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REGULATED EXPRESSION OF TRANSGENES IN EMBRYONIC STEM CELL-DERIVED NEURAL CELLS

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Abstract

Discovery and characterization of gene promoters, enhancers and repressor binding elements is an important research area in neuroscience. Here, the suitability of embryonic stem cells and their neural derivatives as a model system for this research is investigated. Three neural transgenic constructs (from the *Mnx1*, *Fabp7*, and *tuba1a* genes) that have been validated in transgenic mice were inserted into embryonic stem cells as stable transgenes. These transgenic embryonic stem cells were differentiated into neural cultures and the pattern of transgene expression across a series of inducing conditions determined. The pattern of expression matched that predicted from transgenic mouse experiments for each of the three transgenes. The results show that embryonic stem cells and their neural derivatives comprise a promising model for investigating the mechanisms that control cell- and temporal- specific neural gene transcription.

Keywords

Nervous system; gene transcription; neural development; cis-regulatory element; gene promoter

INTRODUCTION

The mechanism that neural cells (stem cells, neurons and glia) in the mammalian CNS use to transcribe genes with appropriate spatial and temporal specificity is a topic of great interest. Many studies aim at locating and functionally validating the promoters, enhancers and repressor binding elements (collectively called cis-regulatory element or **cis-REs**) for genes of interest (reviewed in Visel et al., 2009). Others seek to discover the detailed mechanism that cis-REs use to regulate transcription by RNA polymerase II. Transgenic mice (**TGM**) are a widely used model system for both types of study because of two attributes (see Nakada et al,2004;Henke et al., 2009; Miesgaes et al.,2009 for exemplary studies). First, since TGM are normal except for the presence of the transgene, regulatory elements are evaluated in normal brain cells, which is the most natural context conceivable. Second, the transgene in TGM integrates into a host chromosome placing it in chromatin. This is more natural than transient transfections, where DNA does not integrate into chromosomes. For these reasons TGM provide more rigorous evidence for transgene function than transient transfections of primary CNS cells or cell lines. However, TGM have practical and theoretical limitations. Practical limitations include the cost of making them and the need for specialized facilities and specially trained personnel. These limit the number of TGM that can be produced in an academic laboratory. The theoretical limitations of TGM arise from the way transgenes are arranged in the genome. Most transgenes integrate into a single chromosomal site as linear concatemers of the injected transgene, with

copy number typically ranging from 5-100 transgenes. Having multiple copies per integration site complicates the interpretation of expression data. Another major limitation is that transgenes integrate randomly in the genome so each transgenic mouse line has a unique integration site. Because chromatin at the integration site influences gene transcription, expression patterns from the same transgene differ from line to line. Some lines have no expression, others have ectopic patterns, yet others have either partial or full versions of the native gene expression pattern. The diversity of patterns from one transgene requires the investigator to make a subjective choice of which line best represents the activity of the transgene. In view of these limitations, the field of CNS transcriptional control needs models that complement the TGM approach. One based on Embryonic Stem Cells (**ESCs**) has promising features.

Several factors make ESCs an attractive model. In contrast to tumor-derived or transformed cell lines, ESCs are genetically normal and can be derived at will from normal animals. ESCs can be differentiated in culture into neural cells including neural stem cells, neurons and glia by a variety of well characterized pathways (reviewed in Cai and Gabel, 2007; Gaspard and Vanderhaeghen, 2009). Neural cells derived in cell culture from ESCs are defined as **ESNs** and a large body of literature shows that ESNs have many of the most important phenotypes of their embryo-derived counterparts. Methods for stable genetic modification of ESCs are practical and well-validated. They include random insertion of transgenes, DNA exchange with site-specific recombinases such as Cre or Flp, and gene targeting via homologous recombination, all of which have been used to engineer valuable ES cell lines. For instance in the cardiac lineage, stably incorporated transgenes expressing dominant negative and dominant positive Jak2 have defined a functional role for Jak2 in cardiac development (Foshay et al, 2005). ESCs stably transformed with a reporter construct able to detect STAT3 transcriptional activation (Xie et al., 2009) have been used to understand signaling through this pathway. Thus the potential exists to make transgenic ESCs with stably integrated transgenes and readout their expression in ESNs to find cis-REs. In principle this is a practical model that avoids some of the drawbacks of TGM. However, there are potential problems that might limit the usefulness of ESCs as a model.

One such problem is that transgenes used to transform ESCs always include a selection cassette (typically PGK neo) because only a small fraction of transfected ESCs stably integrates a transgene; these must be selected for. The selection cassette has a strong promoter that might interfere with the expression of the transgene being investigated. Another potential confound is insertion site influence on transgene expression. As described above, most transgenic mice have concatenated transgenes at the insertion site while transfected ESCs carry single integrated transgenes not concatemers. It is possible that expression of single copy transgenes in the ESC system would be lost beneath the “noise” of the insertion site. Another worrisome possibility is inactivation of transgenes in ESCs. ESCs express a set of genes that scan the genome for inserted retroviruses and inactivate them (Matsui et al, 2010; Rowe et al., 2010). These might mistake transgenes for retroviruses and inactivate them as well. In the production of transgenic ESCs, transgenes spend 2 weeks in ESCs during selection and expansion so even a slow inactivation pathway might shut them down. For all these reasons it is conceivable that, in spite of their initial appeal, ESNs are not a practical system for investigating cis-REs of neural genes. Clearly, new experiments are needed to validate them as a model.

In this study we test the hypothesis that ESCs are a valid model for studying cis-REs of neural genes. We focus on 3 genes (*Mnx1*, *Fabp7* and *tuba1a*) for which promoters and enhancers were defined in TGM (Gloster et al., 1994; Feng and Heintz, 1995; Nakano et al., 2005). All 3 have expression patterns in the transgenic mouse brain that resemble native gene expression and in this sense are validated transgenes. We made transgenic ESCs with

each transgene and investigated their pattern of expression in a series of differentiated cultures. All 3 transgenes were expressed in patterns that were predictable from transgenic mice. The data support the hypothesis that ESCs and derived ESNs are a valid model for studying promoters and enhancers of CNS genes. Although the potential pitfalls described above may still impose some limitations, we conclude the ESC model is suitable for investigating cis-REs of many neural genes.

MATERIALS AND METHODS

Mnx1, Fabp7 and tuba1a transgenes

The three transgenes consist of mouse genomic DNA driving luciferase and a PGK-neo cassette for selection in ESCs (Figure 1). All transgenes were constructed from the same parental plasmid, pANeoLuc. pANeoLuc has an AscI site for cloning genomic DNA, a luciferase reporter gene, and the PGK Neo cassette. Sequences of oligonucleotides used in plasmid construction are in Supplementary Table I. These sequences can be used to follow details of transgene construction. By aligning the primers to the UCSB Genome Browser the precise sequences of genomic DNA in each transgene can be found. pANeoLuc was constructed in 2 steps from the luciferase reporter plasmid pGL3 (Promega). First the multicloning site of pGL3 was removed with KpnI and HindIII and replaced by an AscI site to make plasmid pALuc by cloning the DNA fragment consisting of oligos AscI upper strand and AscI lower strand. In the second step, the floxed PGK Neo cassette of plasmid pL452 (Liu et al., 2003) was amplified by PCR with primers flanked by BamHI upstream and SalI downstream and cloned into pALuc cut with BamHI and SalI to create pANeoLuc.

The Mnx1 transgene consisted of 2 pieces of genomic DNA knit together by PCR and cloned into the AscI site of pANeoLuc. BAC RP24-21014 of the CHORI collection, which contains the Mnx1 gene was used as template for PCR. The upstream genomic fragment defined by primers Mnx-1 and Mnx1-2 is 3.5 Kb and contains the enhancer located 9 Kb upstream of the transcription start site (TSS) described in the literature (see Lee et al, 2004; Nakano et al, 2005). The second is the 1.1 Kb promoter sequence immediately upstream of the TSS defined by primers Mnx1-3 and Mnx1-4. After knitting PCR the transgene was cloned into the AscI site of pANeoLuc and recombinants tested by PCR and restriction digests to find clones with the correct orientation.

The Fabp7 transgene consisted of 1.6 Kb of DNA upstream of the TSS and 30 bp of the 5'UTR (see Feng and Heintz,1995). The transgene was amplified from mouse genomic DNA isolated from RW4 ESCs. Sequences of the primers are given in Supplementary Table I (F Fabp7 AscI and R Fabp7 AscI). This segment was cloned into the AscI site of pANeoLuc and clones with the correct orientation recovered.

The tuba1a transgene consisted of 1.1 Kb upstream of the gene (see Gloster et al,1994) amplified from mouse genomic DNA isolated from RW4 ESCs by PCR. Its location is given by primers F tubulin AscI and R tubulin AscI. This was cloned into the AscI site of pANeoLuc and clones with the correct orientation recovered.

The structure of each transgene was confirmed by sequencing and restriction enzyme digests.

ES cells

The RW4 ES cell line was used throughout. Undifferentiated ES cells were maintained by routine methods of the lab (Bain et al. 1995,1998).

Transgenic ES cells

Prior to electroporation into ESCs, transgenes were linearized at a NotI site 200 bp upstream of the AscI site. 10⁷ ES cells were transfected with 50µg of plasmid DNA using a BioRad electroporator set at 250Volts and 960µFarads using techniques previously reported (Zhang et al, 2008). Transgenic cells were selected with 200µg/ml Geneticin (Invitrogen) for 6-7 days; all experiments had over 100 clones. Six or seven days after electroporation, clones from a single dish were dissociated with trypsin and combined to create polyclonal cultures. The polyclonal cultures were treated as a cell line, expanded and differentiated.

Differentiation of ES cells

Polyclonal ESCs were differentiated into ventral spinal neural cells with retinoic acid and sonic hedgehog agonist as described previously (Wichterle et al 2002; Xian et al., 2005). Cultures with other induction conditions were also tested. For all types of cultures ESCs were first cultured in defined (DFK5) medium in low attachment plates for 2 days as unattached embryoid bodies. The 2 day old embryoid bodies were transferred to adhesive plates for 4 days for further differentiation in DFK5 with the following additions: 1- no added factors (-/-), 2- RA alone (RA/-), 3-Shh alone (-/Shh), 4-RA+Shh (RA/Shh), 5-BMP4. Final concentrations of factors were: RA 2µM (Sigma); Shh agonist Hh-Ag 1.4 (Curis) 30 nM; BMP4 (StemGent) 5ng/ml. RA+Shh results in induction of ventral spinal cells whereas other conditions produced cell types described in the Results.

Luciferase assays

Differentiated cultures were washed with PBS, scraped from the surface, suspended in PBS and then briefly sonicated (2 sec at power setting=15% (Branson Model 250 digital sonifier; 1/8" microtip) to break cells. An aliquot of sonicated cell suspension was set aside for protein assay and the rest was made 1X in cell lysis buffer (Promega) designed to stabilize luciferase. Samples were snap frozen in a dry ice/ethanol bath and kept frozen until assay. BCA protein assays (Pierce) and luciferase assays (Promega) were as described by the manufacturers.

Immunofluorescence

Cultures were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature, washed with PBS, permeabilized with 0.1% Triton X-100 and blocked with 1% normal donkey serum. Primary antibody- goat anti-luciferase polyclonal (anti-luciferase pAb, Promega G745A) was used at a 1:50 dilution in PBS/1% normal donkey serum following manufacturer's directions - Secondary antibody- Cy3 donkey anti-goat (Jackson ImmunoResearch) diluted at 1:200. Cultures were visualized with a Nikon TE 2000S inverted microscope. Confocal images were from a Zeiss LSM510 with a C-Apochromat 40X, 1.2 NA objective. The fluorophore was excited with a 543nm HeNe laser and fluorescence emission observed with a 560nm long pass filter.

Clonal expression

The Mnx1 polyclonal transgenic line described above was resolved into single clones by co-plating them with a large excess of RW4 cells to act as a feeder and selecting Mnx1 transgenic clones with neomycin (Geneticin 200ug/ml). Clones were picked, expanded and differentiated. Luciferase assays were performed as above.

RESULTS

Experimental design

The goal of this study is to test the hypothesis that ES cells (**ESCs**) and ES cell-derived neural cells (**ESNs**) provide a model for investigating promoters, enhancers and repressor binding elements (**cis-REs**) of CNS genes. We chose 3 transgenes which have the expression pattern of the corresponding native genes in Transgenic Mice (**TGM**) and made stable transgenic ESC lines carrying them. Transgenic ESCs were differentiated in vitro into ESNs and expression of the transgenes measured. We reasoned that the ESN model would be validated if expression in ESNs occurred in a pattern predicted from the TGM data. The results confirm that each transgene is regulated in a way predicted by their expression pattern in mice and thus support the hypothesis.

The transgenes we chose were from the *Mnx1* (Hb9), *Fabp7* (BLBP) and *tuba1a* genes. Each transgene has been analyzed in TGM (Gloster et al., 1994; Feng and Heintz, 1995; Nakano et al, 2005) where each is expressed in a pattern very similar to the corresponding native gene. Structures of the transgenes are shown in Figure 1. Next we chose the method of making ESCs with stable chromosomally integrated transgenes. Transgenic ESCs can be made by random insertion of transgenes, by site specific recombination and by gene targeting based on homologous recombination. Of these, random insertion is the simplest and easiest to perform, and since our aim is a practical method, we chose random insertion of the transgenes. To further simplify experiments, transgenic clones selected by neo were dissociated and pooled to create polyclonal cultures. This is far less laborious than analyzing individual clones, while still being a stringent way of testing the pattern of transgene expression.

Polyclonal transgenic ESC cultures were differentiated by an established method using RA and Shh to induce a ventral spinal fate (Wichterle et al., 2002). Cell types found in these cultures include Olig2+ progenitors and motor neurons (Wichterle et al, 2002, Miles et al., 2004; Xian et al., 2005; Soundararajan et al., 2006). To further characterize regulation of transgene expression, sister cultures were differentiated with no factors, with RA alone, or with Shh alone. Finally, some cultures were treated with BMP4 to suppress neural differentiation entirely (Zhang et al., 2010). Differentiated cultures were harvested and luciferase expression measured in a luminometer assay and normalized to total protein. Results were displayed as bar graphs with luciferase units/ μ g protein on the y-axis. All experiments were replicated in at least 2 fully independent experiments starting with undifferentiated polyclonal transgenic ESCs.

Mnx1

(motor neuron and pancreas homeobox1; also known as Hb9) is a transcription factor expressed predominantly in the nervous system (Arber et al.,1999). In the spinal cord expression is restricted to motor neurons and a nearby set of glutaminergic interneurons (Lee et al, 2004; Nakano et al, 2005; Wilson et al.,2005). Normal expression in the spinal cord is dependent on both RA and Shh signaling (Novitch et al. 2003). A transgene consisting of 9 Kb of contiguous DNA upstream of the TSS drives appropriately patterned expression in transgenic mice (Wichterle et al 2002, Lee et al 2004) and a derived transgene consisting of a 3.5 Kb portion (–9 Kb to –6.5Kb) of the 9 Kb transgene in front of a minimal promoter recapitulates the expression of the 9 Kb transgene (Lee et al.,2004; Nakano et al, 2005). The native *Mnx1* gene is expressed in ESNs in a pattern similar to TGM expression: both RA and Shh are required to induce *Mnx1* and expression is largely confined to motor neurons.

A transgene with the 3.5 Kb upstream element in front of the 1 Kb native promoter was tested in ESNs, (Figure 1). Polyclonal transgenic cultures were made as described above and

the pattern of luciferase expression across 5 types of cultures determined (Figure 2). Cultures without added factors (−/−) express a very low level of luciferase. Cultures with RA alone (RA/−) have slightly higher, but still low, expression and cultures treated with Shh alone (−/Shh) express even less. In contrast, cultures with both RA and Shh (RA/Shh) express a high level of luciferase. Cultures with BMP4, which suppresses neural differentiation, have the lowest level of all cultures.

The first conclusion is that expression dramatically depends on culture conditions. The highest (RA/Shh= 138,000 units/μg protein) is 106-fold higher than the lowest (BMP4=1300 units/μg protein). Since the cultures are genetically identical, the difference is due to regulation in the broadest sense of the word. It is likely that regulation is due to differential gene transcription, although we can't exclude mRNA stability or translational efficiency as factors. The second conclusion is that expression in the cultures is predicted by expression of *Mnx1* transgenes in mice, which is largely restricted to the ventral spinal cord (Nakano et al., 2005). In the intact animal both RA and Shh signaling are required for differentiation of the ventral cord and *Mnx1* expression (Novitsch et al., 2003). In the ESNs both RA and Shh are required to turn on the native *Mnx1* gene and induce motor neurons (Wichterle et al., 2002). This predicts that RA and Shh are necessary to turn on transgenes in ESNs, which the data shows. The slight elevation of expression in (RA/−) cultures may be due to the presence of low levels of Shh made by ESNs. Because the cultures are polyclonal, results reflect the averaged contribution of all transgenes. We conclude that the average of sites shows appropriate regulation. In summary, results with the *Mnx1* transgene demonstrate that ESNs express this transgene in an appropriate and predicted pattern.

An underlying premise of these experiments is that individual clones carrying the same transgene but at different chromosomal sites have varying levels of expression. This assumption is the reason for using pools of transgenic clones rather than monoclonal cultures. To verify this assumption, expression was measured in 4 *Mnx1* clones (Figure 3). Data shows that there is indeed a wide range of expression across the 4 clones and that pooling clones is an important part of this approach.

Luciferase assays are quantitative and specific but don't reveal the cell type(s) that is expressing. To find out, we investigated the cellular localization of transgene expression in pooled polyclonal *Mnx1* transgenic cells with an antibody to luciferase (Figure 4). We stained RA+Shh induced cultures since they had the strongest expression. As a specificity control we stained non-transgenic ES cells of the RW4 strain (which was used to make the transgenic cells). The pattern of staining of transgenic cultures strongly supports the idea that expression is predominantly in neural cells. RA+Shh induced cultures consist of clumps of cells, many with bundles of axons growing on the gelatin substrate. The typical clump contains intensely stained cells with small cell bodies and fine processes emerging from them (Figure 4A and 4D). Stained cells tend to be clustered within a clump with intervening region having only background staining. Some clumps lack stained cells. Some of the bundles of axons emerging from clumps (Figure 4B) are stained intensely while others are negative. This staining pattern is consistent with luciferase expression in neural cells. The heterogeneity of staining might be explained by 2 factors. First, the expression native *Mnx1* gene occurs in a subset of cells. Second the cultures are polyclonal with expression level varying from clone to clone (Figure 3). It may be that only the highest expressing clones are above the threshold for antibody detection.

There is substantial background staining in the transgenic cultures so it is essential to have a stringent control to distinguish specific staining from background. A genetic control where ESNs that are not transgenic are stained provides such a control since all aspects of the cultures are the same in the experimental and control with the exception of the luciferase

gene. The RW4 parental ESC line was used to avoid any differences due to ES cell strains. While control cultures have background staining (Figure 4C), stained cells with neuronal morphology were completely absent. Control cultures had abundant axons emerging from the clumps and these were not stained. The genetic controls convincingly prove that the stained cells with neural morphology in the *Mnx1* transgenic cultures are expressing luciferase.

Fabp7

(Fatty acid binding protein7; also known as *Blbp*) This gene is involved in the regulation of fatty acid metabolism and is specifically expressed in the CNS and PNS during early development (Feng and Heintz, 1995; Anthony et al., 2004). Most of the expression is in radial glial cells, the great majority of which express the gene. A transgene consisting of 1.6 Kb of DNA immediately upstream of the TSS and the first 30 bp of the 5'UTR is expressed in transgenic mice with a pattern very similar to the native gene (Feng and Heintz, 1995). Evidence from our lab (data not shown) and others (Liour and Yu, 2003; Liour et al. 2006; Kim et al, 2009) show the gene is expressed in ESNs, including radial glia. These attributes make *Fabp7* an appropriate gene for testing our hypothesis.

A luciferase transgene analogous to the published *Fabp7* transgene was constructed and tested in ESNs. Polyclonal transgenic *Fabp7* ESCs were made as for *Mnx1* and expression of the transgene assayed (Figure 5). There are large differences in expression of the *Fabp7* transgene across the set of conditions. With no added factors ($-/-$) expression is very low, whereas expression with RA alone (RA/ $-$) is high. This differs from *MNX1* where RA/ $-$ expression is low. Cultures with Shh alone ($-$ /Shh) have low expression whereas expression with RA and Shh (RA/Shh) is high, at approximately the same level as RA alone. Expression in cultures with BMP4 is very low. This pattern is what we'd expect from expression of the native *Fabp7* gene and its transgene in mice. Native *Fabp7* is expressed in most radial glia of the CNS as is the 1.6Kb transgene in mice. Treatment of ESCs with RA induces radial glial that express *Fabp7* (Liour et al.2006) so it is predicted that cultures with no added factors would not express whereas RA-treated cultures should express strongly. Both predictions are borne out by the data. Shh alone doesn't induce neural cells in ESN cultures and should not turn on the transgene. Since Shh doesn't suppress differentiation of radial glia in ESNs it is predicted that RA+Shh cultures express at high levels, as they do. BMP4 suppresses neural differentiation and, as expected, treated cultures don't express the transgene. In summary, expression of the *Fabp7* transgene in ESNs conforms to predictions based on the transgenic mouse.

Tuba1a

(tubulin, alpha 1A) The native *tuba1a* gene is turned on in young neurons throughout the CNS and PNS but not by astrocytes or oligodendrocytes (Gloster et al.,1994). Expression continues into early post-natal life and is then strongly down-regulated. A 1.1 Kb promoter region from the rat recapitulates these key aspects of expression in transgenic mice (Gloster et al., 1994). Based on this background we set out to determine if a *tuba1a* transgene is regulated appropriately in ESNs. A transgene with 1.1 Kb of upstream DNA from the mouse was constructed using methods described for *Mnx1* and *FABP7* (Figure 1). Production of polyclonal transgenic cultures and testing their luciferase expression was as in previous experiments (Figure 6). Expression is low without factors ($-/-$), high with RA alone (RA/ $-$) or RA and Shh ($+/+$). Expression is low with Shh alone ($-$ /Shh) and in presence of BMP4. These results are predicted from the expression patterns in normal and transgenic mice. The $-/-$ cultures should express very little because they contain few if any neurons. RA/ $-$ and RA+Shh induced cultures have neurons and should turn on the transgene. Shh alone ($-$ /Shh)

and BMP4 cultures don't have neurons and don't express the transgene. This agreement between predictions and data strongly supports our hypothesis.

DISCUSSION

This work addresses the need for model systems to complement transgenic mice (TGM) for research on promoters, enhancers and repressor binding elements (**cis-REs**) of mammalian neural genes. The immediate goal was to learn if transgenes are expressed appropriately in embryonic stem cell-derived neural cells (**ESNs**). Three neural transgenes (Mnx1, Fabp7, and tuba1a) expressed with correct spatial specificity in TGM were tested in ESNs. All 3 are neuro-specific in TGM. In the ESN model there is little expression in control cultures without inducers or cultures with BMP4, both of which have few neural cells. Thus the transgene's broad specificity for the neural lineage is retained in the ESN model. In addition there are differences among the transgenes across the types of neural cultures. Fabp7 and alpha tubulin are expressed in radial glia and young neurons respectively. Cultures with RA alone have radial glia and neurons and express the Fabp7 and alpha tubulin transgenes. The Mnx1 transgene is specifically expressed in the ventral spinal cord in TGM. Expression of ventral spinal cord genes in ESNs requires RA+Shh (Wichterle et al., 2002; Xian et al., 2005). As predicted, expression of the Mnx1 transgene is very low with RA alone but high with RA+Shh providing an example of transgene expression in ESNs with neural sub-type specificity as well as overall neural specificity. We conclude the potential pitfalls described in the Introduction don't prevent appropriate expression of transgenes and that, with certain limitations discussed below, the ESN model is suitable for finding novel promoters and enhancers on neural genes.

The results also have implications regarding a general aspect of gene regulation, namely the impact of surrounding chromatin on random inserted transgene expression. Experiments were done with pooled clone cultures derived from at least 100 independent transgenic clones so measured expression represent the average of many different randomly inserted transgenes. Unlike TGM experiments, where lines with low or inappropriate expression are ignored, these experiments take all integration sites into account. The results lead to the conclusion that many of the integration sites are permissive for expression. They suggest that chromatin flanking a transgene does not impose its own expression pattern on the transgene because, if it did, there'd be substantial expression in non-neural cultures. It is likely that integration sites influence the level of expression, but they are unlikely to influence its tissue specificity. Experiments on BAC transgenes in ESNs support a similar conclusion (Xian et al., 2005; Zhang et al., 2008; Placantonakis et al., 2009). Since the average site permits specific expression, refinements of the system where transgenes are inserted into defined sites by site-specific recombination should be feasible.

The ESN system presently has 2 major limitations. First, although multiple developmental pathways are being modeled with ESNs (reviewed in Cai and Grabel, 2007; Gaspard and Vanderhaeghen, 2010), they still represent a small fraction of brain regions, so many neural populations can't be studied with the ESN approach. Hopefully new research will widen the applicability of ESNs. The second limitation is related to the random integration method we employed. For the 3 transgenes studied, it is clearly sensitive enough to detect the signal of specific expression above the noise due to multiple integration sites. We can't exclude the possibility that some promoters and enhancers are too weak to give signals above the noise. ESCs with strategically placed site specific recombination cassettes may be the best tool to reduce integration site noise and increase the sensitivity of the system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of the transgenes

A- The parental plasmid pANeoLuc showing the AscI cloning site, luciferase gene and PGK neo selection cassette.

B- Promoters and enhancers inserted into the AscI site to produce final transgenes. The Mnx1 insert consists of a 3.5 Kb enhancer and 1.1 kb promoter. The Fabp7 insert is a 1.6 Kb promoter and the tuba1a insert is a 1.1 Kb promoter. DNA sequences in Supplementary Table I and can align the elements with the mouse genome.

Figure 2.

Mnx1 transgene expression is induced by RA+Shh - Bar graph showing expression levels with different inducing conditions. Factors present during differentiation are indicated under bars. Height of bars gives average expression in terms of luciferase units/ μ g of protein. Standard deviation of replicate cultures is shown by lines above the bar.

Figure 3. Mnx1 transgene expression varies among individual clones- 4 clones of Mnx1 transgenic ESCs were differentiated with RA/Shh; differentiated cells were assayed for luciferase.

Figure 4.

Antibody to luciferase stains cells with neural appearance- All images are from cultures stained with anti-luciferase antibody. A, B and D: *Mnx1* transgenic polyclonal cultures induced with RA+Shh by the 2-/4+ regimen. C: RW4 (non-transgenic controls) ESC induced with RA+Shh by the 2-/4+ regimen. A- Stained cells with neural appearance (arrowheads) seen with standard fluorescence microscope. B- Two bundles of intensely stained axons emerging from the periphery of a clump (arrowheads). C- RW4 control culture by standard fluorescence microscopy. Clumps have background staining but cells with neural morphology and axons are never seen. D- Confocal microscopy of *Mnx1* transgenic cells induced by RA+Shh - A group of cells with neural morphology (arrowheads) stained with anti-luciferase. Calibration bars: A-C 100 μ M; D 50 μ M

Figure 5.
Fabp7 expression- Culture conditions are as in Figure 2. Expression is high in both conditions where RA is present.

Figure 6.

Tuba1a expression- Bar graph analogous to Figure 2- Note the RA/- and RA/Shh cultures both have high expression