Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 361 (2007) 109–115
BBRC
www.elsevier.com/locate/ybbrc

A novel gene trapping for identifying genes expressed under the control of specific transcription factors

Chie Naruse, Yoshiyasu Fukusumi, Dai Kakiuchi, Masahide Asano *

Division of Transgenic Animal Science, Advanced Science Research Center, Kanazawa University, Takara-machi 13-1, Kanazawa 920-8640, Japan

Received 22 June 2007
Available online 10 July 2007

Abstract

Gene trapping is a powerful method for identifying novel genes and for analyzing their functions. It is, however, difficult to select trapped genes on the basis of their function. To identify genes regulated by transcription factors that are important in the mesodermal formation, we selected trapped ES clones by infection of adenoviral vectors expressing *Pax1*, *Brachyury*, and *Foxa2*. Among 366 trapped genes, seven seemed to be controlled by these transcription factors in the first screening. The trapped genes were identified by 5′ RACE, and a Northern blotting revealed that expressions of three trapped genes were regulated by these transcription factors. Expression patterns of *Cx43* and *HP1γ* implicated their functional relationships to *Foxa2* in the formation of the notochord and the neural tube. Furthermore, *Wtap* mutant mice derived from the trapped clone showed defects in the mesendoderm formation. Our results indicate that trapped ES clones could be selected effectively using transcription factors.

Keywords: Gene trapping; Mouse; Transcription factor; Mesoderm

Gene trapping is one of random mutagenesis approaches that enable us phenotype-driven genetic screens based on phenotypes of mutant mice. Recently, large-scale phenotype-driven genetic screens in mice have been proceeding using this method by big mouse facilities and strong financial support [1–3]. Although gene trapping is a powerful method for identifying novel genes and for analyzing their functions, it is difficult to select trapped genes on the basis of in vivo phenotypes of mutant mice by small-scale screening in a small-scale laboratory. Nevertheless, a gene trap method is useful for small-scale screening if trapped genes are appropriately selected in vitro. Trapped genes that are responsive to developmental signals can be identified using differentiation factors that induce differentiation of embryonic stem (ES) cells in vitro [4–7]. We tried previously to identify trapped genes whose expressions were regulated by soluble differentiation factors such as retinoic acid, cyclic AMP and basic fibroblast growth factor. Although the selection by these soluble factors was sufficient to exclude ubiquitously expressed genes, it was difficult to focus on such genes that are expressed under the control of these factors or that function in particular developmental stages [8].

In this report, we tried to isolate novel genes whose expressions are regulated during mesodermal formation using a novel gene trap screening with transcription factors. Trapped ES clones were infected with adenoviral vectors expressing *Pax1*, *Brachyury*, or *Foxa2* (*Hnf3β*). These transcription factors are known to play important roles in mesodermal formation and differentiation during early embryogenesis. *Pax1* is expressed in the paraxial mesoderm and essential for development of the sclerotome [9,10]. *Brachyury* is expressed autonomously in the axial medoderm and involved in movement and elongation of the axial mesoderm during gastrulation [11,12]. *Foxa2* is expressed in the definitive endoderm and the axial mesoderm, and involved in development of node, notochord, neural tube and gut tube [13–15].

We obtained 366 individual trapped ES clones by gene trapping using the ROSANβ-geo retroviral vector [8],

* Corresponding author. Fax: +81 76 234 4241.
E-mail address: asano@kiea.m.kanazawa-u.ac.jp (M. Asano).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.06.161

and trapped genes whose transcription levels were altered after the introduction of these transcription factors were selected. Among 366 trapped genes, we could select three genes regulated by these transcription factors in the transcription level. Especially, we could identify *Wilmus' tumor-associating protein* (*Wtap*) that was a novel essential gene involved in gastrulation and mesendodermal formation (Fukusumi et al., submitted). Our results indicate that trapped ES clones could be selected effectively in our novel gene trap screening.

Materials and methods

Adenoviral vectors expressing transcription factors. The replication-defective adenoviral vectors expressing *Pax1*, *Brachyury* and *Foxa2* were generated using Adenovirus Expression Vector Kit (TAKARA, Japan) and Adenovirus Cre/loxP Kit (TAKARA) based on a COS-TPC method. Because the adenoviral vector produced by this kit is deficient in *E1* and *E3* genes, it is only able to proliferate in cells that have *E1* gene, e.g. 293 cells. The *Pax1* cDNA fragment was cloned by RT-PCR using a forward primer (5′-ACGTCAGTATCCCGCGTTC-3′) and a reverse primer (5′-TCCCTTCTCGGTGTTTGAAG-3′). The *Brachyury* and the *Foxa2* cDNA fragments were gifts from Dr. Herrmann (Max-Planck-Institut, Germany) [16] and Dr. Sasaki (RIKEN, Japan) [17], respectively. *Pax1*, *Brachyury* and *Foxa2* cDNA fragments were inserted into the SwaI site of the pAxCALNLw cosmid vector, respectively. The cosmid vectors and the DNA-TPC were transfected into 293 cells using a calcium phosphate method, and recombinant adenoviral vectors were obtained. These vectors have a stuffer sequence that is to be removed by Cre recombinase for transcription of inserted cDNA (Fig. 1A).

Gene trapping and selection of trapped clones using transcription factors. The summary of the screening protocol is illustrated in Fig. 1B. Gene trapping using the ROSANβ-geo retroviral vector, a modified ROSAβ-geo vector [18], was performed as described previously [8]. E14-1 ES cells [19] were infected with the retroviral trap vector and selected with 250 μg/ml of G418. After G418-resistant clones were isolated and cultured in 24-well plates, about 2×10^4 cells of each clone were passaged into 96-well plates. Then, they were infected with 20 multiplicity of infection (MOI) of AxCALNL(*Pax1*), AxCALNL(*Brachyury*), or AxCALNL(*Foxa2*) aden-

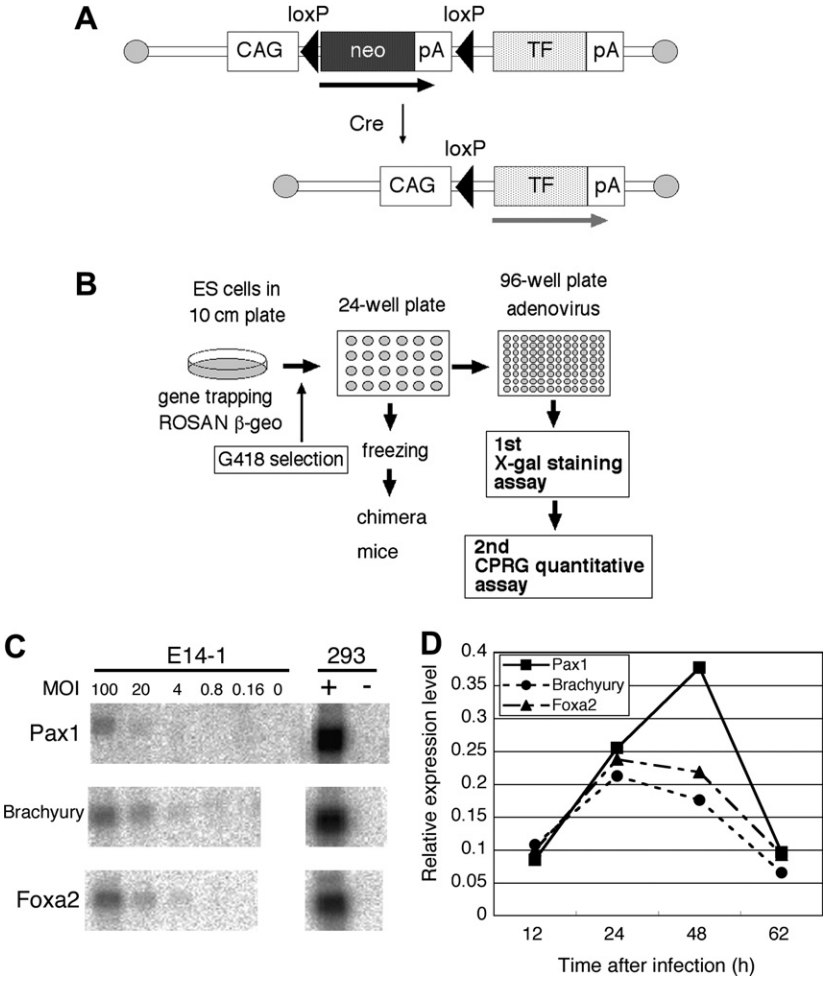

Fig. 1. A novel gene trapping with screening using adenoviral vectors expressing transcription factors. (A) AxCALNL(TF) adenoviral vector and removal of the stuffer sequence (neo-pA) by Cre recombinase for the expression of a transcription factor (TF) are shown. CAG; cytomegarovirus enhancer and chicken β-actin promoter, neo-pA; neomycin resistance gene and SV40 poly A additional signal, TF-pA; a transcription factor and rabbit β-globin poly A additional signal. (B) Schematic screening diagram of a gene trapping using transcription factors. A fraction of ES cells was used for infection with the adenoviral vectors and screening, and the rest of ES cells were frozen to make chimera mice. (C) E14-1 and 293 cells were infected with adenoviral vectors expressing transcription factors and Cre recombinase. Total RNA was isolated 43 hr after the infection, and Northern hybridization with *Pax1*, *Brachyury*, and *Foxa2* probes was performed. (D) Relative expression levels of mRNA of transcription factors in ES cells after infection with adenoviral vectors.

oviral vectors, and simultaneously with 20 MOI of AxCANCre (Cre expressing adenoviral vector). As a negative control, trapped ES clones were infected with 20 MOI of AxCANCre alone. AxCANCre was a gift from Dr. Saito (Univ. Tokyo, Japan). After 3 days of infection, β-galactosidase activity was measured using X-gal staining and a chrolophenol red-β-D-galactopyranoside (CPRG) assay method as described previously [8]. Each examination was performed in duplicate or triplicate.

Isolation of cDNA of trapped genes and Northern blotting. The 5′ end of cDNA of trapped genes was isolated by 5′ RACE as described previously [8] based on the 5′ RACE system (Invitrogen, USA). For Northern blotting, wild-type ES cells were infected with adenoviral vectors expressing transcription factors and Cre recombinase. As a negative control, wild-type ES cells were infected with the Cre expressing adenoviral vector alone. Total RNA was prepared from the ES cells using the acid guanidinium thiocyanate–phenol–chloroform method [20], and electrophoresed on a 0.8% denatured agarose gel and transferred to a nylon membrane. Hybridization was carried out according to the standard methods using ^{32}P-labeled DNA probes made with the Random primer extension labeling system (Perkin-Elmer, USA). Signals were quantified using Image Guage (Fujifilm, Japan). The amount of mRNA of each genes was quantified by Northern blotting and normalized to β-actin mRNA. Relative expression levels to the negative control were calculated.

Production of chimeric and mutant mice. Chimeric mice were generated from trapped ES clones using a modified aggregation method [21] as described previously [22]. Mice were kept under specific pathogen-free conditions in an environmentally controlled clean room at the Institute of Experimental Animals, Advanced Science Research Center, Kanazawa University. Experiments were conducted according to guidelines for the care and use of laboratory animals and safety guidelines for gene manipulation experiments in Kanazawa University.

X-gal staining of embryos. X-gal staining was performed as described previously [8]. After staining, they were re-fixed with 10% formaline and embedded in paraffin according to the standard procedure. Sections of 7–8 μm were prepared and counter-stained with nuclear Fast Red.

Results and discussion

Generation of adenoviral vectors expressing transcription factors

First, we tried to produce adenoviral vectors expressing *Pax1*, *Brachyury*, and *Foxa2* using AxCAwt. However, these transcription factors seemed to have cell toxicity when expressed strongly in 293 cells, and we could not obtain expected adenoviral vectors (data not shown). Then, we utilized AxCALNLw that has a stuffer sequence that is to be removed by Cre recombinase for transcription of the inserted cDNA (Fig. 1A). Transcription factors in the vector were not transcribed in 293 cells without Cre recombinase, and we successfully produced adenoviral vectors containing transcription factors. Finally, 2.9×10^8 pfu/ml of AxCALNL(*Pax1*), 5.0×10^8 pfu/ml of AxCALNL(*Brachyury*), and 6.6×10^8 pfu/ml of AxCALNL(*Foxa2*) monoclonal adenoviral vectors were obtained.

To validate the expression of transcription factors from adenoviral vectors, 293 cells and ES cells (E14-1) were infected with variable MOI (0–100) of these adenoviral vectors. Northern blotting revealed that mRNA levels of transcription factors corresponded well to the amounts of infected adenoviral vectors (Fig. 1C), and expression levels of transcription factors were significantly increased when ES cells were infected with more than 20 MOI of adenoviral vectors. Moreover, these transcription factors from adenoviral vectors were expressed to the maximum level between 24 and 48 h after infection (Fig. 1D). Transcription activation or repression by the transcription factors is thought to occur within 1 day during mesodermal formation. Therefore, we decided to infect trapped ES clones with adenoviral vectors at 20 MOI, and to measure β-galactosidase activity 3 days after infection.

Screening of ES cells using transcription factors

Three hundred and sixty-six of trapped ES clones were screened using adenoviral vectors. Trapped ES clones were infected simultaneously with 20 MOI of AxCALNL expressing transcription factors and with 20 MOI of AxCANCre expressing Cre recombinase. Then, expression levels of trapped genes were compared between before and after the introduction of the transcription factors by means of X-gal staining and CPRG quantitative assay. The intensity of X-gal staining appeared to be altered in 43 clones. Among them, β-galactosidase activities of seven trapped clones were altered when quantified with CPRG assay (Table 1). The trapped clones whose β-galactosidase activities increased more than 1.5 times or decreased less than two thirds were selected for the next screening.

Next, we determined cDNA sequences of these trapped genes using 5′ RACE method (Table 1). Unfortunately, we could not identify any already-known gene regulated by Pax1, Brachyury or Foxa2 in this screening. If more

Table 1
Trapped clones selected with the screening and alteration of β-galactosidase activity by introduction of transcription factors

Clone	Relative β-galactosidase activity[a]			Gene name	Insertion site
	Pax1	Brachyury	Foxa2		
4A2	1.81	1.63	1.35	*Homer2*	Intron 1
5B4	0.85	0.91	0.58	*Wtap*	Intron 1
9C6	0.66	0.58	0.58	*INFaR2*	ND
10A4	32.94	26.25	0.54	*HMG-1*	Intron 1
10C2	0.41	0.34	0.38	*Cbx3/HP1γ*	Intron 1
15C3	0.72	0.67	0.87	*Cx43*	Intron 1
17B6	0.80	0.62	1.10	*Cnbp*	Intron 1

[a] Relative β-galactosidase activities measured by CPRG assay in ES cell clones introduced with transcription factors normalized to those without transcription factors.

trapped clones are analyzed, such trapped genes might be found. We found that the ROSANβ-geo retroviral trap vector was inserted into intron 1 of almost all trapped genes (Table 1). Because an initiation codon often exists downstream of intron 1, the expression of trapped genes was expected to be interrupted efficiently when we use this retroviral trap vector.

We selected four trapped genes, *Connexin 43* (*Cx43*), *Wtap*, *Heterochromatin protein* (*HP*) *1γ* and *Cellular nucleic acid binding protein* (*Cnbp*), for further study. In the first screening we examined the expression of trapped genes using β-galactosidase activity in trapped ES clones. Then, quantitative Northern hybridization analyses were performed to quantify mRNA levels of trapped genes in wild-type ES cells when each transcription factor was introduced. As a negative control, wild-type ES cells were infected with only Cre-expressing adenoviral vectors. As a result, expression of three trapped genes (*Cx43*, *Wtap* and *HP1γ*) was confirmed to be regulated by these transcription factors in wild-type ES cells (Fig. 2). The expression of *Cnbp* did not alter by these transcription factors. The expression of *Cx43*, *Wtap* and *HP1γ* was down-regulated in ES cells, especially when *Foxa2* was introduced into ES cells. They were downregulated within 12 h after introduction of *Foxa2*. *Foxa2* is a member of a folkhead box gene family and activates transcription of target genes. Therefore, *Cx43*, *Wtap* and *HP1γ* might not be direct target genes of *Foxa2*, but might be early response genes to *Foxa2*.

Expression patterns of selected trapped genes compared with those of Foxa2

Cx43 is a gap junction membrane channel protein [23]. HP1γ is involved in maintenance of heterochromatin [24]. Wtap was cloned by yeast two-hybrid assays to identify factors binding to Wilms' tumor 1 protein [25], and thought to be involved in a spliceosome and so on [26]. However, the function of Wtap is still unclear. We succeeded in generating *Cx43*, *HP1γ* and *Wtap* mutant mice derived from the trapped clones. Because the ROSANβ-geo trap vector contains *β-galactosidase* gene driven by the promoters of trapped genes, X-gal staining patterns could mimic the expression patterns of trapped genes *in vivo*. Then heterozygous mutant embryos at various stages were stained with X-gal. Because expression of the three trapped genes was strongly affected by Foxa2 (Fig. 2), *in vivo* expression patterns of these genes were compared with those of *Foxa2*. Expression of *Cx43*, *HP1γ* and *Wtap* monitored by X-gal staining was detected in whole embryos from E6.5 to 7.5 when gastrulation occurred (Fig. 3A–C, J and K; Fukusumi et al., submitted). The expression patterns of *Cx43* were described previously [27], however, its expression pattern in gastrulation stage is unclear. Then, we analyzed it in detail.

Foxa2 expression is detected from E6.5 at anterior primitive streak and node [17,28]. Considering the results that

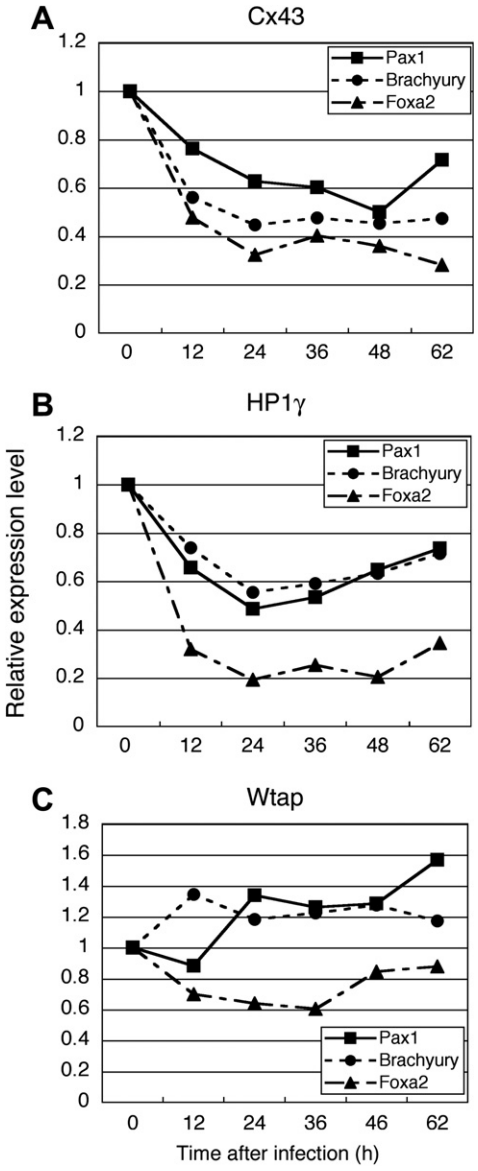

Fig. 2. mRNA transcriptions of the selected trapped genes were regulated by Pax1, Brachyury or Foxa2. Wild-type ES cells were infected simultaneously with transcription factor-expressing and Cre-expressing adenoviral vectors. As a negative control, wild-type ES cells were infected with the Cre-expressing adenoviral vector alone. Total RNA was collected at each time point after the infection, and Northern hybridization was performed using (A) *Cx43*, (B) *HP1γ*, (C) *Wtap* probes. Relative amounts of mRNA at each time point in ES cells infected with the Cre-expressing adenoviral vector and the transcription factor-expressing adenoviral vector to those infected with the Cre-expressing adenoviral vector alone are shown.

the expression of *Cx43*, *HP1γ* and *Wtap* was repressed by Foxa2 in ES cells (Fig. 2), their expression would not be detected in tissues where *Foxa2* was expressed. However, at E6.5, *Cx43*, *HP1γ* and *Wtap* appeared to be expressed at all embryonic cells (Fig. 3A and J; Fukusumi et al., submitted). It might reflect the difference between ES cells and embryonic cells.

At E7.5, *Foxa2* is expressed in the notochord and definitive endoderm. *Foxa2* mRNA is also detected in the ventral neural plate at this time [17,28]. *Cx43* and *Foxa2*

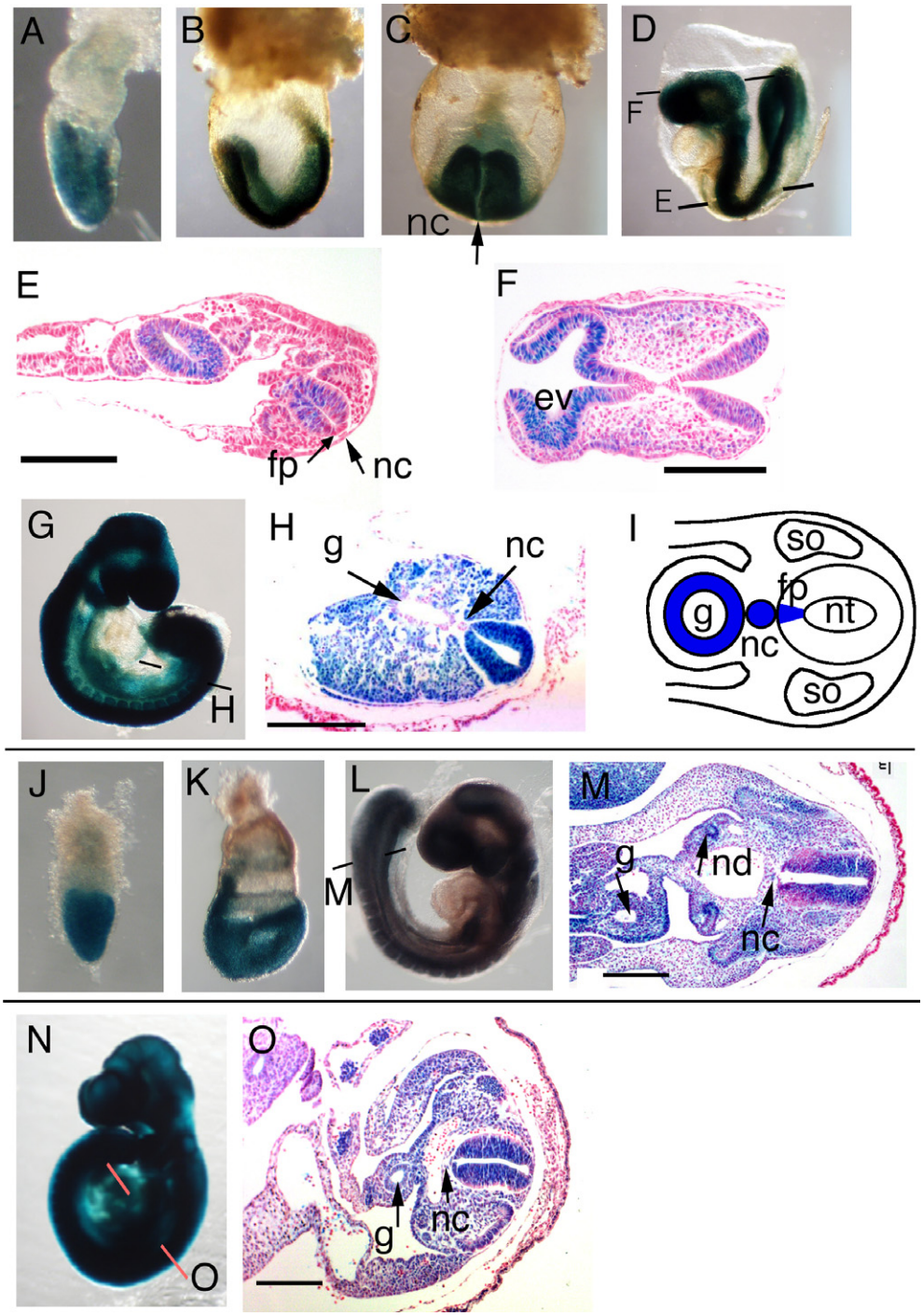

Fig. 3. Gene expression patterns of the trapped genes. X-gal staining patterns of embryos at each developmental stage are shown. (A–H) *Cx43*, (J–M) *HP1γ*, (N,O) *Wtap* heterozygous mutant embryos. Embryonic stages are E6.5 (A,J), E7.5 (B,C,K), E8.5 (D–F), E9.5 (G–I,L–O). The sectioning planes are indicated in D, G, L and O. (I) Expression pattern of *Foxa2* at E9.5. Scale bars indicate 200 μm. Abbreviations are as follows: ev, eye vesicle; fp, floor plate; g, gut; nc, notochord; nd, nephric duct; nt, neural tube; so, somite.

expression patters appeared mutually exclusive, expectedly. At E7.5, *Cx43* was expressed in whole embryo except notochord (Fig. 3C). After E8.5, *Foxa2* is detected in floor plate, notochord and gut (Fig. 3I), but *Cx43* could not be detected in these tissues (Fig. 3E, F and H). *HP1γ* was expressed in neural tube except for ventral region. Regional differentiation of neurons is controlled by the concentration gradient of *Sonic hedgehog* (*Shh*) from notochord and floor plate, resulting in delimited distribution of transcriptional factors such as Lim homeobox proteins [29]. Foxa2 regulates *Shh* expression and neuronal patterning [30], therefore, *HP1γ* might be involved in neuronal differentiation as a downstream factor of Foxa2. Since *HP1γ* represses the transcription epigenetically, the expression

of Lim homeobox genes, for example Isl2, might be repressed by HP1γ. *Wtap* seemed to be expressed in all cells in these stages and we could not found any correlation between *Foxa2* and *Wtap* at least in their expression patterns.

Phenotypes of mutant mice of selected trapped genes

Foxa2 is essential for development of node and notochord, and *Foxa2* null mutant embryos die at E10.5–11.5 with severe defects in node, notochord, neural tube and gut tube [13,14]. *Cx43* mutant mice show defective cardiogenesis and die at birth as reported previously [31]. Although *HP1γ* mutant mice did not show any significant defects in early embryogenesis, they showed infertility (Naruse et al., submitted). *Cx43* and *HP1γ* mutant mice did not show any significant defects that suggest reciprocal relationship to *Foxa2*. The reason why they did not show any abnormal phenotypes in early developmental stages might be compensation of *Cx43* and *HP1γ* functions by their other family members.

Wtap mutant embryos became abnormal around gastrulation stage without expression of mesoderm and endoderm markers, and died by E10.5. Moreover, *in vitro* ES cell differentiation analysis suggested that *Wtap* was involved in differentiation of mesoderm and endoderm itself (Fukusumi et al., submitted). The defective cell lineages were similar in *Foxa2* null mutant embryos. However, *Wtap* appeared to be upstream of *Foxa2*, rather than induced by Foxa2 (Fukusumi et al., submitted). It is possible that there is a negative feedback loop between *Foxa2* and *Wtap*.

In conclusion, we screened 366 trapped ES clones and selected four genes whose expressions were regulated by the transcription factors. We generated three mutant mice and at least two of them showed interesting phenotypes in mesodermal formation. Although this is a small-scale screening, our results show that our novel gene trap screen using a transcription factor is rather effective to identify intentional genes.

Acknowledgments

We thank Dr. Bernhard G. Herrmann (Max-Planck-Institut, Freiburg, Germany) for *Brachyury* cDNA; Dr. Hiroshi Sasaki (CDB, RIKEN, Japan) for *Foxa2* cDNA; Dr. Izumu Saito (IMS, Univ. Tokyo, Japan) for AxCAN-Cre adenovirus vector; all of the members of the Division of Transgenic Animal Science for excellent technical assistance and animal care. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports Science and Technology of Japan (Grant no. 14034218 and 18051008).

References

[1] A.S. Nord, P.J. Chang, B.R. Conklin, A.V. Cox, C.A. Harper, G.G. Hicks, C.C. Huang, S.J. Johns, M. Kawamoto, S. Liu, E.C. Meng, J.H. Morris, J. Rossant, P. Ruiz, W.C. Skarnes, P. Soriano, W.L. Stanford, D. Stryke, H. von Melchner, W. Wurst, K. Yamamura, S.G. Young, P.C. Babbitt, T.E. Ferrin, The International Gene Trap Consortium Website: a portal to all publicly available gene trap cell lines in mouse, Nucleic Acids Res. 34 (2006) D642–D648.

[2] F. Schnutgen, S. De-Zolt, P. Van Sloun, M. Hollatz, T. Floss, J. Hansen, J. Altschmied, C. Seisenberger, N.B. Ghyselinck, P. Ruiz, P. Chambon, W. Wurst, H. von Melchner, Genomewide production of multipurpose alleles for the functional analysis of the mouse genome, Proc. Natl. Acad. Sci. USA 102 (2005) 7221–7226.

[3] W.C. Skarnes, H. von Melchner, W. Wurst, G. Hicks, A.S. Nord, T. Cox, S.G. Young, P. Ruiz, P. Soriano, M. Tessier-Lavigne, B.R. Conklin, W.L. Stanford, J. Rossant, A public gene trap resource for mouse functional genomics, Nat. Genet. 36 (2004) 543–544.

[4] R.K. Baker, M.A. Haendel, B.J. Swanson, J.C. Shambaugh, B.K. Micales, G.E. Lyons, In vitro preselection of gene-trapped embryonic stem cell clones for characterizing novel developmentally regulated genes in the mouse, Dev. Biol. 185 (1997) 201–214.

[5] P. Bonaldo, K. Chowdhury, A. Stoykova, M. Torres, P. Gruss, Efficient gene trap screening for novel developmental genes using IRES beta geo vector and in vitro preselection, Exp. Cell Res. 244 (1998) 125–136.

[6] S. Gajovic, K. Chowdhury, P. Gruss, Genes expressed after retinoic acid-mediated differentiation of embryoid bodies are likely to be expressed during embryo development, Exp. Cell Res. 242 (1998) 138–143.

[7] P. Carroll, Y. Renoncourt, O. Gayet, B. De Bovis, S. Alonso, Sorting nexin-14, a gene expressed in motoneurons trapped by an in vitro preselection method, Dev. Dyn. 221 (2001) 431–442.

[8] C. Naruse-Nakajima, M. Asano, Y. Iwakura, Involvement of EphA2 in the formation of the tail notochord via interaction with ephrinA1, Mech. Dev. 102 (2001) 95–105.

[9] J. Wallin, J. Wilting, H. Koseki, R. Fritsch, B. Christ, R. Balling, The role of Pax-1 in axial skeleton development, Development 120 (1994) 1109–1121.

[10] I. Rodrigo, R.E. Hill, R. Balling, A. Munsterberg, K. Imai, Pax1 and Pax9 activate Bapx1 to induce chondrogenic differentiation in the sclerotome, Development 130 (2003) 473–482.

[11] V. Wilson, L. Manson, W.C. Skarnes, R.S. Beddington, The T gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation, Development 121 (1995) 877–886.

[12] V. Wilson, R. Beddington, Expression of T protein in the primitive streak is necessary and sufficient for posterior mesoderm movement and somite differentiation, Dev. Biol. 192 (1997) 45–58.

[13] S.L. Ang, J. Rossant, HNF-3 beta is essential for node and notochord formation in mouse development, Cell 78 (1994) 561–574.

[14] D.C. Weinstein, A. Ruiz i Altaba, W.S. Chen, P. Hoodless, V.R. Prezioso, T.M. Jessell, J.E. Darnell Jr., The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo, Cell 78 (1994) 575–588.

[15] S.J. Kinder, T.E. Tsang, M. Wakamiya, H. Sasaki, R.R. Behringer, A. Nagy, P.P. Tam, The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm, Development 128 (2001) 3623–3634.

[16] D.G. Wilkinson, S. Bhatt, B.G. Herrmann, Expression pattern of the mouse T gene and its role in mesoderm formation, Nature 343 (1990) 657–659.

[17] H. Sasaki, B.L. Hogan, Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo, Development 118 (1993) 47–59.

[18] G. Friedrich, P. Soriano, Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice, Genes Dev. 5 (1991) 1513–1523.

[19] R. Kuhn, K. Rajewsky, W. Muller, Generation and analysis of interleukin-4 deficient mice, Science 254 (1991) 707–710.

[20] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.

[21] A. Nagy, J. Rossant, R. Nagy, W. Abramow-Newerly, J.C. Roder, Derivation of completely cell culture-derived mice from early-passage embryonic stem cells, Proc. Natl. Acad. Sci. USA 90 (1993) 8424–8428.
[22] M. Asano, K. Furukawa, M. Kido, S. Matsumoto, Y. Umesaki, N. Kochibe, Y. Iwakura, Growth retardation and early death of beta-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells, EMBO J. 16 (1997) 1850–1857.
[23] K. Willecke, J. Eiberger, J. Degen, D. Eckardt, A. Romualdi, M. Guldenagel, U. Deutsch, G. Sohl, Structural and functional diversity of connexin genes in the mouse and human genome, Biol. Chem. 383 (2002) 725–737.
[24] S.I. Grewal, S. Jia, Heterochromatin revisited, Nat. Rev. Genet. 8 (2007) 35–46.
[25] N.A. Little, N.D. Hastie, R.C. Davies, Identification of WTAP, a novel Wilms' tumour 1-associating protein, Hum. Mol. Genet. 9 (2000) 2231–2239.
[26] A. Ortega, M. Niksic, A. Bachi, M. Wilm, L. Sanchez, N. Hastie, J. Valcarcel, Biochemical function of female-lethal (2)D/Wilms' tumor suppressor-1-associated proteins in alternative pre-mRNA splicing, J. Biol. Chem. 278 (2003) 3040–3047.
[27] C.P. Ruangvoravat, C.W. Lo, Connexin 43 expression in the mouse embryo: localization of transcripts within developmentally significant domains, Dev. Dyn. 194 (1992) 261–281.
[28] A.P. Monaghan, K.H. Kaestner, E. Grau, G. Schutz, Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm, Development 119 (1993) 567–578.
[29] H. Roelink, J.A. Porter, C. Chiang, Y. Tanabe, D.T. Chang, P.A. Beachy, T.M. Jessell, Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis, Cell 81 (1995) 445–455.
[30] D.J. Epstein, A.P. McMahon, A.L. Joyner, Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms, Development 126 (1999) 281–292.
[31] A.G. Reaume, P.A. de Sousa, S. Kulkarni, B.L. Langille, D. Zhu, T.C. Davies, S.C. Juneja, G.M. Kidder, J. Rossant, Cardiac malformation in neonatal mice lacking connexin43, Science 267 (1995) 1831–1834.