature (°C)	Cycle number
Rex1	CGTGTAACATACACCATCCG	GAAATCCTCTTCCAGAATGG	129	ProMega	60	35
Fgf5	AAAGTCAATGGCTCCCACGAA	CTTCAGTCTGTACTTCACTGG	465	ProMega	60	35
Pax6	GCTTCATCCGAGTCTTCTCCGTTAG	CCATC TTGCTTGGGAAATCCG	312	platinum	60	30
Wnt1	GATTGCGAAGATGAACGCTGTTTC	TCCTCCACGAACCTGTTGACGG	266	ProMega	55	40
Neurod1	CTTGGCCAAGAACTACATCTGG	GGAGTAGGGATGCACCGGGAA	209	ProMega	60	35
Foxa2	TGGTCACTGGGGACAAGGGAA	GCAACAACAGCAATAGAGAAC	289	ProMega	60	35
Tcf1	CGAAGATGGTCAAGTCGTAC	GGCAAACCAGTTGTAGACAC	461	ProMega	60	35
Hnf4	ACACGTCCCCATCTGAAGGTG	CTTCCTTCTTCATGCCAGCCC	270	ProMega	60	35
Brachyury	CATGTACTCTTTCTTGCTGG	GGTCTCGGGAAAGCAGTGGC	313	ProMega	60	35
Gata1	CATTGGCCCCTTGTGAGGCCAGAGA	ACCTGATGGAGCTTGAAATAGAGGC	289	ProMega	60	35
Csf1r	GCGATGTGTGAGCAATGGCAGT	AGACCGTTTTGCGTAAGACCTG	341	ProMega	60	35
Sox17	GCCAAAGACGAACGCAAGCGGT	TCATGCGCTTCACCTGCTTG	211	platinum	60	35
Afp	GCTCACACCAAAGCGTCAAC	CCTGTGAACTCTGGTATCAG	410	platinum	60	40
Alb1	GCTACGGCACAGTGCTTG	CAGGATTGCAGACAGATAGTC	266	ProMega	60	35
Hhex	AGTGGCTTCGGAGGCCCTCTGTAC	GCCGGATCCTGACTGTCATCCAGCATTAA	540	ProMega	60	35
Ttr	AGTCCTGGATGCTGTCCGAG	TTCCTGAGCTGCTAACACGG	440	ProMega	60	35
Aat	AATGGAAGAAGCCATTCGAT	AAGACTGTAGCTGCTGCAGC	484	ProMega	55	35
Tat	ACCTTCAATCCCATCCGA	TCCCGACTGGATAGGTAG	205	ProMega	55	35
Cps1	ATGACGAGGATTTTGACAGC	CTTCACAGAAAGGAGCCTGA	126	ProMega	60	35
Ipf1	CCACCCCAGTTTACAAGCTC	TGTAGGCAGTACGGGTCCTC	325	platinum	60	35
Mixl1	GCACGTCGTTCAGCTCGGAGCAGC	AGTCATGCTGGGATCCGGAACGTGG	305	ProMega	60	35
Kdr	CACCTGGCACTCTCCACCTTC	GATTTCATCCCACTACCGAAAG	239	ProMega	60	35
Myf5	GAAGGCTCCTGTATCCCCTCAC	GTTCTCCACCTGTTCCCTCAGC	384	ProMega	60	35
Skeletal actin	TTATCGGTATGGAGTCTGCGGG	CACAGCACGATTGTCGATTGTGG	393	platinum	60	35
Sftpc	CATCGTTGTGTATGACTACCAGAG	GAATCGGACTCGGAACCAGTATC	207	platinum	60	35
β-Actin	ATGAAGATCCTGACCGAGCG	TACTTGCGCTCAGGAGGAGC	443	ProMega	60	25

containing 10% plasma derived serum (Antech, Tyler, TX), 5% PFHM-II and the following cytokines; Kit ligand (KL, 1% conditioned medium), interleukin 3 (IL3, 1% conditioned medium), thrombopoietin (Tpo, 5 ng/ml), erythropoietin (Epo, 2 U/ml), interleukin-11 (IL-11, 5 ng/ml), granulocyte-macrophage colony stimulating factor (GM-CSF, 3 ng/ml) and macrophage colony-stimulating factor (M-CSF, 5 ng/ml). Primitive erythroid colonies were counted at day 4, whereas macrophage and multilineage colonies were counted at day 7 of culture. Kit ligand was derived from media conditioned by CHO cells transfected with a KL expression vector (kindly provided by Genetics Institute). IL3 was obtained from medium conditioned by X63 AG8-653 myeloma cells transfected with a vector expressing IL3 (Karasuyama and Melchers, 1988); GM-CSF, M-CSF and TPO were purchased from R&D Systems.

Gene expression analysis

Total RNA was extracted using a RNeasy mini-kit and treated with RNase free DNase (Qiagen, Valencia, CA). Total RNA (2 μg) was reverse-transcribed into cDNA with random hexamers using Omniscript RT kit (Qiagen). PCR was performed with Taq polymerase (Promega, Madison, WI) or platinum Taq (Invitrogen, Carlsbad, CA) in PCR buffer, 2.5 mM, 0.2 μM dNTPs. Cycling conditions were as follows; 94°C for 5 minutes followed by 25-40 cycles of amplification (94°C denaturation for 1 minute, annealing for 30 seconds, 72°C elongation for 1 minute), with a final incubation at 72°C for 7 minutes. Details of primer sequences, annealing temperature and cycle numbers for each PCR reaction are shown in Table 1.

FACS analysis and cell sorting

EBs generated from the GFP-Bry ES cells under different conditions were analyzed using a FACScan (Becton Dickenson) or sorted on a MoFlo cell sorter (Cytomation Systems, Fort Collins, CO).

Immunostaining

Foxa2 staining of brachyury+ cells was carried out in microtiter wells. Cells (1×10^5 ml) were centrifuged in 96-well plates, the supernatant removed and the cells then fixed for 30 minutes in 100 μl of

paraformaldehyde solution (4%). After fixation, the cells were washed twice with PBS, permeabilized in PBS containing 0.2% triton for 5 minutes at room temperature, washed with 150 μl of PBS with 0.1% tween 20 and 0.02% azide and then blocked with PBS containing 10% horse serum for 10 minutes at room temperature. Following the blocking step, the cells were washed again with PBS with 0.1% tween (wash media) and then incubated with an anti-Foxa2 antibody (goat polyclonal P-19, Santa Cruz) in PBS containing 10% FCS and 0.1% tween (staining media) for 30 minutes at room temperature. The stained cells were washed and then incubated with a Cy3-labeled anti-goat IgG (Jackson Immunoresearch, West Grove, PA) in staining solution for 30 minutes at room temperature. After the second staining step, the cells were washed and then covered with 10 μl of vectastain mounting media containing DAPI. The cells in the mounting media were transferred to a slide and covered with a coverslip. For the albumin staining, day 10 EBs were partially dissociated to small aggregates by a 30-second trypsin treatment and cultured on HCl-treated, gelatin-coated glass cover slips for 4 days. For skeletal muscle staining, intact 10-day-old activin stimulated EBs were cultured on coverslips for 4 days. After culture, the cells were processes as described above for the Foxa2 staining. Fixed and blocked cells were incubated for 1 hour with either anti-albumin (Biogenesis, Kingston, NH), anti-skeletal myosin or α-actinin (Sigma) primary antibodies. Expression of these proteins was visualized using a Cy3-conjugated anti-mouse or rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA). Tissue sections were stained with either an anti-Foxa2 (Lake Placid, New York) or anti-Ifabp (Green et al., 1992b) primary antibody. Secondary antibody treatment and color development were carried out using Vectastain ABC kits (Vector Laboratory Burlingame California).

In situ hybridization

The murine cDNA for Sftpc (758 bp) was used as a template for generating riboprobes. In situ hybridization was performed on paraformaldehyde fixed, paraffin wax-embedded sections as previously described (Deterding and Shannon, 1995) with the exception that ^{33}P-UTP was used for labeling the probe.

Fig. 1. Effects of serum on endoderm development and hepatocyte differentiation. EBs were differentiated either in serum for the entire six-day period (serum) or initiated in serum for 2.5 days and then passaged to serum-free cultures for the remaining 3.5 days (SF). (A) RT-PCR expression analysis of different aged EBs. (B) FACS analysis of GFP-Bry expression in EBs differentiated in serum-containing (serum) or serum-containing followed by serum-free media (serum/SF). (C) Hematopoietic progenitor analysis of EBs generated under different conditions. Numbers represent colonies per 1×10^5 cells plated. Data represents mean±s.e.m. (n=3). Ep, primitive erythroid colonies; Mac, macrophage colonies; Mix, multilineage colonies. (D) RT-PCR analysis of replated cultures from day 10 EBs generated in serum/SF (S/SF) cultures. EB were replated for 4 days on matrigel with dexamethasone (10^{-7} M) in the presence of serum. S, replated cells from day 10 EBs differentiated in the presence of serum for the entire time; FL, day 14 fetal liver; AL, adult liver.
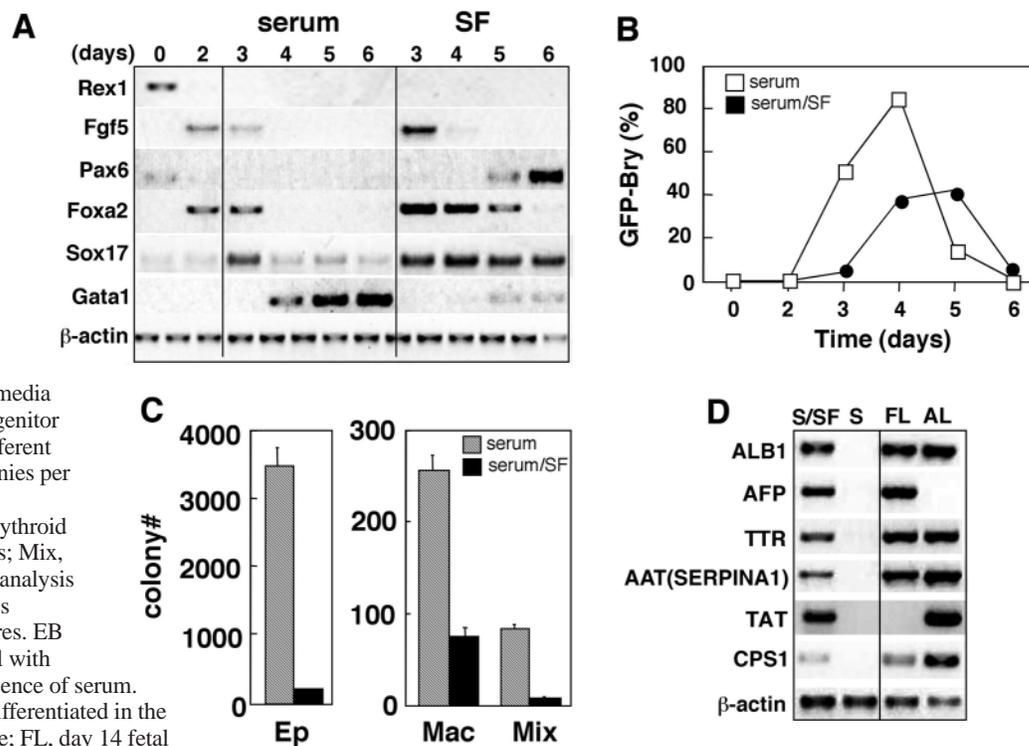
Transplantation of EBs under kidney capsule

Cell aggregates were harvested from the matrigel cultures by cell scraping and then transplanted under the kidney capsule of 5-week-old female SCID-beige mice. Three to four weeks following transplantation, the recipients were sacrificed, the kidneys removed and fixed in 4% paraformaldehyde. After fixation, kidneys were embedded in paraffin wax and sectioned for H/E and D-PAS staining, for immunostaining and for in situ hybridization.

Results

Restricted exposure of EBs to serum enhances expression of genes associated with definitive endoderm development

As an initial approach to evaluate endoderm development in EBs, we monitored expression of *Foxa2* and *Sox17* and compared these patterns with those of brachyury, *Gata1* and *Pax6*, genes that are indicative of mesoderm (Wilkinson et al., 1990), hematopoietic (Orkin, 1992) and neuroectoderm (Hill et al., 1991) development, respectively. For these studies, we used an ES cell line in which the green fluorescent protein (GFP) has been targeted to the brachyury locus (GFP-Bry) (Fehling et al., 2003). With this cell line, brachyury expression can be easily measured and quantified by expression of GFP using FACS analysis. Prior to the onset of differentiation, ES cells expressed *Rex1* (*Zfp42* – Mouse Genome Informatics), a gene that encodes a transcription factor found in ES cells but not in their differentiated progeny (Rogers et al., 1991) (Fig. 1A). Within 2 days of differentiation, EBs downregulated *Rex1* and upregulated *Fgf5* a gene expressed by the primitive ectoderm of the epiblast but not by the inner cell mass or ES cells (Haub and Goldfarb, 1991; Hebert et al., 1991; Rathjen et al., 1999). *Fgf5* expression persisted within the EBs for 48

hours, which is indicative of the presence of epiblast-like cells at this stage of development. Brachyury expression, defining mesoderm induction and development, was upregulated between days 2 and 3 of differentiation, reached peak levels by day 4 and then declined to undetectable levels by day 6 (Fig. 1B). The expression pattern of *Foxa2* was very similar to that of *Fgf5*, restricted to the day 2-3 window of differentiation. *Sox17* expression was upregulated in day 3 EBs and then persisted at low levels throughout the remainder of the time course. *Gata1* was expressed from days 4-6 of differentiation, defining the onset of hematopoiesis within the EBs. Analysis of day 6 EBs revealed the presence of primitive erythroid and definitive hematopoietic progenitors, confirming the establishment of the hematopoietic program at this stage (Fig. 1C). In contrast to the genes associated with mesoderm and endoderm development, *Pax6* was not expressed at any stage of differentiation, suggesting that these conditions do not support differentiation to the neuroectoderm lineage.

The temporally restricted expression patterns of *Foxa2* and *Sox17* are an indication that endoderm development is initiated, but may not be maintained in the presence of serum that was originally selected for optimal hematopoietic development in EBs. To determine if endoderm differentiation could be extended in the absence of serum, EBs stimulated with serum for 2.5 days were transferred to serum-free cultures for an additional 3.5 days and then analyzed for *Foxa2* and *Sox17* expression. As shown in Fig. 1A (SF), removal of the serum-stimulus at 2.5 days of differentiation prolonged expression of *Foxa2* and *Sox17* and significantly reduced *Gata1* expression compared with EBs maintained in serum. The restricted 2.5-day exposure to serum also resulted in lower levels of brachyury expression and decreased hematopoietic potential in the resulting EBs (Fig. 1B,C). The conclusion from these

observations is that restricted exposure to serum followed by a serum-free culture period promotes the induction and development of endoderm and suppresses formation of mesoderm and derivative populations within EBs.

Development of the hepatocyte lineage from EBs expressing endoderm genes

Previous studies have demonstrated that ES cells can generate cells with hepatocyte characteristics indicating that derivatives of definitive endoderm can develop in this model system (Hamazaki et al., 2001; Jones et al., 2002; Yamada et al., 2002a). To determine if the EBs generated using the serum/serum-free protocol could differentiate to hepatocyte-like cells, they were subsequently cultured under conditions known to support development of this lineage (Hamazaki et al., 2001; Kamiya et al., 2001; Kamiya et al., 2002). Early hepatocyte development was marked by the presence of α-fetoprotien (AFP), albumin (ALB1), transthyretin (TTR) and alpha1-antitrypsin (AAT; SERPINA1 – Mouse Genome Informatics) expression, while further maturation of the lineage was defined by expression of tyrosine aminotransferase (TAT) and carbamoyl phosphate synthetase I (CPS1), genes that encode enzymes found in mature hepatocytes (Hamazaki et al., 2001; Yamada et al., 2002a). When 10-day-old EBs (2.5 days serum-containing medium, 7.5 days SF) were replated on matrigel-coated plates in cultures containing serum and dexamethasone for 4 days (serum hepatocyte conditions), expression of all genes could be detected, indicating differentiation to the hepatocyte lineage (Fig. 1D, S/SF). Additional manipulations of the culture conditions revealed that EBs could be replated on matrigel at 6 rather than 10 days of differentiation and that the replated cultures could also be maintained in serum free conditions with bFGF (serum-free hepatocyte conditions) (not shown). The expression of genes associated with hepatocyte development and maturation in cells from these replated cultures is a strong indication that definitive endoderm is induced in EBs generated by a 2.5-day exposure to serum. Control cultures of EBs maintained in serum for the entire 10-day period prior to replating did not give rise to cell populations that expressed any of these hepatocyte genes, suggesting that endoderm potential is not maintained under a continuous serum stimulus (Fig. 1D,S).

Endoderm develops from brachyury+ cells

To define the relationship between cells with endodermal characteristics and mesoderm in the ES/EB system, brachyury+ (GFP-Bry+) and brachyury− (GFP-Bry−) cells were isolated from day 2.5 serum-stimulated EBs on the basis of GFP expression and assayed for endoderm potential (Fig. 2A). Cells from each fraction were allowed to reaggregate for 24 hours in serum-free media, as we have recently demonstrated that such aggregates will continue to differentiate with further culturing (Fehling et al., 2003). The aggregates from each fraction were cultured for an additional 2.5 days in serum-free conditions (total of 6 days of differentiation) and then replated for 4 days in serum-free hepatocyte conditions (Fig. 2A). Expression analysis of various endoderm genes was performed on the initial GFP-Bry sorted populations, the day 6 EBs and the replated cultures. *Foxa2* and *Mixl1* were expressed in the day 2.5 EBs and this expression segregated to the brachyury+ fraction (Fig. 2B; d2.5). *Sox17* and *Hhex* expression in the day

6 EBs (Fig. 2B; d6) and in the day 10 replated cultures (Fig. 2B; d10) as well as *Afp* and *Alb1* expression in the replated cultures was also restricted to cell populations derived from the brachyury+ cells. By contrast, *Pax6* and *Neurod1* (Lee et al., 1995), genes associated with neuroectoderm development were expressed in the brachyury−-derived cell populations. These findings indicate that endoderm develops from a brachyury+ population, whereas neuroectoderm derives from brachyury− cells.

To further evaluate the endodermal potential of the brachyury+ population, freshly isolated cells from day 2.5 EBs and cells from the day 10 replated cultures were stained with specific antibodies for the presence of Foxa2 and albumin, respectively. Between 5 and 10% of the brachyury+ cells isolated from day 2.5 EBs stained positive with an anti-Foxa2 antibody, suggesting that a significant number of cells in these EBs had endoderm potential (Fig. 2C). Few, if any, of the brachyury− cells were positive. Similarly, albumin-positive cells were detected only in the cultures derived from brachyury+ cells. As a final step in this analysis, cells from the respective brachyury fractions were cultured in serum hepatocyte conditions and analyzed for the markers indicative of hepatocyte development and maturation. *Ttr*, *Serpina1*, *Tat* and *Cps1* expression was all restricted to the cell populations that developed from the brachyury+ fraction isolated from day 2.5 EBs (Fig. 2D). Taken together, these data demonstrate that endoderm-derived cell populations develop from brachyury+ cells, adding further support to the existence of mesendoderm.

Activin A induces mesoderm and endoderm in EBs

To define the regulation of endoderm induction in the EB model in more detail, we next attempted to replace the serum-stimulus with specific factors. As activin has been shown to have both mesoderm and endoderm-inducing potential in the *Xenopus* model (Smith et al., 1990; Jones et al., 1993; Gamer and Wright, 1995; Ninomiya et al., 1999), we tested it for its ability to exert these effects in the EB system in the absence of serum. Activin induced brachyury expression to almost the same extent observed with serum induction (Fig. 3A), although the kinetics of this expression pattern was altered, being delayed by ~48 hours (compare Fig. 3A with Fig. 1B). Molecular analysis of the activin-induced EBs demonstrated the expression of *Foxa2* and *Mixl1* by day 5 of differentiation, *Sox17*, *Hhex* and *Hnf4* (Duncan et al., 1994) by day 6 and *Ipf1* by day 7. *Gata1* was not expressed at any of the time points in the activin stimulated EBs, although low levels of *Kdr* (Flk1) were found at days 6 and 7 of differentiation. None of these genes was expressed in EBs differentiated in the absence of activin (–activin). *Pax6* displayed an inverse pattern of expression and was present in the EBs generated in the absence of activin, but not in those differentiated in its presence. These findings indicate that, at the dose used, activin can stimulate brachyury expression and endoderm differentiation in EBs in the absence of serum.

To determine the optimal amount of activin required for endoderm differentiation, we next generated EBs in different concentrations of this factor for 6 to 7 days and then analyzed them for gene expression by PCR and Foxa2 protein expression by immunostaining. EBs differentiated for 6 days with as little as 1 ng/ml of activin expressed significant levels of GFP-Bry (10%) (Fig. 3C). The number of GFP-Bry+ cells increased in
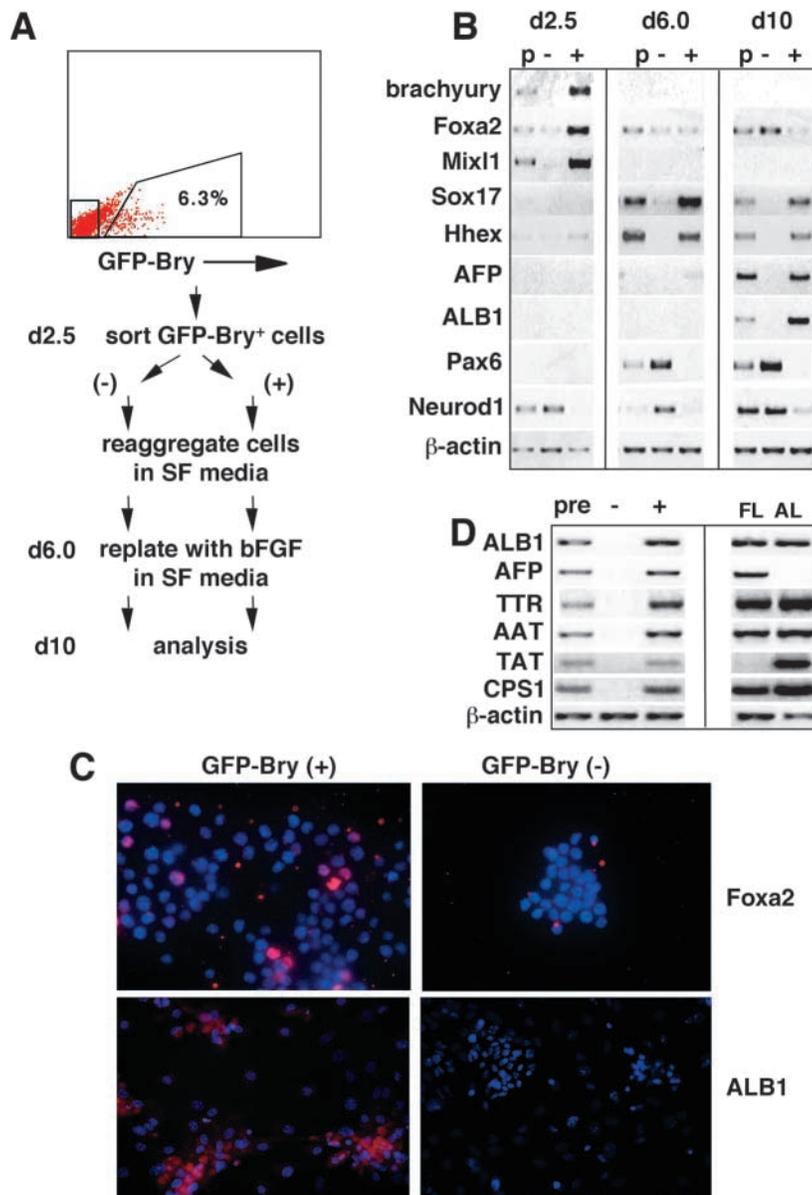
A

GFP-Bry →   6.3%

d2.5   sort GFP-Bry⁺ cells

(-)   (+)

reaggregate cells
in SF media

d6.0   replate with bFGF
in SF media

d10   analysis

B

| | d2.5 | d6.0 | d10 |
| | p - + | p - + | p - + |

brachyury
Foxa2
Mixl1
Sox17
Hhex
AFP
ALB1
Pax6
Neurod1
β-actin

D

	pre - +	FL AL
ALB1		
AFP		
TTR		
AAT		
TAT		
CPS1		
β-actin		

C   GFP-Bry (+)   GFP-Bry (-)

Foxa2

ALB1

Fig. 2. Endoderm potential of GFP-Bry⁺ cells. (A) FACS profile of day 2.5 EBs and reaggregation and culture protocol for differentiation of the sorted cells. (B) RT-PCR expression analysis of pre-sorted (p), GFP-Bry⁻ and GFP-Bry⁺ cells. d2.5, cells analyzed immediately after isolation by sorting; d6.0, reaggregated EBs; d10, cells from replated EBs. (C) Immunostaining of GFP-Bry⁺ and GFP-Bry⁻ cells. Upper panels shows day 2.5 EB sorted cells stained with an antibody to Foxa2. Positive cells are red/pink in color. Nuclei of all cells are stained with DAPI (blue). Bottom panels show cells from 10-day old cultures stained with an antibody to albumin. Positive cells are indicated by red color. (D) RT-PCR expression analysis of genes associated with liver maturation in populations derived from GFP-Bry⁺ and GFP-Bry⁻ day 2.5 EB cells. Cells from the pre-sorted population as well as those from the GFP-Bry⁺ and GFP-Bry⁻ fractions were reaggregated and cultured in SF conditions for 8 days. At this stage, the reaggregated EBs were replated into serum hepatocyte conditions for 4 days, harvested and analyzed.

a dose-dependent fashion, reaching a plateau of 50-60% of the total EB population in cultures stimulated with 30 ng/ml of activin. Gene expression analysis at day 7 revealed differences between the populations stimulated with different concentrations of activin. Undetectable or low levels of *Foxa2*, *Sox17*, *Hhex* and *Hnf4* were induced in EBs by culturing in the presence of 1 and 3 ng/ml of activin, respectively (Fig. 3D). EBs stimulated with 10, 30 and 100 ng/ml of activin displayed readily detectable levels of these four genes. *Ipf1* showed a much more restricted expression pattern and was detected only in EBs stimulated with 30 and 100 ng/ml of factor. Once again, *Pax6* showed an inverse pattern to that of the endoderm genes and was found in unstimulated EBs and in those generated in the low concentrations of activin. *Pax6* was not expressed in EBs differentiated in the presence of 30 or 100 ng/ml of the factor. The findings from the anti-Foxa2 immunostaining confirmed the RT-PCR analysis. More than 50% of the total EB population generated in the presence of 100 ng/ml of

activin expressed Foxa2 protein (Fig. 3E), whereas only 10% of the EB cells induced with 3 ng/ml were positive. Few, if any, positive cells were found in EBs differentiated in the absence of activin. Together, these findings support the notion that different concentrations of activin induce different developmental programs in EBs. High concentrations of this factor stimulate an endodermal program that eventually constitutes more than 50% of the entire EB population and induces fates represented by *Ipf1* expression that are not induced at lower concentrations.

Mesoderm and endoderm potential of activin-induced cell populations

As expected from the lack of *Gata1* expression, no hematopoietic progenitors were detected in day 5 activin-treated serum-free EBs (Fig. 4A, –transfer). To determine if the activin-induced brachyury⁺ population contained mesoderm with hematopoietic potential, day 5 or 6 EBs stimulated with different concentrations of activin were transferred to serum containing medium for 3 days and then assayed for hematopoietic progenitors. Day 5 EBs differentiated in the presence of 3 or 100 ng of activin generated primitive and definitive hematopoietic progenitors following the 3-day exposure to serum (Fig. 4A, +transfer). Significantly lower numbers of progenitors were present in activin-stimulated day 6 EBs and none was detected in day 5 EBs initiated in the absence of activin. The development of hematopoietic progenitors in EBs stimulated with 3 and 100 ng/ml of activin demonstrates that both low and high concentrations of this factor can induce mesoderm with hematopoietic potential. The reduced hematopoietic activity in day 6 EBs suggests that this potential is transient.

As a further assessment of the developmental potential of activin-treated cells, day 6 EBs differentiated in the presence of different concentrations of this factor in the absence of serum were transferred to serum-free media (–activin) for 4 days, and then replated in serum hepatocyte conditions for an

Fig. 3. Effects of activin A on GFP-Bry expression and endoderm induction in EBs. (A) Kinetics of GFP-Bry expression in EBs differentiated in SF cultures in the presence (+activin, 100 ng/ml; open circles) or absence (–activin; closed squares) of activin. (B) Temporal analysis of gene expression in EBs differentiated in the presence (100 ng/ml) or absence of activin in SF cultures. C, controls; top six lanes, day 3 serum-stimulated EBs; lower three lanes, day 6 serum-stimulated EBs. (C) FACS analysis demonstrating the effect of different activin concentrations on GFP-Bry expression in day 6 EBs. (D) RT-PCR analysis demonstrating the effect of different activin concentrations on gene expression profiles in day 7 EBs. (E) Immunostaining demonstrating the presence of Foxa2 protein in day 6 EBs differentiated in the absence (0 ng/ml) or presence (3 ng/ml or 100 ng/ml) of activin. Pink color indicates Foxa2-positive cells.
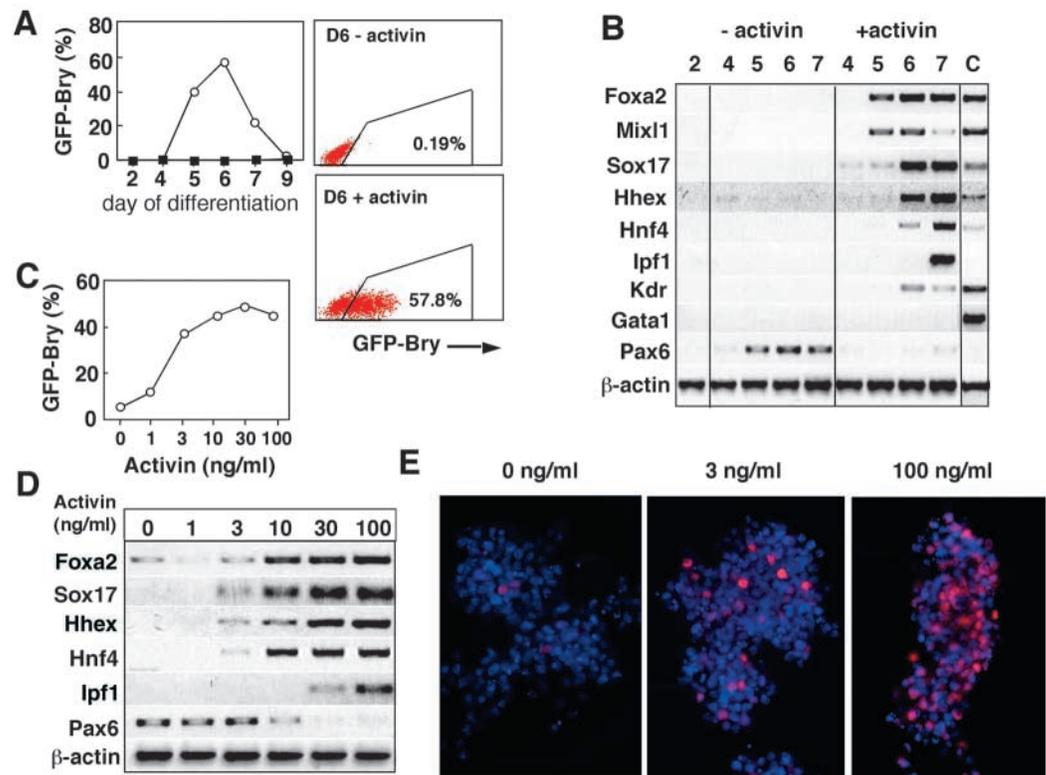
additional 4 days. At day 14 of culture, the cells from each group were harvested and subjected to PCR expression analysis. Expression of *Myf5* and skeletal actin were monitored to evaluate skeletal muscle development as an additional mesoderm-derived lineage (Buckingham, 2001). Surfactant protein C (Sftpc), a gene expressed specifically in embryonic distal lung endoderm and adult type II pneumocytes (Wert et al., 1993) and *Tcf1* (HNF1α), a gene expressed in the developing and adult liver (Blumenfeld et al., 1991) were included as markers of endoderm differentiation in addition to *Afp* and *Alb1*. *Myf5* and skeletal actin were expressed in cultures stimulated with as little as 1 ng/ml of activin, and this expression was detected over a broad range of factor concentrations (Fig. 4B). Expression of both genes was, however, downregulated at the highest concentration of activin. Cultures stimulated with low amounts of activin contained groups of cells with the morphology of skeletal muscle. Immunostaining demonstrated that these cells expressed both skeletal myosin and α-actinin (Lane et al., 1977), indicating that they are of the skeletal muscle lineage (Fig. 4C). Evaluation of the proportion of replated EBs that generated skeletal muscle outgrowths was consistent with the gene expression analysis; those stimulated with between 3 and 30 ng/ml displayed the most robust skeletal muscle development (Fig. 4B). No endoderm genes were expressed at low activin concentrations, whereas all were readily detected in cultures stimulated with the highest concentrations of the factor. Expression of *Pax6* was restricted to untreated cultures and those stimulated with low concentrations of factor. The findings from this analysis confirm and extend those from the previous experiment by demonstrating that different
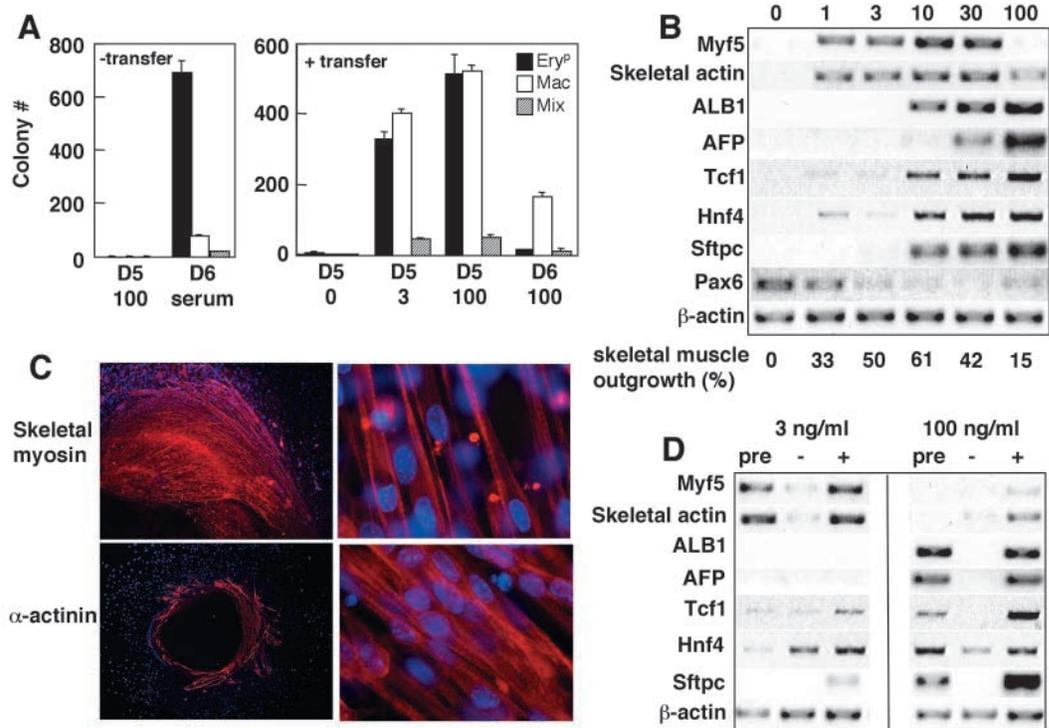
concentrations of activin induce different developmental programs, with low concentrations favoring a mesodermal fate and high concentrations supporting endoderm and some mesoderm differentiation. In addition, they show that the endodermal cells induced by activin are able to differentiate and give rise to cells that express hepatocyte and lung markers.

As a final characterization of the activin-stimulated cells, brachyury[+] and brachyury[–] populations isolated from EBs stimulated with low and high concentrations of factor were reaggregated and cultured as described and then analyzed for expression of the skeletal muscle and endoderm genes. As shown in Fig. 4D, both *Myf5* and skeletal actin expression was restricted to the population generated from the brachyury[+] population isolated from EBs stimulated with 3 ng/ml of activin. Similarly, the endoderm genes were expressed in the brachyury[+]-derived cells isolated from EBs generated in the presence of 100 ng/ml of factor. These findings further support the concept that both mesoderm and endoderm develop from a brachyury[+] population.

In vivo potential of activin-induced GFP-Bry[+] populations

To evaluate the developmental potential of the activin-induced cells in vivo, cells from the GFP-Bry[+] and GFP-Bry[–] fractions isolated from day 5 EBs induced with 100 ng/ml of factor were reaggregated and cultured as EBs for 8 days (serum free), replated in serum hepatocyte conditions for 4 days and then transplanted under the kidney capsules of SCID-beige mice. The extended culture time as EBs was included to promote maturation of the endoderm populations. Three weeks after transplantation, the mice were sacrificed and the kidneys

Fig. 4. Induction of mesoderm and endoderm derivatives in EBs differentiated in the presence of activin A. (A) Left panel: comparison of the hematopoietic progenitor potential of EBs differentiated in the presence of activin and serum. D5100, day five EBs differentiated in SF cultures in the presence of 100 ng/ml activin; D6 serum, day 6 EBs differentiated in the presence of serum. Right panel: hematopoietic progenitor potential of activin-stimulated serum-free EBs (day 5 or 6) following an additional 3 days of culture in serum containing medium (+transfer). EBs were generated in SF cultures in either 0 ng/ml, 3 ng/ml or 100 ng/ml of activin. Numbers represent colonies per 5×10^4 cells plated. Data represents mean±s.e.m. (n=3). (B) Expression analysis of cultures from EBs

differentiated in the presence of different activin concentrations. Day 6 EBs differentiated in variable concentrations of activin were transferred into SF media without activin for 4 days and then replated in serum hepatocyte conditions for an additional 4 days. At day 14, replated EBs were harvested and analyzed by RT-PCR. Prior to harvesting, the proportion of EBs with visible skeletal muscle outgrowth was evaluated (indicated below panel). Numbers on top of panel indicate the activin concentration used (ng/ml). (C) Immunostaining demonstrating expression of skeletal myosin and α-actinin in skeletal muscle outgrowths generated from EBs differentiated in the presence of 3 ng/ml activin. EBs were generated as in section (B) above. At day 10, EBs were plated on gelatin-coated coverslips, cultured for 4 days and then stained with antibodies to skeletal myosin and α-actinin. (D) Expression analysis of GFP-Bry+ and GFP-Bry− populations isolated from EBs differentiated for 5 days in the presence of 3 ng/ml or 100 ng/ml of activin. Cells from the pre-sorted, the GFP-Bry+ and GFP-Bry− populations were reaggregated in SF cultures in the absence of activin for 8 days. At day 13, the reaggregated EBs were replated in hepatocyte conditions for 4 days and then harvested for RT-PCR analysis.

analyzed. Grafts from the GFP-Bry+ population were relatively small and homogenous in sized and their growth was restricted to only part of the kidney (Fig. 5A). By contrast, grafts from the GFP-Bry− fraction were very large, ~100 times the size of those from the GFP-Bry+ cells, heterogeneous in appearance and often engulfed the entire kidney (Fig. 5A). Histological analysis indicated that the GFP-Bry+ grafts contained both endoderm and mesoderm derivatives. Endoderm was represented predominantly by the presence of ductal structures that consisted of cells with the morphology of gut epithelial cells (Gu) and tall columnar cells (C), possibly representing bronchial epithelium (Fig. 5B). Cells with the morphology of hepatocytes were not detected in these grafts. With respect to mesoderm derivatives, skeletal muscle (SM) and adipocyte tissue were detected and in some instances bone (B) was also present. The cells within these structures appeared to be well differentiated, indicating the presence of mature populations. By contrast, grafts from the GFP-Bry− cells displayed morphological characteristics of ES cell-derived teratomas and consisted of derivatives of all three germ layers. The GFP-Bry−derived grafts contained substantial populations of neural tissue (brain; Br), skeletal muscle, gut-like epithelial cells as well as many regions of immature cells, including neural tube-like structures (NT) that were not found in the grafts from GFP-Bry+ cells.

To further characterize the cell populations in the GFP-Bry+ grafts, sections of tissue were analyzed for expression of markers indicative of endoderm development by in situ hybridization and immunostaining. These analyses revealed the presence of Foxa2-positive cells in many of the duct structures, confirming their endoderm origin (Fig. 6A,B). Some of the structures expressed intestinal fatty acid-binding protein (Ifabp), which is indicative of intestinal development (Sweetser et al., 1987) (Fig. 6C,D). Sftpc was expressed in some branching acinar structures in the grafts, suggesting differentiation to a distal lung epithelial phenotype (Fig. 6E,F). Periodic acid staining following diastase digestion (D-PAS) demonstrated the presence of mucin (M) in many of duct structures (Fig. 6H), indicating that they may represent goblet cells found in gut epithelium. The presence of these different markers demonstrates the development of foregut and hindgut derivatives from the GFP-Bry+ ES-derived cells. As expected from the in vitro analysis, grafts from GFP-Bry+ cells induced with a low concentration of activin (3 ng/ml) consisted predominantly of skeletal muscle, with little or no endoderm derivatives (Fig. 6I). The findings from these renal transplantation experiments confirm our in vitro studies and demonstrate that GFP-Bry+ populations induced with high levels of activin display the potential to generate derivatives of the endoderm lineage.

Fig. 5. Analysis of kidney capsule grafts of activin-stimulated GFP-Bry+ and GFP-Bry− EB-derived populations. GFP-Bry+ and GFP-Bry− cells isolated from day 5 EBs differentiated in the presence of 3 or 100 ng/ml activin were reaggregated and cultured as EBs for an additional 8 days in SF cultures in the absence of factor. The reaggregated EBs were replated in hepatocyte conditions for 4 days and then harvested and transplanted under the kidney capsule of SCID-beige mice. Three weeks following transplantation, the mice were sacrificed and the kidneys harvested for analysis. (A) Photograph showing the size of the grafts from the GFP-Bry+ and GFP-Bry− populations. Size is indicted by the ruler (mm) at the bottom of the figure. (B) Histological analysis of grafts from the GFP-Bry+ and GFP-Bry− populations. SM, skeletal muscle; Gu, gut epithelial-like structure, B, bone; C, columnar cells; Br, neural tissue; NT, neural tube-like structure.
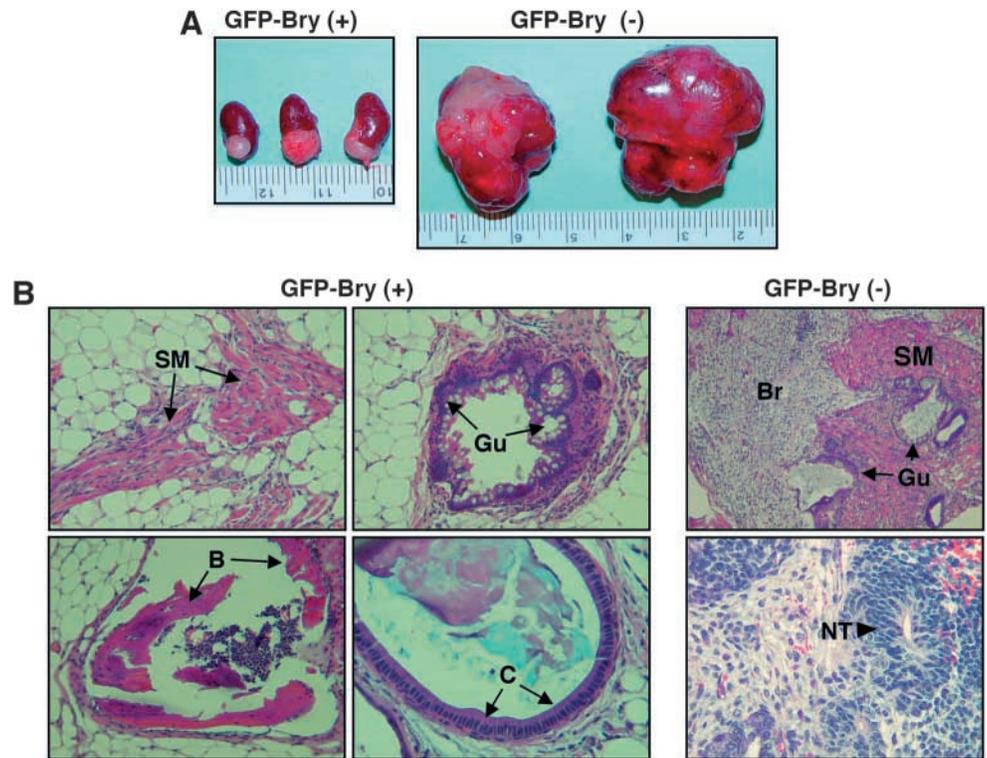

Fig. 6. Analysis of endoderm derivatives in kidney grafts of GFP-Bry+ populations. (A,B) Immunohistochemistry showing expression of Foxa2 protein in tubular structures present in the grafts. (C,D) Immunohistochemistry demonstrating expression of intestinal fatty acid binding protein (Ifabp) in the gut-like structures present in grafts. (E,F) In situ hybridization indicating expression of surfactant protein C (Sftpc) in the grafts: (E) Bright-field exposure, arrow indicates positive area, (F) dark-field exposure. (G) Hematoxylin and Eosin, and (H) D-PAS staining of consecutive sections demonstrating muscin (M) in the gut epithelial-like structure in the grafts. (I) Skeletal muscle (SM) in a graft from GFP-Bry+ cells induced with 3 ng/ml of activin. K, kidney of recipient.
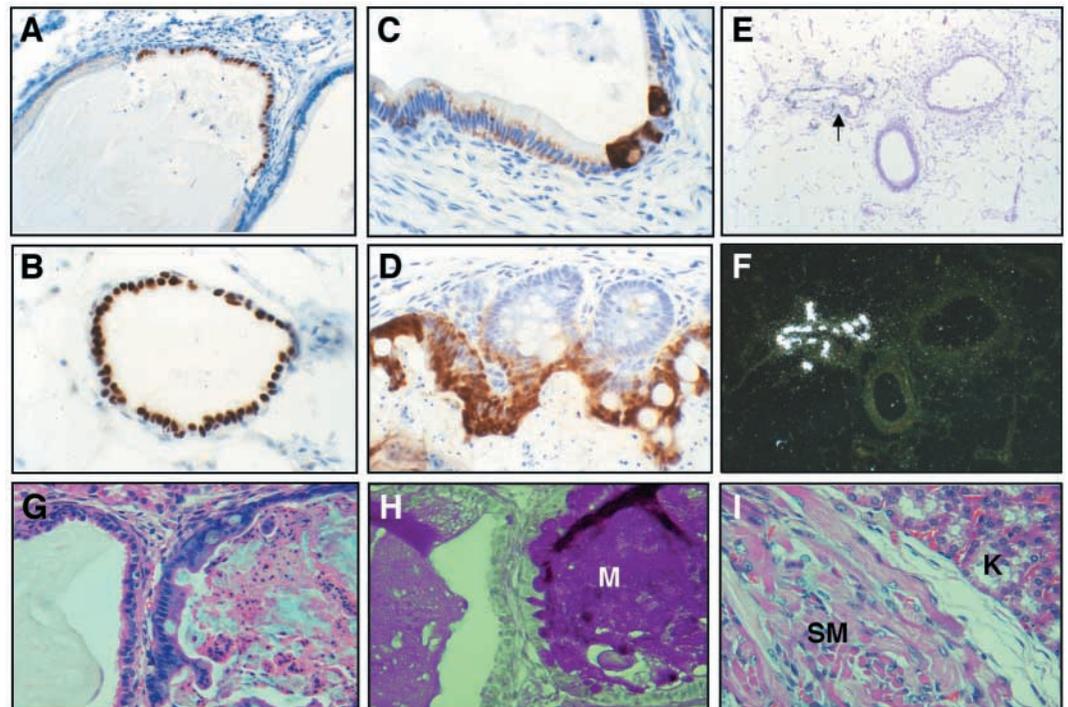
Discussion

Access to cell populations representing early stages of development from ES cells differentiated in culture offers an unprecedented opportunity to define the key events regulating the establishment of the primary germ cell layers. Understanding lineage development at this stage is an important first step for the use of this model for basic studies in developmental biology as well as for a source of specific cell populations for cell-replacement therapy. In this report, we provide the first comprehensive analysis of definitive endoderm development in the ES/EB model and demonstrate that restricted exposure to serum factors or differentiation in the

presence of activin will induce the development of this lineage. Using an ES cell line with GFP targeted to the brachyury locus we show that endoderm develops from a brachyury$^+$ cell, suggesting that it derives from a mesendoderm progenitor.

A comparison of endoderm development in serum-containing and serum-free cultures highlights the importance of providing appropriate inducing factors for the generation of differentiated progeny from ES cells. Although both protocols result in the generation of endoderm, the extent of endoderm induction within the EBs, based on Foxa2 protein analysis, appears to be significantly higher in the serum-free/activin cultures. In addition, activin consistently induced the expression of *Sftpc* and *Ipf1*, genes indicative of lung and pancreas specification that were seldom detected using our serum-induction protocol. The upregulation of *Ipf1* following activin treatment suggests that this protocol may provide a novel approach for the generation of pancreatic β cells from differentiating ES cells. A number of recent studies have described the development of insulin-expressing cells in ES differentiation cultures (Lumelsky et al., 2001; Hori et al., 2002; Blyszczuk et al., 2003). However, the interpretation of these studies is complicated by the fact that the analysis was carried out in mixed lineage cultures. The ability to separate neuroectoderm from mesoderm and endoderm by brachyury expression will enable us to define the origin of the *Ipf1*-expressing cells in the developing EBs, and ultimately follow their differentiation to a β cell fate.

The mesoderm potential induced by activin and serum also differed. A 6-day exposure to serum induced hematopoietic progenitor development (Fig. 1C), but little if any skeletal muscle potential (A.K. and G.K., unpublished). By contrast, activin induced both skeletal muscle and hematopoietic mesoderm development. However, unlike the serum-induced hematopoietic program that resulted in the generation of progenitors, the program induced by activin appears to consist of hematopoietic mesoderm that has not yet advanced to the progenitor stage. Progression to the progenitor stage of development was observed following a 3-day culture period in serum. Previous studies have demonstrated the generation of skeletal muscle from ES cells differentiated in serum-containing cultures (Rohwedel et al., 1994). Our activin induction protocol represents a significant advancement over these early studies in that it is faster and more efficient. The combination of activin induction with the isolation of brachyury$^+$ populations provides a novel approach for the generation of large numbers of skeletal myocytes for cell replacement studies.

The demonstration that activin can induce mesoderm and endoderm differentiation in the ES cultures in the absence of serum is consistent with numerous studies that have defined a requirement for factors of the TGFβ family in the early induction of these lineages (Smith, 1993; Gurdon et al., 1994; Schier and Shen, 2000). Although it is well established that activin can induce mesoderm and endoderm in different model systems, targeting studies in mice would suggest that it is not the endogenous factor that regulates these developmental decisions in the early embryo (Vassalli et al., 1994; Matzuk et al., 1995). Rather, most evidence suggests that nodal and nodal-like factors function in the capacity to regulate early mesoderm and endoderm development in vivo (Schier and Shen, 2000; Whitman, 2001). In a recent study, we have shown that nodal is expressed in GFP-Bry$^+$ populations isolated from early EBs, suggesting that it may also be regulating lineage

fates in this model (Fehling et al., 2003). The overlapping activities of activin and nodal early in development may reflect the fact that they can bind the same receptors and thus initiate the same signaling events (Schier and Shen, 2000).

The response to varying concentrations of activin in the ES system is also consistent with studies in *Xenopus* that have demonstrated that different concentrations of activin will induce different fates in animal cap cells in culture (Green and Smith, 1990; Green et al., 1992a; Jones et al., 1993; Gamer and Wright, 1995; McDowell et al., 1997; Ninomiya et al., 1999). In the *Xenopus* model, high concentrations of this factor were shown to induce dorsal mesoderm and endoderm, whereas low concentrations induce ventral mesoderm. In the ES/EB model, endoderm was induced most efficiently at high concentrations, whereas low and intermediate levels of activin induced mesoderm. The differential response to activin in the ES/EB model is consistent with its functioning as a morphogen in which different concentrations can induce different fates in a given cell. Alternatively, different concentrations of activin could be stimulating distinct subpopulations of progenitors, those with endoderm potential requiring a higher activin concentration than those with mesoderm potential. Distinguishing between these possibilities will require the establishment of conditions that will enable the analysis of the fate of single cells following induction.

The observation that endoderm derives from GFP-Bry$^+$ cells suggests that this germ layer develops from a bi-potential mesendoderm population that co-expressed brachyury and Foxa2. This ES-derived GFP-Bry population could be similar to cells in the anterior region of the primitive streak of the mouse embryo that express these genes (Wilkinson et al., 1990; Monaghan et al., 1993; Sasaki and Hogan, 1993) and give rise to the first endodermal cells (Wells and Melton, 1999). Studies in other species have identified populations with mesendoderm potential and have provided evidence that although most endoderm is derived from this population, only a subset of mesoderm, including that fated to blood and cardiac muscle arises from these precursors (Rodaway and Patient, 2001). In this study, we demonstrated that the skeletal muscle lineage develops from an activin-induced brachyury$^+$ population and in a recent report we showed that serum-induced brachyury$^+$ cells isolated at the same stage of development display hematopoietic and endothelial potential (Fehling et al., 2003). In addition to these lineages, beating cardiac myocytes were often found in the cultures of brachyury$^+$ cells maintained in hepatocyte differentiation conditions (A.K. and G.K., unpublished). Which, if any, of these mesoderm lineages shares a common precursor with endoderm will require clonal analysis. Access to the brachyury$^+$ cells from the EBs will enable us to not only address this question, but also define and characterize the regulatory mechanisms involved in the induction, patterning and tissue-specific differentiation of endoderm and mesoderm.

The findings from the kidney capsule transplantation experiment further support our in vitro studies and demonstrate that the activin-induced EB cells displayed endoderm and mesoderm potential in vivo. The identification of Sftpc- and Ifabp-expressing cells in the grafts clearly demonstrates the potential of these cells to generated endoderm-derived tissues. Although we could reproducibly generate hepatocyte-like cells in culture, this lineage did not persist or expand in the grafts under the kidney capsule. The lack of hepatocyte differentiation

may indicate that the environment of the kidney capsule is not optimal for the differentiation and growth of this lineage. Ultimately, functional analysis of the ES cell-derived hepatocyte-like cells will require transplantation into animal models of liver failure, such as the fumarylacetoacetate hydrolase (FAH)-deficient mouse (Grompe et al., 1993). The development of teratomas from the brachyury⁻ fraction is consistent with our previous findings demonstrating that undifferentiated ES cells segregate to the negative population (Fehling et al., 2003). The fact that teratomas developed from the GFP-Bry⁻ cells even after extensive time in culture, highlights the importance of establishing methods for removing residual ES cells from the cultured population prior to engraftment. Isolation of brachyury⁺ cells prior to culture significantly reduced the development of multilineage teratomas.

In summary, the findings reported here demonstrate that ES cells can generate definitive endoderm-derived cell populations under defined conditions in culture. Access to activin-induced brachyury⁺ cells provides a unique population of enriched endoderm progenitors for future studies aimed at their tissue specific differentiation, as well as the identification and characterization of genes involved in these processes. Finally, defining the events involved in the development of endoderm from mouse ES cells in this study represents an important first step in generating similar populations from human ES cells.

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