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[27] Gene Trap Vector Screen for Developmental Genes in Differentiating ES Cells

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Introduction

A powerful approach to screen for genes involved in developmental processes is to generate mutations by random insertion of gene trap vectors into mouse embryonic stem (ES) cells. Insertion of foreign DNA elements into coding or regulatory regions of the genome frequently disrupts or alters expression of flanking genes. The insertion serves to “tag” the endogenous genes, allowing for subsequent cloning. The availability of mouse ES cells, together with the development of “entrapment vectors,” has made it possible to efficiently introduce insertional mutations in ES cells and to screen for candidate genes in ES cells and embryos derived from the ES cells. ES cells are undifferentiated, pluripotent cells derived from the inner cell mass (ICM) of blastocysts.^{1–3} When introduced into morula- or blastocyst-stage embryos, they contribute to the formation of somatic tissues as well as functional germ cells.^{4,5} Furthermore, they can be induced to differentiate *in vitro* into embryoid bodies (EBs), either spontaneously by removal of the feeder layer of mouse embryonic fibroblasts (MEFs) and leukemia inhibitory factor (LIF), or directed by removal of differentiation inhibitors and addition of specific growth factors and cytokines. Examples of specific ES differentiation protocols are found elsewhere in this volume. Upon differentiation, ES cells give rise to a variety of different cell lineages, including parietal and visceral endoderm, mesoderm, cardiac and skeletal myocytes, hepatocytes, hematopoietic and endothelial cells, dendritic cells, neurons, and glial cells.^{5–19} The process of EB formation *in vitro* closely mimics early postimplantation development, as has been demonstrated by the temporal appearance of differentiated cell lineages and expression of stage- and lineage-specific markers.

The essential feature of entrapment vectors is a promoter-less reporter gene, usually the *E. coli lacZ* gene, whose expression becomes dependent on transcription initiated from cis-acting regulatory sequences of a targeted cellular gene. Therefore, candidate genes can be identified based upon the pattern of reporter gene expression rather than a mutant phenotype (reviewed in Refs. 20, 21, 22). Three types of entrapment vectors have been previously developed, enhancer trap, promoter trap, and gene trap vectors that contain a splice acceptor site positioned 5' to the reporter gene, allowing for the generation of fusion transcripts of the reporter gene to upstream exon sequences upon integration into an intron.^{23–27} Entrapped endogenous genes can be rapidly cloned by PCR-based methods, such as inverse PCR or 5'RACE, or by cloning of genomic DNA flanking the vector insertions. New vector designs include polyadenylation site vectors to efficiently trap genes that are not expressed in undifferentiated ES cells,²⁸ and secretory-trap vectors to identify secreted and transmembrane proteins expressed in ES cells.²⁹ Retrovirus- or plasmid-based entrapment vectors have been used in mutagenesis screens, expression- and induction-trapping screens, or sequence-based screens in ES cells and embryos derived from the ES cells. Several consortia presently perform large-scale gene trap projects and provide ES cell lines and insertion site sequences as public resources [University of Manitoba Institute of Cell Biology (<http://www.esells.ca>); The Gene Trap Project of the German Human Genome Project (<http://tikus.gsf.de>); The BayGenomics Gene Trap Project (<http://baygenomics.ucsf.edu>); The CMHD Gene Trap Project (<http://www.cmhd.ca>)]. In addition, specialized smaller screens have been performed by a number of individual groups.

An excellent review by Stanford *et al.* (2001)²¹ provides detailed information on entrapment vectors and different approaches for screening.

Here, we describe an expression screen to identify genes that are expressed at specific stages of mouse development by using retroviral entrapment vectors. ES cell clones with entrapment vector integration into putative developmentally regulated genes can be selected by virtue of their stage-specific reporter gene expression, and by colocalization with stage- and lineage-specific markers upon *in vitro* differentiation into embryoid bodies (Fig. 1). Selected clones can be further examined *in vivo*, either by generating transgenic lines or “transient” chimeric embryos. Finally, genomic integration sites can be cloned and used to screen stage-specific embryonic cDNA libraries. This approach allows us to efficiently identify and preselect *in vitro* for insertions into genes that are restricted in their temporal and spatial distribution in the postimplantation embryo and that may not be expressed in pluripotent embryonal cells.^{30,31}

This chapter focuses on the strengths of the *in vitro* differentiation screen to preselect for entrapment integrations into developmentally regulated genes (Fig. 1). First, we discuss infection of ES cells with retroviral entrapment vectors. Second, screening for reporter gene expression during EB differentiation will be described. The third section provides protocols for colocalizing reporter gene expression and developmental markers. The protocols described in this chapter use commercially available reagents, they are reproducible and efficient.

Retroviral Entrapment Vector Infection of ES Cells

Bifunctional Retroviral Vectors

The design and use of retroviral- or plasmid-based entrapment vectors has been reviewed in detail elsewhere.^{20,21} We chose to use bifunctional retroviral entrapment vectors with an alkaline phosphatase (AP) reporter gene (Fig. 2) to identify genes that are differentially expressed during early mouse development. Retrovirus vectors efficiently infect ES cells and stably integrate into their genomic DNA. Other advantages of retroviral vectors are that infection at a low multiplicity leads to single copy insertions, their conservative integration via their long terminal repeat sequences, and their propensity to integrate into 5' untranslated or first intron genomic sequences. The human placental AP gene was chosen as a reporter, because we found significantly less variability in its expression in ES cells and EBs as compared to *lacZ* (R. Battaglino and H. Stuhlmann, unpublished). In order to allow for selection of cells carrying the integrated entrapment vector independent of AP reporter expression, the *neo^R* gene was transcribed from an internal phosphoglycerokinase (*pgk-1*) promoter that is functional in a variety of undifferentiated and differentiated cells.

The entrapment vectors (Fig. 2) contain 5' and 3' Moloney murine leukemia virus- (Mo-MLV-) derived sequences, including the two LTRs and about 1-kb of *gag* sequences that are important for efficient packaging of the retroviral genome. The plasmid constructs contain a 300-bp deletion of enhancer and promoter sequences in the 3' LTR. Following infection and reverse transcription the deletion is copied into both LTRs in the provirus. Expression of the AP reporter gene requires initiation of transcription from a promoter of an endogenous gene 5' to the retroviral integration site and termination at the retroviral polyadenylation sequence in the 3' LTR. In addition, the LTRs contain an insert of a bacterial suppresser tRNA gene, *sup F*, to allow cloning of proviral and flanking genomic host sequences in a suitable bacterial host. With the PT-AP (promoter trap-AP) vector, integration close to a cis-acting cellular regulatory sequence and in the sense orientation leads to read-through transcription into the retroviral genome. Depending on the position of the first ATG in the cellular gene relative to the initiator ATG in AP, either a chimeric protein with the AP at its C-terminus, or an authentic AP protein will be made. In the GT-AP (gene trap-AP) construct, the initiator ATG in AP is deleted, and a 170-bp fragment containing the splice acceptor site from the Mo-MLV *env* mRNA is inserted

upstream of the AP gene. Integration of the entrapment vector within an intron of a transcribed cellular gene generates a spliced fusion transcript and, if in-frame, leads to expression of a chimeric AP protein. The construct PT-IRES-AP contains a 600-bp internal ribosomal entry site (IRES) element from encephalomyocarditis virus (EMCV) upstream of the AP gene. Integration of the PT-IRES-AP vector into a transcribed gene leads to cap-independent translation of an authentic AP reporter protein from a fusion transcript, independent of the reading frame.

Virus producing lines are generated by electroporation of retroviral entrapment vector constructs into retrovirus packaging lines; these are genetically engineered to provide helper virus function in trans but are incapable to produce wild-type virus. In our hands, all three entrapment vectors gave rise to stable producer lines with comparable titers, between 2 to 5×10^4 /ml on NIH3T3 fibroblasts, and 0.5 to 1×10^3 /ml on ES cells, respectively.

Propagation and Maintenance of ES Cells

We have successfully used the ES cell lines R1⁵ and W4³² for retroviral infection, *in vitro* differentiation, and ES cell chimera formation. Both are derived from the mouse inbred strain 129Sv, they have been shown to contribute with high efficiency to the germ line upon blastocyst injection and to chimera formation by morula-ES cell aggregation, and they maintain these characteristics at high passage numbers. We found that conditions for optimal growth and differentiation are very similar for the R1 and W4 cell lines. Stocks of ES cells at low passage numbers are kept in liquid nitrogen, and cells are propagated for fewer than five passages prior to an experiment. To retain the cells in an undifferentiated state, both lines require growth on primary mouse embryonic fibroblast feeders (MEFs) and the presence of LIF in the culture medium. Equally important is the quality of the fetal bovine serum (FBS). We routinely test several lots of serum for maintenance of the undifferentiated phenotype upon at least five serial passages, cloning efficiency, and cell toxicity at a serum concentration of 30%. We have obtained very good success with ES cell-qualified FBS from HyClone and Gemini.

Culture Medium and Solutions—All reagents for culturing ES cells and MEFs should be “tissue culture grade” or “tissue culture tested.” Preferably, only sterile tissue culture grade plastic ware and pipettes should be used. For culture of ES cells, we use Dulbecco's modified Eagle's medium (DMEM) with high glucose and glutamine (Mediatech Cellgro) supplemented with 15% heat-inactivated fetal bovine serum (FBS, HyClone or Gemini), 20 mM HEPES (50X stock, pH 7.3, GIBCO), 0.1 mM nonessential amino acids (100X stock, GIBCO), 0.1 mM β -mercaptoethanol (1000X stock, GIBCO), penicillin and streptomycin (100X stock, GIBCO), and 500 u/ml LIF (GIBCO). ES cells are cultured on gelatin-coated plastic plates or flasks (0.7% solution; gelatin from porcine skin, approximately 300 bloom, Sigma No. G1890). Cells are passaged every 2–3 days. For this, dishes are rinsed twice with phosphate buffered saline (PBS), and cells are carefully dissociated with Trypsin–EDTA (0.05% solution, GIBCO) to obtain a single cell suspension. Trypsin digestion is stopped by adding an equal amount of culture medium, and cells are transferred to a new plate at a dilution 1:5 or 1:10. Culture medium for MEFs is DMEM supplemented with 10% heat-inactivated FBS, 20 mM HEPES, and penicillin and streptomycin.

Preparation of Mouse Embryonic Fibroblast Feeder Cells—We routinely use the transgenic line DR-4 as a donor for multiple drug-resistant MEFs. The DR-4 mice bear selectable genes *neo^R*, *puro^R*, *hyg^R*, and a deletion of the X-linked *Hprt* gene,³³ which confers resistance to G418, puromycin, hygromycin, and 6-thioguanine. DR-4 mice can be purchased from The Jackson Laboratory (Stock Tg(DR4)1Jae, Stock No. 003208). MEFs are isolated from mouse embryos at day 13.5 or 14.5 of gestation. For maintenance of ES cells in the absence of drug selection, MEFs from CD-1 embryos can be used. Each preparation of primary MEFs

has to be tested for mycoplasma before expansion and coculture with ES cells. We are routinely using a PCR-based and an ELISA-based mycoplasma detection kit, both of which detect the most common mycoplasma species found in contaminated mammalian cell culture systems.

Embryos are dissected from their implantation sites and transferred to a dish with sterile PBS and rinsed once in PBS. Under a stereomicroscope in a sterile hood, heads and soft tissues (liver, intestine, kidneys, lung, and heart) are removed with watchmaker forceps. The embryo carcasses from one litter (6–10 embryos) are placed into a fresh 10 cm dish, and just enough Trypsin/EDTA is added to cover them (about 5 ml for 10 embryos). The carcasses are minced into fine pieces by using sterile scalpels, and incubated for 30 min in the 37°C incubator. MEF culture medium is added, and the digested tissues are dissociated by repeated cycles of vigorous pipetting and collecting the supernatant cell suspension. Cells from combined supernatants are plated into T165 flasks (10 flasks for 10 embryo carcasses) in MEF culture medium. Cells reach near confluence usually 2 days after plating, at which time they are frozen (2 vials per embryo) at -80°C and transferred to liquid N₂ 1 day later.

To expand primary MEFs, frozen cells from one vial are quickly thawed at 37°C in MEF culture medium and divided into three T165 flasks. Cells are expanded on 150 mm plates in intervals of 2–3 days two more times at a dilution of 1:3. Cells from the final passage (total of 27 × 150 mm plates) are harvested upon reaching complete confluence, pelleted and resuspended in 45 ml ice-cold MEF culture medium. The cells are mitotically inactivated using a γ -irradiation source. Dimethyl sulfoxide (DMSO, 5 ml) is added to the cell suspension, and cells are frozen down in aliquots (0.2 ml aliquots for T25 flasks feeder layer, 0.5 ml for T75 flask or 100 mm dish, and 1.5 ml for 3 × T75 flasks or 3 × 100 mm dishes, respectively). The MEF feeder cells can be stored at -80°C for several months. It is crucial that the MEFs are actively dividing during their expansion and that they do not reach senescence. In our hands, the expansion protocol described above generates reproducibly MEFs that support maintenance of undifferentiated ES cell cultures.

Culture of ES Cells on MEFs—ES cells are routinely cultured in ES medium on mitotically inactivated MEF feeder layers in gelatin-coated flasks. A fresh vial of inactivated MEFs is thawed out for each ES cell passage. The MEFs can be plated either before passage of ES cells, or at the same time. Culture medium is changed every day.

To thaw ES cells, a frozen vial is quickly warmed in a 37°C water bath. Cells are transferred to a 15-ml conical tube filled with 5 ml ES medium and pelleted for 5 min at 1000 rpm. Cells are resuspended in 1 ml ES medium and transferred into a gelatin-coated T25 flask with MEF cells and 5 ml ES cell medium. Culture medium is changed after ES cells have attached in order to remove dead cells (usually 2–3 hr after thawing cells).

ES cells should be passaged when they are about 80% confluent. It is crucial to dissociate cells well to achieve a single cell suspension (otherwise the ES cells will spontaneously differentiate), and to seed the cells at appropriate densities. For regular passages, the following protocol is used: Cells in a T25 flask are rinsed 2× and trypsinized with 0.5 ml Trypsin–EDTA for 2 min at 37°C. The cells are detached from the bottom by knocking against the flask, and incubated for another 2–4 min at 37°C. After adding 1.5 ml ES cell medium, cells are completely dissociated by pipetting several times with a 1-ml pipette against the bottom of the flask. An aliquot of the suspension is counted, and 1.5×10^6 cells are added to a new T25 gelatin-coated flask with a MEF feeder layer. Alternatively, ES cells are plated in a new flask at a 1:5 or 1:10 dilution. The cells should be passaged every 2–3 days. ES cells are frozen down from ~80% confluent cultures at 5×10^6 cells/ml in 0.5-ml aliquots per freezing vial. For this, cells are washed, dissociated with Trypsin–EDTA as described above, and counted. Cells are pelleted in a 15 ml conical tube and resuspended in ES cell culture medium supplemented with

10% (v/v) DMSO at a concentration of 5×10^6 /ml. The cell suspension is frozen in 0.5-ml aliquots over night at -80°C and transferred to liquid N_2 the next day.

Retroviral Entrapment Vector Infection and Selection of ES Cells

Titration of Virus Supernatant on ES Cells—Our experience and that of other groups is that infectious titers from retroviral producer lines are between 10- and 50-fold lower on ES cells than on NIH 3T3 fibroblasts. Therefore, virus titers should be determined on ES cells. Virus supernatant from producer lines is freshly harvested and passed through a $0.4\text{-}\mu\text{m}$ syringe filter to remove cells. Alternatively, supernatant can be stored as frozen stocks in aliquots at -80°C . Since infectious titers drop to about 50% during each freezing/thawing cycle, we only thaw virus supernatant once. Serial dilutions (10^0 to 10^{-4}) are made in PBS supplemented with 3% FBS and $4\text{-}\mu\text{g/ml}$ polybrene in an ice-water bath. Two milliliters from each dilution are added to a 100 mm dish (plated the day before with 0.5×10^6 ES cells in the presence of $4\text{-}\mu\text{g/ml}$ polybrene). ES cell medium (8 ml) is added 4 hr later, and selection medium (culture medium supplemented with 0.35-mg/ml G418) is added after 36-hr incubation. Seven days later, cells are fixed with 100% methanol and stained with GIEMSA (1:10 dilution in H_2O). To determine entrapment efficiencies, plates infected in parallel with virus dilutions (10^0 and 10^{-1}) are selected for G418 resistance, and clones are stained 7 days later for AP activity (see below). Under a stereomicroscope, the total number of G418-resistant colonies, and the number of AP positive colonies per plate are counted.

Infectious titers are scored as number of G418-resistant colony forming units per ml. The entrapment efficiencies are determined as percentage of AP-positive clones out of the total number of G418-resistant clones. In our hands, the trapping frequency with the PT-AP virus was 1–3% in fibroblasts and in ES cells, and similar frequencies were found with the GT-AP virus. The intensity of the purple AP stain varied somewhat among positive clones but was similar in most cells within one particular clone, consistent with the notion that many entrapment events lead to the expression of AP fusion proteins with varying enzymatic activities. Significantly higher entrapment efficiencies (5–13% in NIH 3T3 cells, and 2–5% in ES cells) were obtained when the IRES element was included upstream of the AP reporter gene (PT-IRES-AP), consistent with the notion that expression of a functional AP gene is independent of the translational reading frame. Histochemical staining for AP was uniformly strong among different clones as well as among cells within the same clone, consistent with the notion that the reporter gene is expressed as an authentic rather than a fusion AP protein.

Because the entrapment efficiencies vary between 1 and 5% in ES cells, large numbers of G418-resistant colonies have to be screened. To obtain between 100 and 500 of G418-resistant ES cell clones from one 100-mm dish, we use two alternative protocols for retrovirus infection, as outlined below.

Retrovirus Infection with Virus Supernatants—This protocol is being used if: (i) the virus titers on ES cells are at least $10^2\text{--}10^3$ /ml; and (ii) single proviral integrations per infected ES cell are desired. Twelve to 18-hr before virus infection, $0.5\text{--}1 \times 10^6$ ES cells are plated per 100-mm gelatin-coated dish with feeder cells in ES cell culture medium, and in the presence of $4\text{-}\mu\text{g/ml}$ polybrene. For a typical experiment in which several hundred colonies are to be isolated, we use four to six 100-mm dishes. The next day, aspirate medium and infect cells with 2-ml of virus supernatant (fresh harvested or frozen stocks, sterile filtrated through $0.4\text{-}\mu\text{m}$ filters). After 4-hr of incubation at 37°C , 8 ml of culture medium is added. Thirty-six hours later, selection medium (ES cell culture medium supplemented with 0.35 mg/ml G418 [dry powder, GIBCO; stock solution 50 mg/ml]) is added. Medium is changed every day, and colonies are picked 7–8 days later (see below).

Retrovirus Infection by Cocultivation—This protocol describes retrovirus infection of ES cells by cocultivation with virus-producing cells. This method is being used if: (i) the virus titers are very low ($<10^2$ /ml), and/or (ii) drug selection of infected ES cells is not possible and infection of a high percentage of ES cells should be achieved. One 100-mm dish with virus producer cells at 70–80% confluence is treated with 5-ml of 10- μ g/ml mitomycin C in culture medium for 3 hr at 37°C. The cells are carefully washed 3 $\times$ with PBS to remove residual mitomycin C, trypsinized, and plated on 100-mm gelatinized dishes with fresh feeder cells in ES cells medium supplemented with 4- μ g/ml polybrene. ES cells are trypsinized, counted, and pelleted as described above. Between $0.5\text{--}1\times 10^6$ ES cells are seeded for cocultivation into one 100-mm dish. Cells are cocultivated for 48 hr, and fresh medium supplemented with polybrene is added every day. The cells are trypsinized and plated at a 1:100 dilution into new dishes in ES medium supplemented with G418 and fresh MEFs added. Medium is changed every day, and colonies are isolated 7–8 days later.

Isolation of ES Cell Clones and Preparation of Master Plates—At the time of their isolation, ES colonies should have a smooth, rounded “hunched-up” shape, with no differentiating cells visible on the borders and no dead cells in the center. Cells from a single ES cell clone are divided into two 96-well plates, one of which will be frozen as a master plate, and the second will be used for expansion and expression screening during *in vitro* differentiation.

The following plates are prepared for the isolation of clones: (i) 96-well plates with flat-bottom wells, gelatin-coated, with MEFs added (use one 0.2-ml frozen aliquot per 96-well plate) in 200- μ l ES cell culture medium per well. Duplicate plates for each clone are prepared. We routinely isolate 400 clones per day; thus eight 96-well plates are needed. (ii) 96-well plates with V-shaped bottom wells, filled with 30- μ l Trypsin–EDTA each. Colonies are picked in a laminar flow hood under a stereomicroscope. Using a 20- μ l pipetman, scrape with the tip in a circle around the ES cell clone to disrupt the layer of MEFs, carefully scoop up and then suck up the ES cell clone in a small volume. Transfer to a V-shaped 96-well plate filled with Trypsin–EDTA. Continue to pick one row or two rows of 8 clones each. It is important not to let the clones incubate in trypsin for extended periods of time. Using a multichannel-pipettor with eight tips attached, cells from one row of eight wells are dissociated by pipetting 20–30 times up and down. Culture medium (75 μ l) is added, and cell suspension (50 μ l each) is plated into the prepared duplicate 96-well plates. Clones are grown until they reach 50–80% confluence. Cells are frozen down either from both plates or, alternatively, cells are frozen from one plate and cells from the duplicate plate are immediately expanded to induce EB formation as described in the following section.

To freeze cells in 96-well plates, cells are washed twice with PBS and dissociated in 20- μ l Trypsin–EDTA using the multichannel-pipettor. Eighty microliters of freezing solution (70- μ l culture medium plus 10- μ l DMSO) is mixed with the cell suspension. The plates are sealed with parafilm, placed in a freezing bag and frozen at -80°C . To thaw clones quickly, 200- μ l warm medium is added to 96-well plates, and plates are placed in the 37°C incubator. After cells are attached, medium is replaced with a freshly prepared suspension of MEFs in 200- μ l culture medium per well. Cells are expanded from 96-well plates to 24-well plates and then to T25 flasks.

Screen for Differential AP Reporter Expression During *In Vitro* Differentiation of ES Cells

Design of Differential Expression Screen

To identify clones that contain entrapment vector insertions into developmentally regulated genes, we designed a screen in which candidate clones are selected by virtue of their stage-specific reporter gene expression upon *in vitro* differentiation into embryoid bodies. Studying the onset of expression of a panel of germ layer and cell lineage marker genes allowed us to determine the time course of differentiation during the formation of embryoid bodies (Table I). Thus, stages equivalent to embryogenesis between implantation and the beginning of gastrulation (4.5–6.5 dpc) occur within the first two days of EB differentiation. Between days 3 and 5, EBs contain cell lineages found in embryos during gastrulation at 6.5–7.0 dpc, and after day-6 in culture, EBs are equivalent to early organogenesis-stage embryos (7.5 dpc).³¹

The screen described here eliminates a bias for insertions into genes that are transcriptional active in undifferentiated ES cells. It also reduces the number of candidate clones to be analyzed later in developing embryos. In a screen of 2400 ES cell clones, 41 (1.7%) displayed a differential reporter gene expression during EB formation. Strikingly, the majority of these insertions (76%) were in genes that showed alkaline phosphatase expression during *in vitro* differentiation but not in undifferentiated ES cells³⁰ (Xiong et al., 1998³⁰). A crucial aspect of this *in vitro* differentiation screen is that the temporal and spatial reporter gene expression during EB formation is a good predictor for its expression in the embryo. Thus, we found that clones with restricted reporter gene expression *in vitro* exhibited similar temporally and spatially restricted AP expression *in vivo*.^{30,31}

The protocol provided below is used for screening a large number of ES cell clones with random retroviral entrapment vector insertions. Prior to inducing spontaneous differentiation, single ES cell clones are pooled in groups of 8–10. Since the trapping efficiency with our entrapment vectors is between 1 and 5%, one expects that about 1 out of two to ten pools will contain one positive clone. Once a positive pool has been identified, individual clones from this pool are rescreened.

Culture and Assay Conditions

Embryoid Body Culture Conditions—Embryoid bodies are cultured in suspension in DMEM (containing high glucose and glutamine) supplemented with 10% heat-inactivated FBS (Gemini or Atlas), 20 mM HEPES (50X stock, GIBCO), and penicillin and streptomycin (100X stock, GIBCO). Suspension cultures are grown in bacterial dishes (LabTech, Nunc). It is crucial to test serum samples for their ability to support good EB formation. In our experience, serum that is optimal for undifferentiated ES cell cultures often is not ideal for *in vitro* differentiation. Criteria for a good serum are formation of intact, spherical EBs of uniform size with smooth borders, absence of sticking to the bacterial surface, and absence of cell death. We have had good success with FBS from Gemini and Atlas.

Alkaline Phosphatase Activity Assay—This protocol has been optimized from a previously published procedure³⁴ to assay for human placental AP activity in ES cells, EBs and embryos. AP expression is detected as a strong, dark purple histochemical stain. Uninfected mouse ES cells and embryoid bodies exhibit a high level of endogenous AP activity, even in the presence of Levamisole, a specific inhibitor for rodent APs. Heat treatment at 70°C for 30 min in the presence of MgCl₂ prior to staining with BCIP/NBT reduces the nonspecific activity to a faint pink background staining while not interfering with the dark purple stain from the human placental AP activity. In our experience, using the BM Purple AP substrate (Boehringer Mannheim/Roche) substantially reduces the background from endogenous AP activities and

is preferred over BCIP/NBT. However, when used in combination with RNA *in situ* analysis, the strong purple precipitate of the BM Purple AP substrate can interfere with detection of silver grains. If this proves to be a problem, the samples should either be stained for a shorter time, or BCIP/NBT should be used instead.

Cells, EBs, or embryos are washed 1× in PBS at room temperature and fixed for 5 min at room temperature in fixation solution. To prepare the fixation solution, 2-g solid paraformaldehyde is dissolved in the fume hood on a heat stirrer for 5–10 min in 100-ml 0.1 M PIPES (pH 6.9), 2 mM MgCl₂, 1.25 mM EGTA, pH 8.0, and subsequently cooled on ice. The fixation solution should be freshly prepared. Samples are washed twice for 5 min each in PBS containing 10 mM MgCl₂. To inactivate endogenous AP activities, samples are heat-treated for 30 min at 70° C in PBS containing 10 mM MgCl₂. Samples are washed for 10 min at room temperature in AP buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 10 mM MgCl₂) and stained in AP staining solution (0.1-mg/ml BCIP; 1-mg/ml NBT; 0.24-mg/ml Levamisole (SIGMA) in AP buffer) or with BM Purple AP substrate. Samples should be stained for at least 4 hr or overnight at room temperature in the dark and are subsequently washed 1× in PBS.

Differentiation of ES Cells into Embryoid Bodies

Individual ES cell clones are expanded from 96-well plates to 24-well plates. About 2 days later, when cells reach 30–50% confluence, clones are pooled in groups of 8–10 and induced to spontaneously differentiate into embryoid bodies. About 1/4 of cells from each clone are replated in the original 24-well plate in the presence of fresh MEFs, and they are fixed and stained for AP activity 1–2 days later when small clumps of undifferentiated ES cells have formed. Pooled cells are plated in 60-mm gelatin-coated plates in the absence of MEFs in ES cell culture medium without LIF. After 2 days in culture, small ES cell clumps are lifted off the plates by gentle trypsinization and transferred with a wide-mouth pipette into suspension culture in EB culture medium. It is important to incubate in trypsin for the shortest time possible to avoid breaking up of the embryoid bodies. Every 48 hr half of the culture supernatant is replaced with fresh culture medium. This is achieved by tilting the plates to let EBs settle down on one side of the plate. After 4 days, “simple embryoid bodies” form, consisting of an outer layer of endodermal cells, a basal lamina and an inner layer of columnar, ectodermal cells. The simple EBs are kept for another 6 days in suspension culture, where they further differentiate into complex “cystic embryoid bodies” containing visible cavitations, pockets of primitive blood islands and rhythmically contracting cardiomyocytes. In parallel, day-4 simple EBs are replated on gelatinized tissue culture plates, where they reattach within 24 hr. Outgrowth of differentiated cells with various phenotypes, including fibroblasts, endothelial-like cells, neurons, contracting cardiomyocytes, differentiated cell types with an uncharacterized phenotype, as well as pockets of undifferentiated cells, are observed during the next 6 days. Aliquots of EBs at day 4, day 7, and day 10 in suspension culture are harvested, fixed and histochemically stained for AP activity. Day-6 cultures of replated EBs are assayed for AP activity as well. Once positive pools are identified, individual clones from these pools are expanded from the frozen master plates and analyzed for AP activity in undifferentiated ES cells, upon EB formation, and in cultures of replated EBs. Only those ES cell clones should be scored positive that display reproducible AP expression in at least 50% of all EBs present in each culture sample. Positive ES cell clones should be retested for their AP expression pattern, expanded and frozen in aliquots for further analysis. Examples for AP staining in EBs from ES cell clones that were infected with retroviral entrapment vectors are depicted in Fig. 3.

Colocalization of AP Reporter Gene and Developmental Marker Expression

Typically, characterization of entrapment events has been dependent on the generation of transgenic lines where reporter gene expression can be examined *in vivo*, or mutagenicity can

be assessed. However, the time-consuming and expensive nature of *in vivo* analysis limits its practicality for large-scale entrapment screens. Here, we describe a method for characterizing the tissue specificity of reporter gene expression *in vitro* in order to preselect candidate ES cell clones for generating transgenic lines. By colocalizing reporter gene expression with RNA *in situ* hybridization signals from a panel of developmental marker genes (listed in Table I) in EBs, information on the cell lineage specificity of entrapment vector insertions can be obtained. In order for this approach to be successful, colocalization has to fulfill three criteria: (i) localization of *in situ* hybridization signals in EBs should be temporally and spatially defined. Leahy *et al.*³¹ have shown this to be true. (ii) It should be possible to examine histochemical staining for AP and *in situ* hybridization with riboprobes on the same section of EBs. We found that AP staining prior to *in situ* hybridization did not interfere with the sensitivity of hybridization. However, a strong AP precipitate can sometimes interfere with signal intensity by “masking” the silver grains. To minimize this problem, we recommend either reducing the time for staining, or using BCIP/NBT as a substrate instead of BM Purple (see above). (iii) Colocalization in EBs should accurately reflect colocalization in the embryo. Below, proof of principle is provided for one particular entrapment insertion identified in our screen.

RNA *in situ* Hybridization

Procedures used for embedding, sectioning, and mRNA *in situ* hybridization are essentially as described previously.^{31,35} Embryoid bodies are fixed in 4% paraformaldehyde and embedded in paraffin at 60°C overnight following a series of dehydrating washes (from 50 to 100% ethanol, final wash for 30 min in xylene). Embedded material is sectioned at 6 µm, deparaffinized in xylene, rehydrated, and digested with Proteinase K (Sigma) for 7.5 min. Sections are treated with acetic anhydride in 0.01 M triethanolamine and dehydrated prior to a 16-hr hybridization with ³⁵S-radiolabeled antisense mRNA probes. [α -³⁵S] UTP methionine (specific activity 1250 Ci/mmol) is obtained from NEN/Dupont (Cambridge, USA). The final probe concentration during hybridization should be 35 cpm/ml in formamide hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10 mM NaH₂PO₄×H₂O pH 8.0, 10% dextran sulfate, 1 × Denhardt's, 0.5-mg/ml total yeast RNA, 10 mM DTT). Posthybridization washes of increasing stringency (50% formamide, 2 × SSC, 0.1 × SSC) and 1 hr RNase A treatment at 37°C are included to reduce background. Slides are dehydrated through a series of ethanol/NH₄OH washes and dried for 1 hr. Slides are dipped in Kodak NBT-2 emulsion, dried over-night and exposed for 5 days to 2 weeks at 4°C. Sections are counter-stained with toluidine blue (Sigma), unless they were stained before as whole-mount for AP, dehydrated and coverslipped.

Colocalization: Proof of Principle

For colocalization analysis, ES cell clones are induced to spontaneously differentiate in suspension culture. Embryoid bodies are harvested between days 4 and 10, fixed and histochemically stained as whole-mounts for AP (see above). *In situ* hybridization is performed on serial sections of paraffin-embedded EBs, using the panel of developmental markers listed in Table I. As an example and proof of principle, we will discuss our results with ES clone 1–13 that contains an insertion of the PT-IRES-AP virus (Fig. 4). Clone 1–13 displayed spotty AP reporter gene expression in embryos, varying between day 4 and 10 in culture. Expression of the AP reporter in embryos derived from clone 1–13 was detected in the visceral yolk sac and developing vascular structures.³⁰ AP reporter expression at various stages of EB differentiation was found to colocalize with signal from the endothelial marker *flk-1*, but not with other developmental markers, including *Brachyury* T (Fig. 4). Furthermore, colocalization of AP reporter activity and *flk-1 in situ* hybridization signal was also observed in sections of 8.0 and 9.5 dpc embryos derived from clone 1–13 (Fig. 4). Thus, reporter gene expression during *in vitro* differentiation accurately reflected expression during embryogenesis, and its colocalization with *flk-1* was predictive of expression *in vivo* in the endothelial lineage.

Conclusions

The genetic screen described in this chapter combines an approach that is unbiased for gene expression in ES cells with an *in vitro* screen in EBs to identify candidate genes with restricted expression during early postimplantation development as well as in particular cell lineages. We have used this approach successfully to isolate previously unidentified genes with lineage restricted expression (Refs. 30, 36, 37; our unpublished results). This general approach is versatile and can be adapted easily for different purposes. For example, different stages of embryonic development could be targeted by using a different time frame during *in vitro* differentiation for the screen. Second, conditions that induce differentiation of ES cells into particular cell lineages could be applied in order to enrich for insertions into genes that are specifically expressed in this lineage (e.g., Refs. 38, 39). Finally, it might be possible to modify the approach to allow for functional screening. For example, one might select for ablation of a particular cell lineage or cell type if a Herpes virus simplex thymidine kinase (TK) gene or a Diphtheria toxin gene is used as a reporter gene.

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Fig. 1.

Schematic overview of a genetic screen for developmentally regulated genes. ES cells are infected with a retroviral entrapment vector (depicted is PT-IRES-AP) and integration events are selected for in the presence of G418. Following selection and isolation of single colonies, ES cell clones are differentiated *in vitro*, and EBs are screened at multiple days of differentiation for temporally and spatially regulated AP gene expression. AP-stained EBs are embedded, sectioned and *in situ* hybridization is performed to assay for colocalization with developmental marker genes.

Fig. 2.

Structure of bifunctional AP entrapment vectors. (A) The constructs contain a human placental AP reporter gene. PT-AP contains a 1.9-kb AP cDNA fragment including its ATG. GT-AP contains a 170-bp XhoI-KpnI fragment with the Mo-MLV *env* splice acceptor site (SA) and the AP cDNA with a deletion of its ATG. PT-IRES-AP contains a 0.6 kb IRES fragment from EMCV and the AP cDNA. All constructs contain a *neo* expression cassette with a *pgk-1* promoter and the *neo^R* gene. The vectors contain 5' Mo-MLV sequences including the 5'LTR, the packaging site ψ , and 1-kb of gag sequences. The 3' end of the vectors contains Mo-MLV sequences including the 3'LTR with the 200-bp *E. coli supF* gene inserted in U3. The 3'LTR also contains a 300-bp deletion of retroviral enhancer and promoter sequences. The relative position of the *supF* insert, the deletion of enhancer and promoter (enh⁻/pro⁻), and the polyadenylation [p(A)] sequence are indicated. (B) The structure of 3' Mo-MLV LTR in AP entrapment vectors is schematically shown. Indicated are the positions of the 200-bp *E. coli supF* insert, enhancer sequences (direct repeats; DR), CAT and TATA transcriptional sequences, and the polyadenylation sequence p(A) in the LTR. The enhancer and promoter sequences are deleted between the PvuII (P) and the SacI (S) sites.

Fig. 3.

Differential AP reporter gene expression in EBs. ES cells were infected with entrapment vectors and differentiated *in vitro* to spontaneously form EBs in suspension culture. Aliquots were harvested and stained for AP activity. (A, B) Day-4 and -7 EBs from uninfected ES cells. (C, D) Day-4 and -7 EBs from a clone infected with PT-AP. (E, F) Day-7 and -10 EBs from a clone infected with GT-AP. (G, H) Day-4 and -7 EBs from a clone infected with PT-IRES-AP. (See Color Insert.)

Fig. 4.

Colocalization of *flk-1* and AP reporter gene expression in EBs and transgenic mice derived from ES cell clone 1–13. *In situ* hybridization on sectioned, AP stained clone 1–13 EBs (A–F) and clone 1–13-derived transgenic embryos (G–L). (A, B) *Brachyury* T riboprobe did not colocalize with AP reporter gene expression. Riboprobes corresponding to *flk-1* colocalized with AP reporter gene expression in (C, D) day-4 EBs; (E, F) day-9 EBs; (G, H) 8.0 dpc embryo (transverse section), insert showing higher magnification of the dorsal aorta (dorsal aorta, da; neural groove, ng; vitteline vein, vv; blood island, bi); (I, J) neural tube of 9.5 dpc embryo; (K, L) somites of 9.5 dpc embryo. Dark field images of A, C, E, G, I, and K correspond to bright field images of B, D, F, H, J, and L, respectively. (See Color Insert.)

TABLE I.
Summary of Marker Gene Expression in Embryoid Bodies