

Differentiation Trapping Screen in Live Culture for Genes Expressed in Cardiovascular Lineages

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We have developed a gene trap vector that transduces an EGFP-neo fusion gene (Eno) to monitor the expression of trapped genes in living cells and embryos. Upon *in vitro* differentiation, most gene-trapped embryonic stem (ES) cell clones exhibited detectable green fluorescence in various specialized cell types, which can be followed in the live culture in real time. Populations of ES cell-derived cardiomyocytes, smooth muscle cells, vascular endothelial cells, and hematopoietic cells were readily recognized by their distinctive morphologies coupled with unique activities, allowing efficient screening for clones with trapped genes expressed in cardiovascular lineages. Applying G418 selection in parallel differentiation cultures further increased detection sensitivity and screening throughput by enriching reporter-expressing cells with intensified green fluorescent protein signals. Sequence analyses and chimera studies demonstrated that the expression of trapped genes *in vivo* closely correlated with the observed lineage specificity *in vitro*. This provides a strategy to identify and mutate genes expressed in lineages of interest for further functional studies. *Developmental Dynamics* 229:319–327, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

The cardiovascular system is the first functional organ system to form in vertebrate embryos. The complexity of these organogenesis events is reflected in the high prevalence of congenital heart diseases, which occur in approximately 8 of every 1,000 live births (Brown and Anderson, 1999). With the development of advanced mutagenesis techniques, particularly gene targeting, considerable progress in cardiovascular research has been made in recent years. Many genes implicated in cardiovascular development, most notably including several transcrip-

tion factors, have been identified and their roles validated in knockout mice. These findings have provided insights into the molecular mechanisms governing the complicated developmental process, as well as the etiologies of many cardiovascular diseases (reviewed in Baldwin and Artman, 1998; McFadden and Olson, 2002).

Gene trapping in mouse embryonic stem cells is a potent mutagenesis tool for both gene discovery and functional analysis. By introducing a promoterless reporter/selectable marker gene into the mouse genome, gene trapping efficiently dis-

rupts endogenous transcription units largely at random and allows for straightforward expression monitoring and cloning of the trapped genes (reviewed in Stanford et al., 2001). In a phenotype-driven gene trap screen (Friedrich and Soriano, 1991), we previously have established the ROSA5 line in which transcription enhancer factor 1 (TEF-1) was disrupted. *TEF-1* null embryos die around E11.5 with thin ventricle walls and impaired trabeculation, suggesting its essential role in cardiogenesis (Chen et al., 1994). A similar screen identified the transcription factor Jumonji as a critical player for

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neural tube formation, hematopoiesis, and cardiac development (Takeuchi et al., 1995, 1999; Kitajima et al., 1999). Alternatively, expression-driven gene trap screens can be conducted to find lineage-specific genes by generating chimeric embryos with trapped clones and examining the reporter expression patterns (Wurst et al., 1995). Although exceedingly informative, *in vivo* gene trap screens are labor-intensive and require vast resources. Taking advantage of the developmental potential of embryonic stem (ES) cells to differentiate into a variety of cell types under appropriate culture conditions (reviewed in Keller, 1995; O'Shea, 1999), several differentiation trapping methods have been developed to preselect gene trap events *in vitro* (Shirai et al., 1996; Baker et al., 1997; Muth et al., 1998; Stanford et al., 1998; Cannon et al., 1999; Hidaka et al., 2000). By using morphologic criteria and/or immunolabeling with lineage-specific antibodies, these approaches attempted to predict if the trapped gene is expressed in certain lineages in the mouse by identifying the reporter-expressing cell types derived from the corresponding trapped clone. Although limited information on spatial-temporal expression patterns can be obtained, these methods significantly enrich for clones with trapped genes expressed in the lineages of interest. The power of differentiation trapping screens was well demonstrated in the rediscovery of *jumonji* based on its expression in ES-cell derived cardiomyocytes, which faithfully reflects the *in vivo* myocardium expression (Baker et al., 1997; Lee et al., 2000).

The cardiovascular lineages are among the most recognizable cell types in the complex embryonic stem (ES) cell differentiation culture. In addition to the specialized structures they form, these cells exhibit characteristic activities that are hallmarks for lineage identification. The most striking activities are the spontaneous, rhythmic contractions of cardiomyocyte aggregates (Wobus et al., 1991). Smooth muscle cells are found in tightly packed bundles of thin fibers showing slow, wave-like contractions (Weitzer et al., 1995;

Drab et al., 1997), which differ markedly from the much thicker, fast-contracting skeletal muscle myotubes (Rohwedel et al., 1994). Vascular endothelial cells form blood island-like pockets and blood vessel-like channels that frequently contain slowly moving or flowing hematopoietic cells, which are round and refractile in appearance (Wang et al., 1992; Bautch et al., 1996). However, morphologic identification of cardiovascular lineages, especially of cardiomyocytes, is challenging with conventional differentiation trapping screens where β -galactosidase is predominantly used as gene trap reporter, as these cellular activities are abolished after fixation as required for X-Gal staining. Although lineage identities can be unambiguously revealed with immunocytochemistry, this approach is rather laborious, and would need multiple replicates to label different cell types. Moreover, because the differentiation culture is highly heterogeneous and dynamic, screening at one end point may miss genes expressed in certain lineages that are either absent or unidentifiable at the time of sampling. To circumvent these obstacles, we have developed a gene trap vector that uses green fluorescent protein as a noninvasive reporter for differentiation trapping screens in live culture.

RESULTS AND DISCUSSION

Construction of a Green Fluorescent Neomycin Phosphotransferase

A human codon-optimized green fluorescent protein variant EGFP (Clontech) was fused in-frame to the neomycin phosphotransferase (*neo*) gene to create a reporter/selectable marker hybrid, Eno (for EGFP-*neo*), in the same manner as previously described for β geo (Friedrich and Soriano, 1991). To determine whether the GFP activity is compromised in the fusion protein, expression vectors with EGFP, Eno, or a commercially available fusion NeoEGFP (Clontech) placed under the controls of the phosphoglycerate kinase 1 (PGK) promoter and the bovine growth hormone polyadenylation signal (*bpA*) were constructed.

Transient expression in NIH3T3 cells indicated that the GFP signal intensity of Eno was only slightly weaker than that of EGFP, yet considerably stronger than that of NeoEGFP. To test the functionality of the *neo* moiety of the fusion protein, PGKEnobpA was electroporated into ES cells in parallel with PGK β geobpA. Similar numbers of G418 resistant clones were obtained, and virtually every PGKEnobpA clone emitted robust green fluorescence. Therefore, Eno encodes a bifunctional protein with both GFP and *neo* activities, neither of which is significantly impaired in the fusion.

Establishment of Differentiation Culture Conditions Suitable for Cardiovascular Lineage Formation

To set up the *in vitro* differentiation conditions that favor the formation of cardiovascular lineages, particularly cardiomyocytes, we first compared two methods for embryoid body (EB) induction: the suspension culture and the hanging drop culture. The induced EBs were transferred to gelatin-coated 24-well plates to allow attachment. EBs formed in the suspension culture varied vastly in size and shape and tended to aggregate with each other. As a result, many EBs were unable to attach or to outspread, and secondary cystic EBs were frequently formed. Although multiple EBs ($n \approx 10$) were seeded into each well, only half of the wells contained spontaneously contracting areas during 20 days of differentiation, most of which appeared after day 12 (i.e., 8 days after plating). In sharp contrast, the hanging drop culture reproducibly yielded EBs with similar size and regular shape, most attaching onto gelatinized surface overnight and forming nice outgrowths. Rhythmical contractions were observed in around one third of individual EB outgrowths on as early as day 7, and the majority of wells contained multiple beating foci by day 14. To examine the representation of cardiovascular lineages in these cultures, we performed indirect immunofluorescence staining on day 20 EB outgrowths by using antibodies against sarcomeric myosin heavy

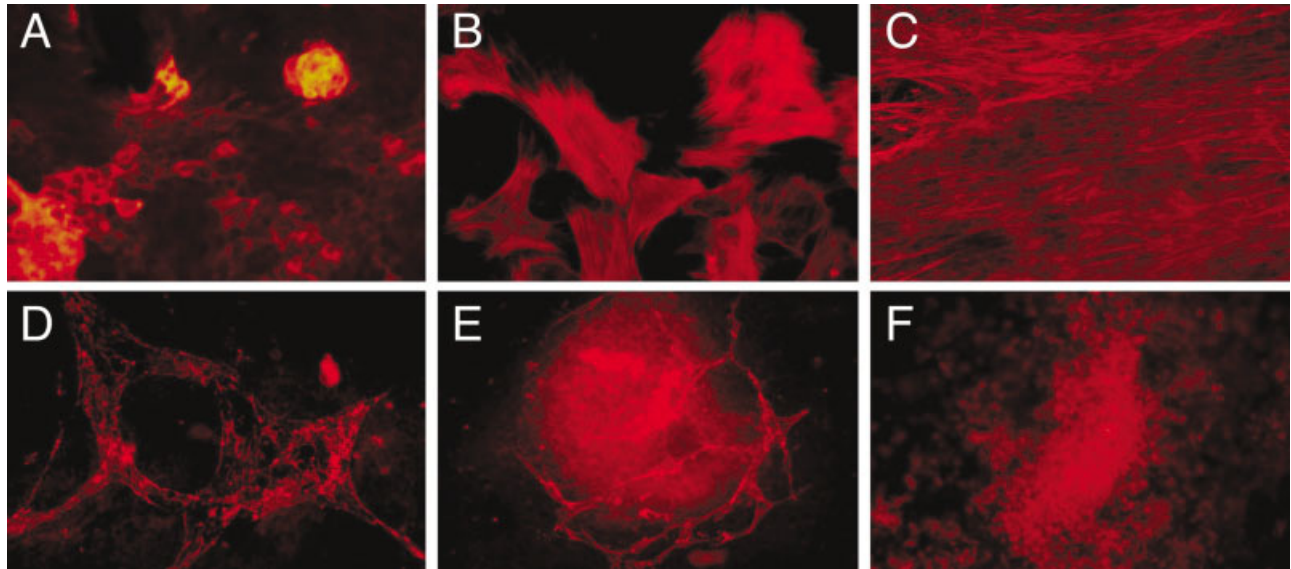


Fig. 1. Representation of cardiovascular lineages in the differentiation culture. Embryoid bodies (EBs) were formed in the hanging drop culture and transferred to gelatinized chamber slides for attachment. EB outgrowths were assayed on day 20 by indirect immunofluorescence staining with lineage-specific primary antibodies. **A:** Cardiomyocytes were labeled with the sarcomeric myosin heavy chain mAb MF-20, which also recognizes skeletal muscle cells (not shown). **B,C:** Individual (B) or bundles (C) of smooth muscle cells were usually found in the periphery or the center of EB outgrowths, respectively (smooth muscle α -actin mAb 1A4). The PECAM-1/CD31 mAb labels vascular endothelial cells and certain hematopoietic cells. **D:** Blood-vessel-like channels lined by vascular endothelial cells. **E:** A blood island-like structure surrounded by vascular networks. **F:** A hematopoietic cell colony with strong PECAM-1/CD31 staining.

chain (Bader et al., 1982), smooth muscle α -actin (Skalli et al., 1986), and PECAM-1/CD31 (Vecchi et al., 1994). Besides abundant cardiomyocytes (Fig. 1A), populations of smooth muscle cells (Fig. 1B,C), vascular endothelial cells, and hematopoietic cells (Fig. 1D–F) were also frequently observed, suggesting that the hanging drop culture is the method of choice to promote the formation of a full spectrum of cardiovascular lineages.

Expression of *Eno* Under α -Cardiac Myosin Heavy Chain Promoter

To mimic a gene trap event in a cardiac-specific gene and examine the behavior of *Eno* in the differentiation culture, the fusion gene was placed downstream of α -cardiac myosin heavy chain (α MHC) promoter (Klug et al., 1996), and a PGK-hygro cassette was incorporated in the vector to derive stable transfectants. After electroporation and hygromycin selection, resistant ES cell colonies were pooled, expanded, and induced to differentiate in the hanging drop culture followed by the attachment cul-

ture. Starting on day 14, G418 selection was applied in one duplicate and maintained for 10 days. Although green fluorescent beating foci were readily observed in unselected EB outgrowths, the GFP signals were typically weak (Fig. 2A,D). In contrast, the 10-day selection eliminated most cells in culture, and masses of rhythmically contracting cells with strong green fluorescence (Fig. 2B,E), as well as individual green fluorescent cells with contractility (Fig. 2C,F) were observed, indicating the accumulation of *Eno* proteins within these cells. These observations suggested that expression of the fusion gene is strictly controlled by the acquired regulatory elements and that applying G418 selection in the differentiation culture can enrich for reporter-expressing cells with intensified GFP signals. Therefore, this pilot experiment provided the proof-of-principle and guidelines for differentiation trapping screens in live culture by using an *Eno*-based vector.

Differentiation Trapping Screen

The gene trap vector SA β geobpA was constructed by replacing β geo

in SA β geobpA (Friedrich and Soriano, 1991) with *Eno*, and gene-trapped clones were obtained with electroporation followed by G418 selection. Because capturing an endogenous promoter is absolutely required to activate *Eno* expression, essentially every G418 resistant clone represents a gene trap event (Fig. 3A). Of the 118 trapped clones isolated, 116 clones emitted green fluorescence of markedly different intensities as assayed at passage 2 (Fig. 3B,C), reminiscent of the high percentage of β -galactosidase-positive clones obtained with SA β geobpA or its retroviral version ROSA β geobpA. This finding was due to a mutation in the neo moiety that reduces its sensitivity (Komada et al., 2000), so that most trapped genes are expressed at relatively high levels in ES cells. As seen with β geo-based gene traps introduced by either electroporation or retroviral infection, in some clones, the GFP fluorescence displayed a mosaic pattern, suggesting that the clonal heterogeneity in reporter expression is reporter independent.

For the differentiation trapping screen, a 96-format hanging drop culture was designed to facilitate

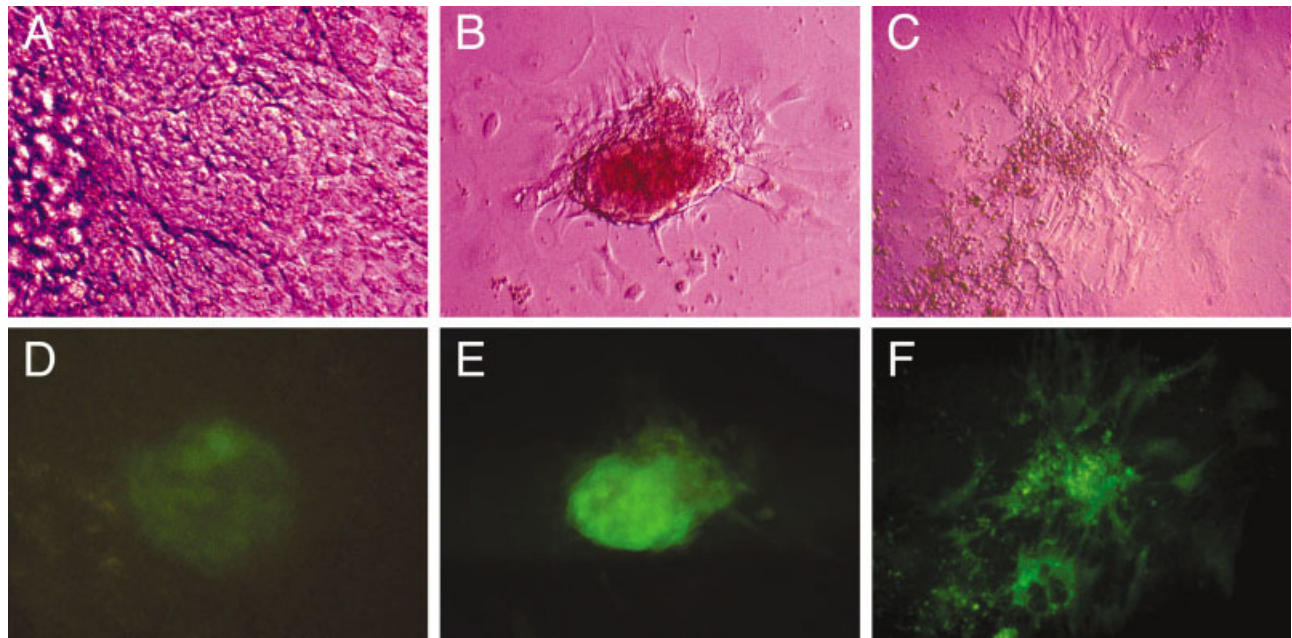


Fig. 2. Expression of α MHC-driven Eno in embryonic stem (ES) cell-derived cardiomyocytes. Embryoid body (EB) outgrowths from transfected ES cells were either selected with G418 on day 14 for 10 days or kept unselected. **A,D:** Green fluorescence was readily detected in beating areas in the unselected culture, but the signal intensity was typically weak. **B,C,E,F:** G418 selection eliminated most cells in EB outgrowths, and much stronger GFP signals were observed in the remaining contractile cell masses (**B,E**) as well as individual cardiomyocytes (**C,F**), indicating the accumulation of Eno proteins under selection.

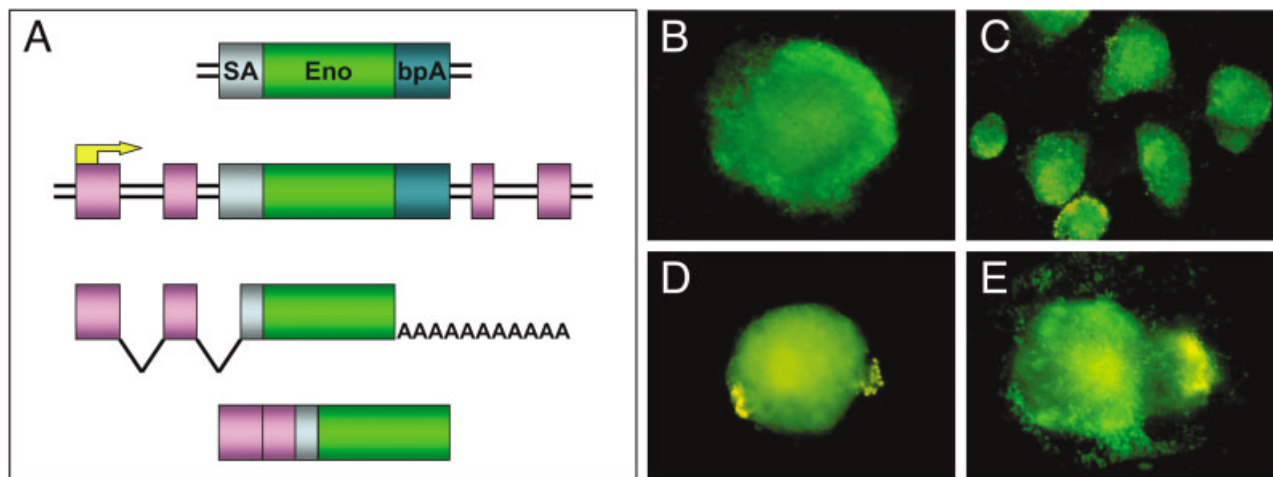


Fig. 3. Gene trapping with SAEnobpA and Eno expression in embryonic stem (ES) cells and embryoid bodies of gene-trapped clones. **A:** The gene trap vector was constructed by placing a promoterless Eno downstream of an adenoviral splice acceptor (SA) and upstream of the bovine growth hormone polyadenylation signal (bpA). Upon integration into an intron of an endogenous gene, the 5' exons were forced to splice into the introduced gene trap to generate a fusion transcript, from which Eno can be expressed independently or as a fusion protein depending on the insertion position. As a result, the endogenous transcription is arrested. **B:** A gene-trapped ES cell clone showing a medium level of green fluorescence. **C:** ES cell colonies of the same clone at passage 2. **D:** A day 4 EB formed in the hanging drop culture. **E:** EB outgrowths formed 1 day after plating. Note the characteristic yellow autofluorescence from dead cells.

the manipulation of multiple clones. The formed EBs were subsequently transferred into duplicate 24-well plates for attachment cultures (Fig. 3D,E), and G418 selection was applied to one set after day 14. With Eno as a vital reporter, we were able

to monitor the expression of trapped genes at desired stages throughout the entire culture period. Although a general reduction in signal intensity was observed after differentiation, the majority of trapped clones continued to exhibit detectable green

fluorescence in a variety of derivative cell types. While most cell types were morphologically indistinguishable, aggregates of beating cardiomyocytes, contracting smooth muscle cell bundles, as well as blood vessels and blood islands comprising

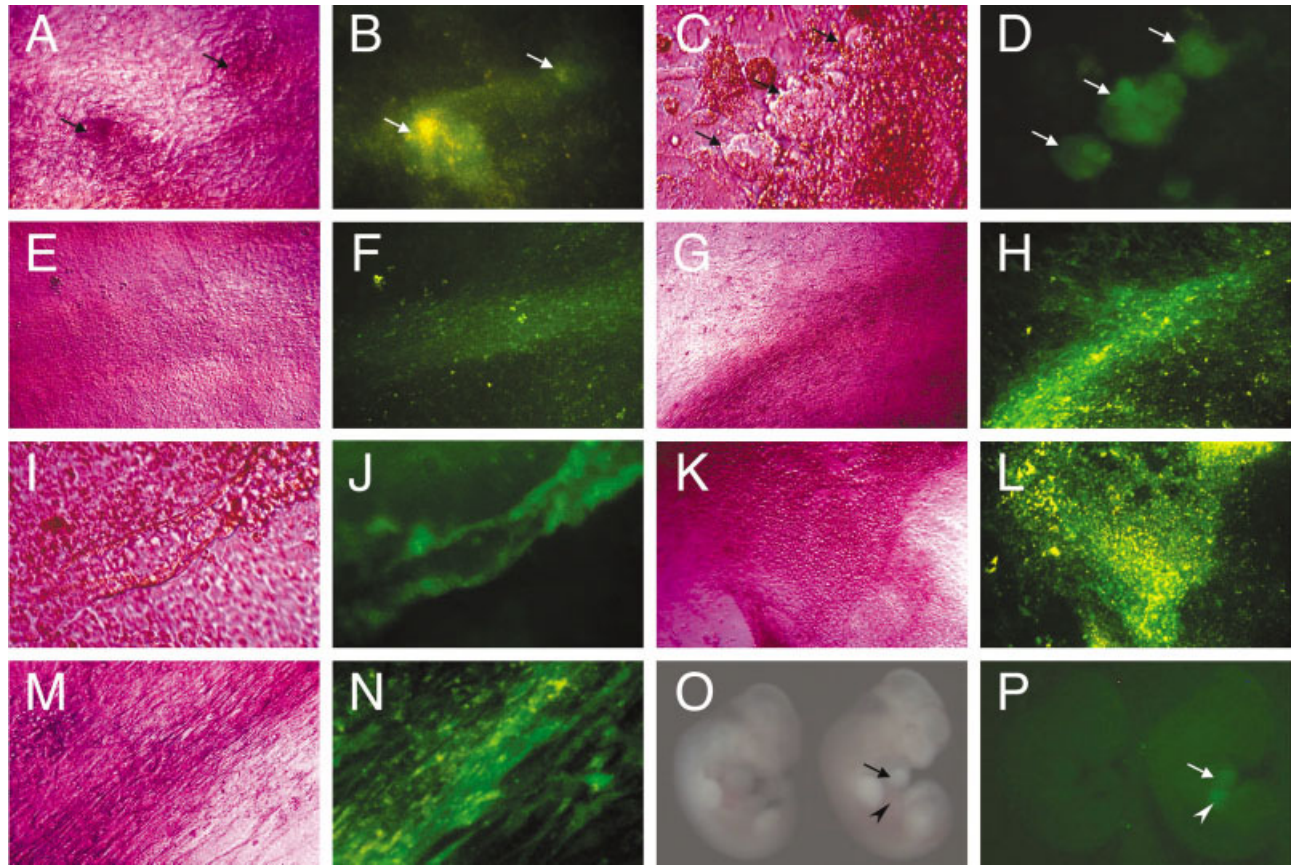


Fig. 4. Differentiation trapping screen in live culture. Embryoid body (EB) outgrowths of gene-trapped embryonic stem (ES) cell clones were cultured in the presence or absence of G418 and observed for Eno-expressing cell types. Shown are unselected (A,B,E,F) or selected (C,D,G–N) EB cultures of representative clones with Eno expression in lineages identifiable with their characteristic morphologies and activities. Detection sensitivity and screening throughput were significantly increased with G418 selection. **A–D:** Beating cardiomyocyte aggregates (arrows). **E–H:** Contracting smooth muscle cell bundles. In both cases, the GFP signals were evidently stronger in the selected culture (C,D,G,H) compared with the unselected culture (A,B,E,F) of the same clone. **I,J:** A blood vessel with Eno expression in vascular endothelial cells. **K,L:** A blood island with Eno expression in hematopoietic cells. **M,N:** Eno-positive contractile skeletal muscle myotubes. **O,P:** An embryonic day 11.5 chimeric embryo (right) produced with clone CC1B11 (shown in A–D) and a wild-type littermate (left). Green fluorescence was detected in the heart (arrows) and the liver (arrowheads) in the chimeric embryo. Forelimbs were removed to facilitate visualization.

vascular endothelial cells and moving hematopoietic cells were readily identified by their characteristic structures and activities. This identification allowed us to screen for clones in which the trapped genes are expressed in the cardiovascular lineages (Fig. 4A–L). Among other lineages, contractile skeletal muscle myotubes were the most prominent (Fig. 4M,N). Consistent with the above observations on the behavior of α MHC-driven Eno, the expression in the same lineage was evident in both selected and unselected cultures in some cases, whereas the green fluorescence was always remarkably brighter in the selected culture (Fig. 4A–H). In many clones, the GFP signals in certain lineages were only detectable in the se-

lected culture; these signals would have been missed if only unselected cultures were set up. Therefore, applying G418 selection significantly increased detection sensitivity and screening throughput. However, a drawback of the selection procedure was that no cells survived in some cases, presumably due to diminished Eno expression upon differentiation, or the deprivation of supporting cell layers underneath expressing cells. Occasionally, undifferentiated ES cells were also enriched, which could form enormous colonies that dominate the culture. Together, these two scenarios resulted in uninformative selected cultures in approximately 30% of trapped clones, for which only the unselected cultures were examined for reporter-expressing cell types.

Of the 118 clones screened, we identified a total of 18 clones that showed detectable GFP signals in beating cardiomyocyte nests. In 35 clones, green fluorescence was observed in blood vessel-like or blood island-like structures, indicating the expression of trapped genes in vascular endothelial cells and/or hematopoietic cells. Eno-positive smooth muscle cells or skeletal muscle cells were found in only a few clones, which was apparently associated with the low occurrence of identifiable populations of these cells in the culture. Applying specialized differentiation culture conditions (Rohwedel et al., 1994; Drab et al., 1997) or conducting the screen in a larger format may be needed to increase the throughput for genes expressed in these lineages.

TABLE 1. In Silico Analysis of Trapped Sequences^a

Clone	Sequence identity	GenBank accession no.	UniGene cluster	Cardiac ESTs/Total ESTs
CC1B5	Mouse 45S pre-ribosomal RNA gene	X82564		
CC1B11	Repeat, ERV-L family			
CC1C5	RIKEN cDNA 6820402O20 gene; transcription factor TZIP homolog (Homo sapiens)	NM_172674	Mm.18898	2/313
CC1F10	Hypoxia-induced gene 1 (Hig1)	NM_019814	Mm.7381	15/307
CC1G5	RIKEN cDNA 6720485C15 gene	NM_026551	Mm.181633	3/237
CC2E4	Hypothetical protein C530030I18; similar to zinc finger protein FLJ10697 (Homo sapiens)	XM_283507		
CC2E5	Stromal antigen 1 (Stag1)	NM_009282	Mm.42135	2/150

^aNumbers of cardiac ESTs and total ESTs of each UniGene cluster were based on UniGene Build #120. EST, expressed sequence tag.

Cloning of Candidate Cardiac Genes and Correlation Between In Vitro and In Vivo Expression

For all of the 18 clones with trapped candidate cardiac-expressing genes, green fluorescent beating foci were observed after 10 days under selection, although the signal intensity varied vastly between clones. Antibody staining with MF-20 in three selected clones further confirmed GFP expression in labeled cardiomyocytes (not shown). To assess in vivo expression patterns of trapped genes, chimeric embryos were generated from five clones and examined at E11.5. As expected, all three lines with detectable green fluorescence showed expression in the heart (Fig. 4O,P). The absence of GFP signals in the remaining two lines might be due to low chimerism or low expression levels at this stage.

We performed 5'-rapid amplification of cDNA ends (RACE) on 10 clones and obtained informative trapped sequences from seven clones (Table 1). In five clones, the trapped sequence matched a known or predicted protein-coding gene. For the four genes with UniGene cluster available, multiple corresponding expressed sequence tags (ESTs) isolated from heart or cardiac muscle cDNA libraries were found in each cluster, indicating the expected heart expression in vivo. Among these, the UniGene cluster for hypoxia-induced gene 1 (*hig1*)

contains the highest number of cardiac ESTs ($n = 15$). *Hig1* was originally identified with microarray analyses as upregulated by hypoxia in normal and cancerous human cervical epithelial cells (Denko et al., 2000) as well as in mouse cerebral cortical neurons (Jin et al., 2002). Given the cardiac expression of HIG1 and its potential localization in mitochondria (Denko et al., 2000), it is appealing to investigate its involvement in cardiomyocyte apoptosis, a main cause of hypertensive heart diseases known to be inducible by hypoxia (Fortuno et al., 2001). Remarkably, two of these genes (trapped in clone CC1C5 and clone CC2E4) encode for putative transcription factors, and one gene (*Stag1*) encodes for a member of a novel family of nuclear proteins (Carramolino et al., 1997). With the availability of the trapped clones, mutant mice could be directly generated to study their potential roles in cardiogenesis and other developmental processes.

The ubiquitously expressed mouse 45S pre-ribosomal RNA gene (rDNA) was trapped in clone CC1B5. This finding might have resulted from the relatively higher levels of rDNA expression in beating cardiomyocytes in the differentiation culture, as accelerated rDNA transcription is required for cardiac growth and hypertrophy (Brandenburg et al., 2001). Nonetheless, due to the complexity of the differentiation culture and the mosaic patterns of reporter expression, ubiquitous genes cannot

be reliably ruled out in differentiation trapping screens (Baker et al., 1997; Stanford et al., 1998; Hidaka et al., 2000). The trapped sequence of clone CC1B11 matched multiple genomic DNA fragments from different chromosomes and over 500 mouse ESTs with a cutoff expect value $\leq 10^{-25}$, suggesting that it is a repetitive element present in many transcripts. RepeatMasker searching (<http://repeatmasker.genome.washington.edu/>) revealed that it belongs to the endogenous retrovirus-like (ERV-L) repeat family (Benit et al., 1999). Of interest, restricted GFP expression pattern was observed in chimeric embryos of this clone (Fig. 4O,P), consistent with the observations that expression of some endogenous retroviruses is developmentally regulated (Schon et al., 2001). The identity of the trapped gene is yet to be defined in further studies. Together, chimera studies and sequence analyses provided evidence on heart expression for most candidate cardiac clones examined, manifesting that expression of the trapped genes in the mouse faithfully recapitulated the observed lineage specificity in culture.

In summary, we have developed an Eno-based vector system for differentiation trapping screens in live culture, which is particularly useful for identifying genes expressed in the cardiovascular lineages that form specialized structures and exhibit characteristic cellular activities. This approach significantly facilitates

lineage identification compared with using morphologic criteria only and is more efficient and cost-effective than performing immunostaining with lineage-specific antibodies. Moreover, the use of GFP as gene trap reporter permits continuous expression monitoring in the dynamic differentiation culture without the need of making multiple replicates for timed samplings, thereby further increases the screening throughput. Clearly, this system can be exploited to identify genes expressed in other lineages with suitable differentiation culture conditions, as well as genes responsive to exogenous stimuli with standard induction trapping methods (Forrester et al., 1996; Komada et al., 2000) or fluorescence activated cell sorting (Whitney et al., 1998; Medico et al., 2001).

EXPERIMENTAL PROCEDURES

Vector Construction

The EGFP coding sequence was amplified from pEGFP-1 (Clontech) by using primers 5'-GAAGATCTTCACCGGTCGCCACCATG-3' and 5'-GAAGATCTTGTACAGCTCGTCATGCC-3' to introduce a *Bgl*II site on both ends. The *Bgl*II-*Bgl*II fragment was used to replace the *Bam*HI-*Bam*HI β gal fragment in PGK β geobpA (Friedrich and Soriano, 1991) to derive PGKEnobpA. To construct PGKEGFPbpA, PGK β geobpA was first digested with *Sma*I and *Xba*I to remove β geo, blunted with T4 DNA polymerase, ligated to an *Nhe*I linker and then digested with *Nhe*I, followed by inserting an EGFP fragment modified in the same manner. PGK-NeoEGFP was constructed by replacing the *Nhe*I-*Nhe*I EGFP fragment in PGKEGFPbpA with the *Nhe*I-*Xba*I NeoEGFP fragment from pNeoEGFP (Clontech). To derive α MHC-Eno/P GK-hygro, an *Spe*I-*Sma*I (partial) EnobpA fragment was released from PGKEnobpA, blunted, and cloned into α MHC-neo/P GK-hygro (Klug et al., 1996) by using a *Not*I linker to replace the *Not*I-*Not*I neo-pA fragment. To engineer the gene trap vector SAEnobpA, the *Spe*I-*Sma*I (partial) EnobpA fragment was subcloned into pBluescript KS II, excised on a *Hind*III-*Xba*I fragment, and used to replace β geobpA in SA β geobpA.

Cell Culture and Transfection

NIH3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Equal molar amounts of PGKEnobpA, PGKEGFPbpA, and PGKNeoEGFPbpA were used to transfect the cells by using Lipofectamine-plus reagents (Invitrogen). Relative GFP signal intensity for each construct was assessed by the average exposure time of 10 representative fields. AK7.1 ES cells (Soriano, 1997) were cultured in DMEM supplemented with 15% fetal bovine serum, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 0.1 mM 2-mercaptoethanol on mitomycin C-treated SNL (neo^r; McMahan and Bradley, 1990) or SNLH (neo^r/hygro^r) feeder cell layers. ES cells were electroporated with linearized plasmids and selected in appropriate antibiotics (200 μ g/ml G418 or 200 μ g/ml hygromycin B) for 10–12 days to derive resistant clones. PGK β geobpA, PGKEnobpA, and α MHC-Eno/P GK-hygro were linearized with *Xho*I, and SAEnobpA with *Sca*I.

In Vitro Differentiation

Embryoid body (EB) induction in the suspension culture (Stanford et al., 1998) or the hanging drop culture (Wobus et al., 1991) was carried out essentially as described, except that 30- μ l drops containing 800–1,000 cells were set up and that standard ES cell medium was used. Resulting EBs were transferred in groups (if formed in the suspension culture) or individually (if formed in the hanging drop culture) into gelatin-coated 24-well plates on day 4 for subsequent attachment cultures. For the differentiation trapping screen, the hanging drop culture method was modified to form EBs in a 96-format. Briefly, gene-trapped ES cell clones were grown to confluence in 96-well plates, trypsinized, and reduced to a single cell level by vigorous pipetting. Serial dilutions were then made in 96-well plates to adjust the cell density to 3×10^4 /ml, based on the cell counts of representative wells. Drops of 30- μ l ES cell suspension were evenly arrayed on the lid of a U-bottom 96-well plate by using a multiple channel pipettor, and the

lid was carefully inverted and placed over the bottom of the 96-well plate preloaded with 10 ml of PBS. The assemblies were left untouched in the incubator for 4 days, and the resultant EBs were then transferred into gelatinized 24-well plates. For each clone, 6 EBs were produced for duplicate attachment cultures, with 3 EBs per well. To enrich for reporter-expressing cells, G418 selection (200 μ g/ml) was applied in one set of differentiation cultures on Day 14 after EB induction and maintained for 10 to 20 days. Medium was changed every 2–3 days for the first 10 days and when needed thereafter.

Chimera Production

ES cells were microinjected into C57BL/6J blastocysts to generate chimeric embryos according to standard procedures.

Immunocytochemistry

EB outgrowths cultivated on chamber slides were fixed in 4% paraformaldehyde for 10 min, followed by a 30-min treatment in 0.3% Triton X-100 and 10% goat serum in PBS. Treated cells were incubated for 1 hr with antibodies against sarcomeric myosin heavy chain (MF-20), smooth muscle α -actin (1A4), or PECAM-1/CD31 in 10% goat serum. Primary antibodies were detected with tetra-rhodamine isothiocyanate-conjugated goat anti-mouse IgG.

Fluorescence Microscopy and Imaging

GFP fluorescence of living cells was observed under an inverted Zeiss epifluorescence microscope using a standard fluorescein isothiocyanate filter, and images were recorded on Kodak P1600 films. To reduce autofluorescence, growth medium was replaced with warm Ca⁺⁺/Mg⁺⁺-containing HBSS (Invitrogen) just before observation. Chimeric embryos were placed in PBS and observed under a dissecting microscope equipped with an epifluorescence condenser and a GFP filter. Images were acquired with a digital Kalman averaging filter to reduce random noise.

Cloning Trapped Genes by 5'-RACE

The 5'-RACE was performed as described (Hildebrand and Soriano, 1999) by using total RNA extracted from gene-trapped ES cell clones. The gene-specific primers used were as follows: EnoR1, 5'-GATGGGGT-GTTCTGCTGGTAGTGG-3'; EnoR2, 5'-GAAGATGGTGCCTCCTGGACGTAG-3'; and EnoR3, 5'-CTCAGG-GTCAGCTTGCCGTAGGTG-3'. RACE products were cloned into the pGEM-T Easy vector (Promega) and sequenced with a splice acceptor exon-specific primer SAER 5'-GAAACACCGCGAAGAGTTG-3'.

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