Development 139, 4484-4490 (2012) doi:10.1242/dev.078790

A fast and sensitive alternative for β-galactosidase detection in mouse embryos

Sakthi Sundararajan[1], Maki Wakamiya[2], Richard R. Behringer[3] and Jaime A. Rivera-Pérez*

SUMMARY

The bacterial *lacZ* gene is widely used as a reporter in a myriad of mouse transgenic experiments. β-Galactosidase, encoded by *lacZ*, is usually detected using X-gal in combination with ferric and ferrous ions. This assay produces a blue indole precipitate that is easy to detect visually. Here, we show that Salmon-gal in combination with tetrazolium salts provides a more sensitive and faster staining reaction than the traditional β-galactosidase assay in mouse embryos. Using a combination of Salmon-gal and tetranitroblue tetrazolium, we were able to visualize the activity of β-galactosidase in embryos at stages when the customary X-gal reaction failed to detect staining. Our studies provide an enhanced alternative for β-galactosidase detection in expression and cell fate studies that use *lacZ*-based transgenic mouse lines.

KEY WORDS: Mouse, Embryo, *lacZ*, β-Galactosidase, X-gal, S-gal, TNBT

INTRODUCTION

The *lacZ* gene is frequently fused to heterologous promoters to reveal the expression of endogenous genes in transgenic mice (Bonnerot and Nicolas, 1993; Goring et al., 1987) and in the detection of genetic recombination events in cell fate mapping experiments (Joyner and Zervas, 2006; Petit et al., 2005; Watson et al., 2008). The most popular substrate for the detection of β-galactosidase is X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a glycoside that is used in combination with potassium ferri- and ferro-cyanide (FeCN). The β-galactosidase assay occurs in two steps. In the initial step, β-galactosidase induces the release of a soluble, colorless indolyl group from X-gal. Subsequently, two indolyl moieties form a dimer that is oxidized to form an insoluble blue indigo precipitate (Cotson and Holt, 1958; Pearson et al., 1963). The dimerization and oxidation steps are facilitated by ferric and ferrous ions, which serve as electron acceptors (Lojda, 1970).

There are multiple chromogenic substrates for β-galactosidase that can substitute for X-gal, these include Salmon-gal (S-gal) (6-chloro-3-indolyl-β-D-galactopyranoside), Magenta-gal (5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside) and Bluo-gal (5-bromo-3-indolyl-β-D-galactopyranoside) (Aguzzi and Theuring, 1994; Brunet et al., 1998; Kishigami et al., 2006; Pearson et al., 1963), as well as fluorescent substrates (Zhang et al., 1991). S-gal has been shown to be more sensitive than X-gal in early mouse embryos when used in combination with ferric and ferrous ions (Kishigami et al., 2006).

The tetrazolium salts NBT (nitroblue tetrazolium), TNBT (tetranitroblue tetrazolium) and INT (iodonitrotetrazolium) are substitutes for potassium ferri- and ferro-cyanide and precipitate, when reduced, to form colored formazan compounds (Altman, 1976). X-gal, in combination with NBT, produces a purple precipitate, combined with INT yields a dark red brick color and mixed with TNBT, an intense dark-brown product (Altman, 1976). This later combination was found to be more sensitive than the classic X-gal/FeCN indigogenic reaction in tissue sections (Gugliotta et al., 1992).

In experiments using the traditional X-gal/FeCN assay, we noticed that the expression pattern of some *lacZ*-based transgenes did not mimic the pattern of expression of endogenous genes. One explanation for these results is that the β-galactosidase assay is not sensitive enough to reveal the full range of expression of the transgene. Because of the potential implications of this possibility in the interpretation of *lacZ*-based transgenic experiments, we embarked on a search for a more-sensitive β-galactosidase assay. To achieve this goal, we assayed S-gal in combination with the tetrazolium salts NBT, TNBT and INT as alternative substrates for β-galactosidase detection and compared them with the classic X-gal/FeCN assay. Here, we report that S-gal in combination with TNBT is the most-sensitive combination and produces faster results than all the different substrate combinations tested for β-galactosidase detection. These results provide a novel alternative for β-galactosidase detection that will facilitate the analysis of reporter lines or cell fate studies and avoid false-negative results.

MATERIALS AND METHODS

Mouse strains and dissection

All mouse strains were maintained as mixed stocks. BAT-Gal and ROSA26 reporter mice were obtained from the Jackson Laboratory (stock numbers 005317 and 003474, respectively). Ttr^{cre} mice were provided by Dr Anna-Katerina Hadjantonakis (Memorial Sloan-Kettering Cancer Center, New York, USA). Heterozygous BAT-Gal embryos were obtained from crosses between homozygous BAT-Gal males and CD-1 females (Charles River Laboratories). Heterozygous $Wnt3^{lacZ/+}$ embryos were obtained by crossing $Wnt3^{LacZ/+}$ males with CD-1 females. $Wnt3^{lacZ/+}$ mice carry an *IRES-lacZ* gene cassette inserted in exon 4 of *Wnt3*. Dissections were carried out in low glucose DMEM (Dulbecco's Modified Eagle Medium) (Invitrogen, catalog number 31600-034) containing 10% heat inactivated fetal bovine serum (Atlanta Biologicals, catalog number S11150), penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen, catalog number 15140-122) and 20 mM HEPES (Fisher, catalog number BP310). The embryos were staged according to the staging method outlined in previous studies (Rivera-Pérez et al., 2010) or described in terms of embryo dissection time (E). The

[1]Department of Cell and Developmental Biology, University of Massachusetts Medical School, 55 Lake Avenue North, S7-228, Worcester, MA 01655, USA. [2]Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, TX 77555-1048, USA. [3]Department of Genetics, The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA.

*Author for correspondence (jaime.rivera@umassmed.edu)

Accepted 1 September 2012

middle of the dark cycle that occurred prior to observing a vaginal plug was considered the beginning of gestation.

β-Galactosidase staining protocol

Embryos dissected between E5.5 and E6.5 were fixed in 0.2% glutaraldehyde, 2% formalin, 5 mM EGTA and 2 mM $MgCl_2$ in 0.1 M phosphate buffer (pH 7.3) for 5 minutes. They were then washed three times in rinse solution: 0.1% sodium deoxycholate, 0.2% IGEPAL, 2 mM $MgCl_2$ and 0.1 M phosphate buffer (pH 7.3) for 20 minutes each. Embryos were then incubated in the dark in staining solution at 37°C. The staining solution consisted of 1 mg/ml X-gal (Lab Scientific, catalog number X-266) and 5 mM potassium ferri- and ferro-cyanide (Sigma, catalog number P9387 and P8131, respectively) or 1 mg/ml Salmon gal (6-chloro-3-indolyl-β-D-galactopyranoside) (Lab Scientific, catalog number X668) and 0.4 mM of either NBT (4-nitro blue tetrazolium chloride) (Roche, catalog number 11383213001), TNBT (5-bromo-4-chloro-3-indoxyl phosphate) (VWR, catalog number 101108-494) or INT (2-[4-iodophenyl]-3-5-phenyl-2H-tetrazolium chloride) (Sigma, catalog number 18377), in rinse solution. Tetrazolium salts were dissolved in absolute ethanol at a concentration of 100 mg/ml (3.3 μl were used per ml of staining solution). The staining reaction was monitored every 10 minutes for the first 30 minutes and then every 30 minutes in order to determine the onset of staining. Embryos were left in the staining solution for a minimum of 20 minutes and a maximum of 3 hours when using S-gal and tetrazolium salts or overnight up to a maximum of 3 days when using X-gal and potassium ferro- and ferri-cyanide. The reactions were carried out in four-well plates containing 800 μl of fix or rinse solution and 500 μl of staining solution. During incubation in the staining solution, the plates were sealed with a plastic film (USA Scientific, catalog number 2921-0000) and wrapped in aluminum foil to avoid evaporation and protect them from light.

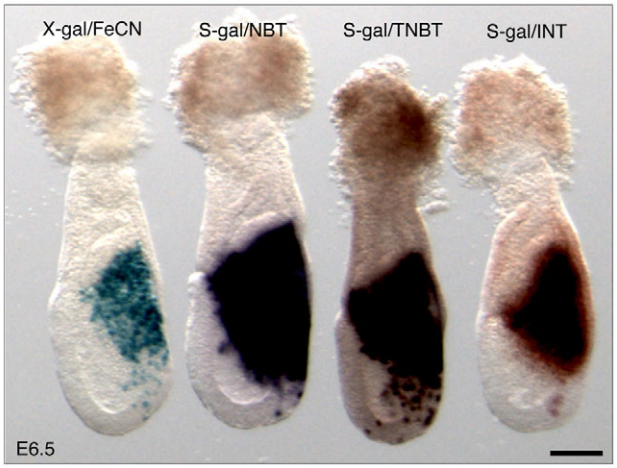

Fig. 1. Detection of BAT-Gal transgene using four different β-galactosidase assays. Heterozygous BAT-Gal embryos dissected at E6.5 were subjected to β-galactosidase assays using the traditional X-gal/FeCN assay and three different combinations of Salmon-gal and tetrazolium salts. All embryos show *lacZ* activity in the primitive streak, indicating activation of the canonical Wnt signaling pathway. Embryos assayed with S-gal and tetrazolium salts combinations produce a stronger and faster color reaction than the traditional X-gal/FeCN assay. Embryos subjected to tetrazolium salts combinations were stained for 3 hours whereas the X-gal/FeCN reaction was allowed to proceed for 3 days. Scale bar: 100 μm.

RESULTS

Detection of β-galactosidase using the traditional X-gal/FeCN assay, and novel S-gal and tetrazolium salts combinations

A previous report showed that S-gal is a superior substitute for X-gal in β-galactosidase assays in mouse embryos (Kishigami et al., 2006). Tetrazolium salts, however, provide a powerful alternative as electron acceptors in the β-galactosidase reaction. Based on this data, we hypothesized that the use of S-gal in combination with tetrazolium salts could provide a better alternative for β-galactosidase detection in mouse embryos. To address this hypothesis, we subjected heterozygous BAT-Gal transgenic embryos to β-galactosidase assays. The BAT-Gal transgene provides a readout of the canonical Wnt signaling pathway in mice. BAT-Gal is readily detected in the primitive streak and its derivatives in gastrulating mouse embryos (Maretto et al., 2003). We obtained heterozygous BAT-Gal embryos by crossing homozygous BAT-Gal males and wild-type CD-1 females. This ensured that all the offspring from these crosses carried one copy of the BAT-Gal transgene and, at the same time, obviated the need to genotype the embryos.

E6.5 embryos were dissected and were assayed for β-galactosidase activity in a mixture of S-gal and one of three tetrazolium salts, NBT, TNBT and INT immediately after dissection. For comparison, embryos from the same litter were assayed using the traditional X-gal/FeCN substrate mixture.

All reagent combinations provided an unambiguous readout of β-galactosidase activity in the primitive streak region; however, there were clear variations in the color and velocity of staining (Fig. 1). The traditional X-gal/FeCN combination showed a clear blue stain devoid of background. This combination, however, took the longest time to develop, requiring 1 day or longer to reveal its full color intensity. The S-gal/NBT mix produced a dark purple precipitate with negligible background. This mixture proved a good substitute for X-gal/FeCN. The most intense staining pattern was observed using the S-gal/TNBT mixture. This mix produced a dark-brown color; however, it also generated the highest levels of background and led to completely blackened embryos when the reaction was allowed to proceed longer than 3 hours (data not shown). The S-gal/INT combination provided a dark-red staining pattern. This was the weakest combination of substrates as it provided a diffuse staining pattern that failed to outline clearly the primitive streak.

Overall, the fastest and strongest staining pattern was obtained using S-gal in combination with TNBT. When compared with S-gal/NBT- and X-gal/FeCN-stained embryos, the S-gal/TNBT embryos showed darker staining in the primitive streak and clearer definition of individual mesendodermal cells at the tip of the egg cylinder (Fig. 1). S-gal/TNBT was also faster to reveal the presence of the BAT-gal transgene, which became evident within 10 minutes of the start of the color reaction.

From these results, we conclude that S-gal/NBT and S-gal/TNBT, in particular S-gal/TNBT, provide a faster and stronger staining pattern than the traditional X-gal/FeCN or S-gal/INT substrates for β-galactosidase assays.

S-gal/TNBT but not X-gal/FeCN can detect the BAT-Gal transgene in embryos dissected at E6.0

Markers of the primitive streak activated by the canonical Wnt signaling pathway, such as brachyury are expressed in the posterior epiblast, the precursor of the primitive streak, as early as E6.0 (Rivera-Pérez and Magnuson, 2005). Moreover, *Wnt3*, a gene required for the formation of the primitive streak, is expressed in the posterior epiblast in embryos dissected at E5.75 (Rivera-Pérez and Magnuson, 2005). These data suggests that the BAT-Gal transgene should be activated in the precursors of the primitive streak of

embryos dissected at E6.0. To determine the onset of detection of the BAT-Gal transgene, we assayed BAT-Gal heterozygous embryos dissected between E5.5 and E6.5 with a mixture of S-gal/TNBT. For comparison, we also assayed embryos from the same litters with the traditional X-gal/FeCN procedure. In all cases, the S-gal/TNBT staining reaction was stopped after 3 hours to prevent excessive background staining and to maximize its effectiveness. The X-gal/FeCN staining was allowed to proceed for 3 days to allow enough time to account for the slow pace of the reaction.

All BAT-Gal/$^{+}$ embryos dissected at E6.5 were positive for either of the two staining assays (Table 1; Fig. 2). However, differences between the two staining procedures became evident at E6.25. The S-gal/TNBT mixture detected the BAT-Gal transgene in almost all of the embryos tested (16/17) whereas the X-gal/FeCN mixture detected staining in about two-thirds of the embryos (13/18). Detection of BAT-Gal became less effective at E6.0. At this stage, no staining was detected in embryos assayed with X-gal/FeCN (0/15), while the S-gal/TNBT mixture detected BAT-Gal activity in 3 out of 16 embryos tested. Embryos dissected at E5.75 or E5.5 did not show evidence of β-galactosidase activity regardless of the staining procedure.

In some cases, we noticed blue staining in the extra-embryonic visceral endoderm of embryos stained with X-gal/FeCN (Fig. 2A, arrows). We believe that this staining represents background staining as sometimes it was also observed in wild-type embryos.

These results show a greater capacity of the S-gal/TNBT mixture to detect BAT-Gal transgene expression than that of X-gal/FeCN.

The S-gal/TNBT assay detects a *Wnt3*^{lacZ} transgene in E5.75 embryos

The experiments above showed that a mixture of S-gal/TNBT provides a faster and more-sensitive alternative than the X-gal/FeCN assay. To confirm these results, we assayed embryos carrying a null *Wnt3*^{lacZ} allele. *Wnt3* is expressed initially in the posterior visceral endoderm at E5.5, expands to the adjacent epiblast by E5.75 and continues to be expressed in these tissues at E6.5 (Rivera- Pérez and Magnuson, 2005). *Wnt3*^{lacZ} mice carry an *IRES-lacZ* cassette inserted in the *Wnt3* locus (to be described elsewhere), allowing the visualization of *Wnt3* expression using a β-galactosidase assay.

To compare the ability of the S-gal/TNBT and X-gal/FeCN assays to detect the *Wnt3*^{lacZ} allele in embryos dissected before E6.5, we generated embryos from crosses between heterozygous *Wnt3*^{lacZ} males and wild-type females and subjected them to β-galactosidase assays at E6.5, E6.25, E5.75 and E5.5 (Table 2). *Wnt3* nulls die at ~E8.5 (Liu et al., 1999). We expected that 50% of the embryos would show β-galactosidase activity as half of the embryos would be heterozygous for the *Wnt3*^{lacZ} allele.

As expected, 50% of the embryos dissected at E6.5 showed β-galactosidase activity regardless of the staining assay (Table 2; Fig. 3). The number of stained embryos conformed to the expected Mendelian ratios, S-gal/TNBT (11/18) (χ^2=0.89, P<0.1) and X-gal/FeCN (8/18) (χ^2=0.22, P<0.1). The number of positive embryos stained with S-gal/TNBT still matched the expected Mendelian ratios at E6.25 (13/25) (χ^2=0.04, P<0.1) but not the number of positive embryos assayed with X-gal/FeCN (χ^2=3.85, P>0.05) showing more sensitivity for the S-gal/TNBT assay. At E5.75, the S-gal/TNBT assay still detected the *Wnt3*^{lacZ} allele at Mendelian ratios (14/33) (χ^2=0.757, P<0.1) but none of the embryos stained with X-gal/FeCN (0/20) showed β-galactosidase activity. The ability of the S-

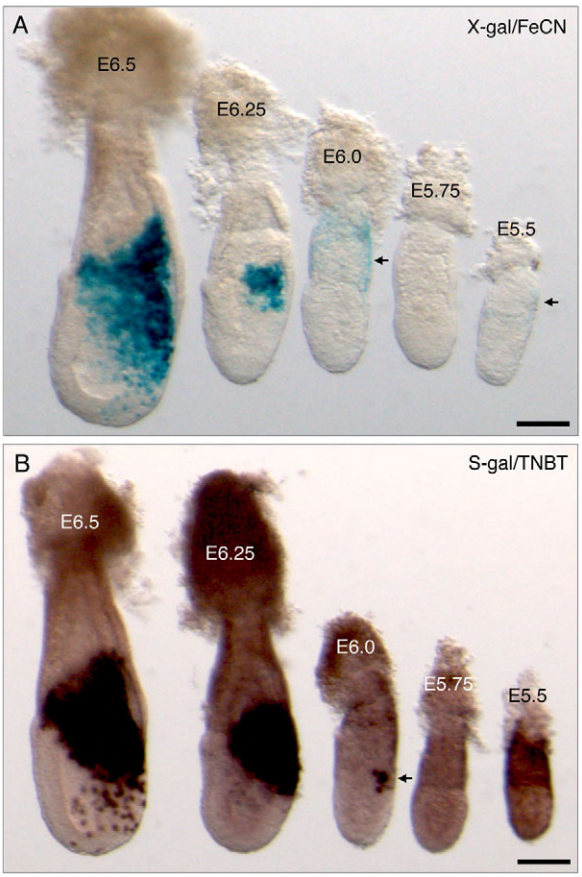

Fig. 2. The S-gal/TNBT assay detects the BAT-Gal transgene at younger stages than the traditional X-gal/FeCN assay. (**A**,**B**) Embryos heterozygous for the BAT-Gal transgene dissected at E6.5, E6.25, E6.0, E5.75 and E5.5 were assayed for β-galactosidase activity using X-gal/FeCN (A) or S-gal/TNBT (B) mixtures. The S-gal/TNBT mix detected the BAT-Gal transgene in embryos dissected as early as E6.0 (arrow in B), whereas the X-gal/FeCN assay detected the transgene only in embryos dissected at E6.25 or later stages. Non-specific blue staining is visible in the extra-embryonic visceral endoderm of embryos stained with X-gal/FeCN at E6.0 and E5.5 (arrows in A). Scale bars: 100 μm.

Table 1. Detection of BAT-Gal transgene using S-gal/TNBT or X-gal/FeCN staining assays

	BAT-Gal/BAT-Gal × CD-1		S-gal/TNBT		X-gal/FeCN	
Dissection time	Number of litters	Number of embryos	Positive	Negative	Positive	Negative
E6.5	2	19	16	0	3	0
E6.25	4	35	16	1	13	5
E6.0	3	31	3	13	0	15
E5.75	3	35	0	16	0	19
E5.5	3	23	0	12	0	11
Total	15	143	35	42	16	50

Table 2. Detection of *Wnt3*^{LacZ} transgene using S-gal/TNBT or X-gal/FeCN assays

	Wnt3$^{lacZ/+}$ × CD-1		S-gal/TNBT		X-gal/FeCN	
Dissection time	Number of litters	Number of embryos	Positive	Negative	Positive	Negative
E6.5	3	36	11	7	8	10
E6.25	4	46	13	12	6	15
E5.75	5	53	14	19	0	20
E5.5	3	29	0	29	-	-
Total	15	164	38	67	14	45

gal/TNBT assay to detect the *Wnt3*^{lacZ} allele tapered at E5.5, when none of the 29 embryos assayed were shown to express *Wnt3*^{lacZ}.

These results provide additional evidence that a S-gal/TNBT combination is a more sensitive alternative to X-gal/FeCN for the detection of β-galactosidase in mouse embryos.

Detection of Cre/lox recombination using S-gal/TNBT assay

ROSA26 reporter (*R26*^{r}) mice (Soriano, 1999) are frequently used to map the activity of Cre transgenes driven by tissue-specific promoters. To test the ability of the S-gal/TNBT to detect Cre-recombination events, we assayed embryos obtained from crosses between *R26*$^{r/r}$ males and *Ttr*^{cre} heterozygous females for β-galastosidase activity. *Ttr*^{cre} mice express Cre under the control of the *Transthyretin* promoter exclusively in the visceral endoderm layer of early post-implantation embryos (Kwon and Hadjantonakis, 2009; Kwon et al., 2008). We expected half of the progeny to be double heterozygous for *R26*^{r} and *Ttr*^{cre}, and half to be heterozygous only for *R26*^{r}. These later embryos serve as control littermates in the experiment.

We assayed 41 embryos dissected at E6.5 for β-galactosidase activity using S-gal/TNBT or X-gal/FeCN mixtures (Fig. 4). We detected β-galactosidase activity in half of the embryos assayed (24/41), irrespective of the detection method used. In the case of S-gal/TNBT, 11 out of 22 embryos and in X-gal/FeCN assays 13 out of 19 embryos. These results indicate that S-gal/TNBT method can be used in place of the traditional X-gal/FeCN technique.

S-gal/TNBT embryos can be subjected to whole-mount in situ hybridization procedures

To test the possibility of using S-gal/TNBT stained embryos in whole-mount in situ hybridization experiments, we subjected BAT-Gal embryos stained using the S-gal/TNBT protocol to in situ hybridization using probes for *Oct4*, a gene expressed in the epiblast of early post-implantation embryos (Rosner et al., 1990), and *Wnt7b*, a gene expressed in the extra-embryonic ectoderm (Kemp et al., 2007). For comparison, we included X-gal/FeCN-stained embryos in the same experiments.

E6.5 embryos were subjected to the S-gal/TNBT assay immediately after dissection and allowed to stain for 20-30 minutes. After staining, the embryos were rinsed in PBS, fixed in 4% paraformaldehyde for 2 hours at room temperature and

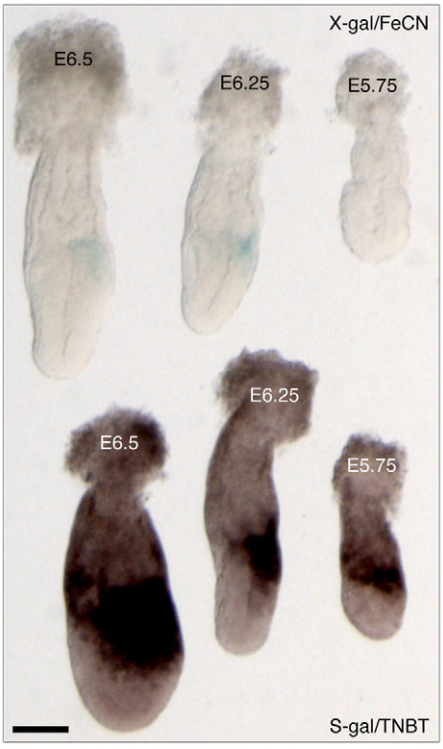

Fig. 3. Detection of β-galactosidase activity in *Wnt3*^{lacZ} embryos. Embryos derived from *Wnt3*^{lacZ} heterozygous males and wild-type females crosses dissected at E6.5, E6.25 and E5.75 were assayed for β-galactosidase activity using X-gal/FeCN or S-gal/TNBT as substrates. S-gal/TBNT detected β-galactosidase activity at the expected Mendelian ratios as early as E5.75. This compared favorably with X-gal/FeCN staining, which detected the *Wnt3*^{lacZ} transgene only in embryos dissected at E6.25 or later. Scale bar: 100 μm.

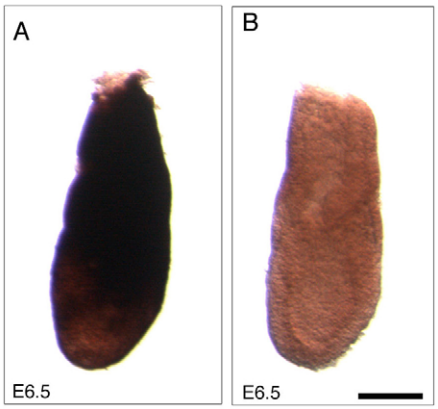

Fig. 4. Detection of Cre/lox recombination using S-gal/TNBT assay. (**A**,**B**) Double heterozygous R26 reporter/*Ttr*^{cre} embryos (A) and R26 reporter heterozygous controls (B) were subjected to β-galactosidase staining using the S-gal/TNBT assay. *Ttr*^{cre} directs Cre expression in the visceral endoderm layer of the conceptus. Cre recombination is evident in the whole visceral endoderm in the double heterozygous but not in the control embryo. Embryos are shown at the same scale. Scale bar: 100 μm.

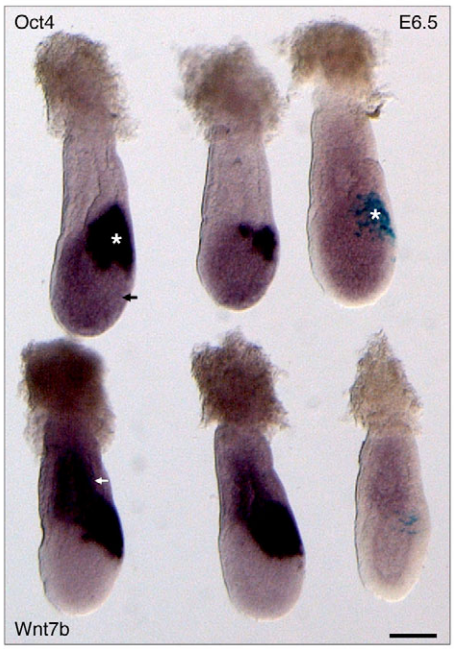

Fig. 5. Whole-mount in situ hybridization of S-gal/TNBT-stained embryos. Heterozygous BAT-Gal embryos dissected at E6.5 were stained with S-Gal/TNBT (dark purple staining) or X-gal/FeCN (blue staining) and then subjected to whole-mount in situ hybridization using *Oct4* and *Wnt7b* probes. *Oct4* expression marks the epiblast (black arrow) and *Wnt7b* the extra-embryonic ectoderm (white arrow). The β-galactosidase activity provided by the BAT-Gal transgene (asterisks) marks the primitive streak region. Scale bar: 100 μm.

subjected to the whole-mount in situ hybridization procedure as previously described (Rivera-Pérez and Magnuson, 2005). X-gal/FeCN embryos were stained for 18 hours and fixed overnight at 4°C. They were then mixed with S-gal/NBT stained embryos and subjected to the whole-mount in situ hybridization procedure.

All S-gal/TNBT embryos tested showed expression of *Oct4* (n=13) and *Wnt7b* (n=5) (Fig. 5) with minimal or no background. The X-gal/FeCN embryos (n=3) also were positive for both probes; however, the staining was weaker, took longer to develop and the background staining was higher.

These results show that it is possible to combine S-gal/TNBT staining with whole-mount in situ hybridization procedures. S-gal/TNBT stained embryos also appear better suited for whole-mount in situ experiments than X-gal/FeCN embryos.

The S-gal/TNBT staining assay can detect *lacZ* activity in midgestation embryos

To determine whether the S-gal/TNBT staining assay was suitable for detecting β-galactosidase activity in post-gastrulation embryos, we assayed *Wnt3*^{lacZ} heterozygous embryos dissected at E10.5 with the same conditions used in embryos dissected at E5.5-E6.5. In embryos stained for 3 hours, the expression pattern of the *Wnt3*^{lacZ} allele was clearly above background (Fig. 6). E10.5 embryos showed expression in the dorsal thalamus, midbrain, hindbrain, neural tube, mandibular process of the first branchial arch, second branchial arch, forelimbs and hindlimbs (Fig. 6A). These expression areas have been previously reported (Barrow et al., 2003; Roelink and Nusse, 1991; Salinas et al., 1994). In addition, *Wnt3*^{lacZ} expression marked the dorsal root ganglia (Fig. 6A). No *lacZ* activity was detected in control wild-type littermates (Fig. 6B).

To determine the utility of the S-gal/TNBT staining in whole-mount preparations of adult tissues, we assayed multiple organs of adult BAT-Gal or *Wnt3*^{lacZ} mice and compared them with X-gal/FeCN staining. Our results revealed little contrast between the rust coloring provided by the S-gal/TNBT mixture and dark-colored tissues (not shown) showing no major advantages over the traditional X-gal/FeCN staining method.

These results demonstrate that it is feasible to use the S-gal/TNBT staining protocol in embryos dissected at midgestation stages but that its utility is dampened by color contrast problems in whole-mount preparations of dark-colored adult organs.

DISCUSSION

The detection of the activity of the *lacZ* gene in mouse embryos is overwhelmingly performed using X-gal in combination with potassium ferro- and ferri-cyanide to detect β-galactosidase. Although this staining assay provides a clear blue precipitate that is easy to detect, it is not the most sensitive or fastest assay available to detect β-galactosidase (Gugliotta et al., 1992; Kishigami et al., 2006). In this study, we tested three tetrazolium salts, NBT, TNBT and INT, in combination with the glycoside Salmon-gal to test their effectiveness in detecting the activity of the

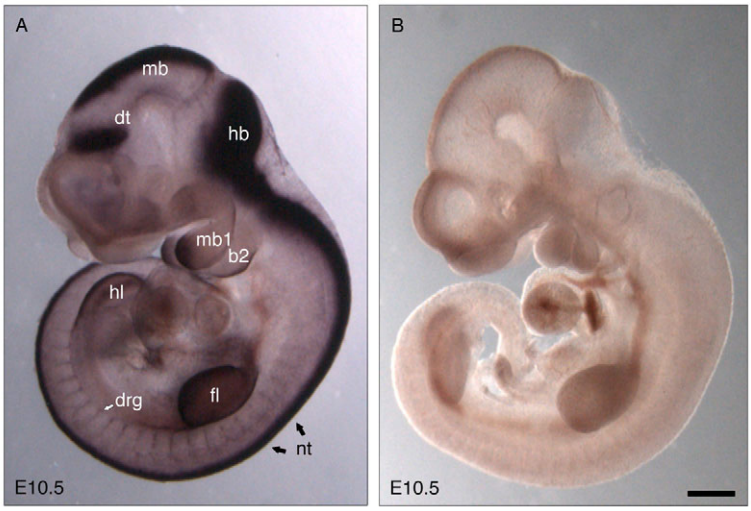

Fig. 6. Detection of *Wnt3*^{lacZ} allele expression in E10.5 embryo using S-gal/TNBT assay. (**A**,**B**) Heterozygous *Wnt3*^{lacZ} embryo (A) stained with S-gal/TNBT mixture and wild-type control (B). *Wnt3*^{lacZ} expression marks the dorsal thalamus (dt), midbrain (mb), hindbrain (hb), mandibular process of the first branchial arch (mb1), second branchial arch (b2) forelimb (fl), hindlimb (hl), the neural tube (nt, arrows) and the dorsal root ganglia (drg). Scale bar: 500 μm.

lacZ gene in mouse embryos. At the same time, we compared this assay with the traditional X-gal/FeCN assay.

In our hands, a combination of S-gal and TNBT proved the most sensitive of the three S-gal/tetrazolium salt combinations that we tested. This assay also proved more sensitive than the traditional X-gal/FeCN assay in detecting *lacZ* activity in two independent transgenic mouse lines. The X-gal/FeCN assay required days to reveal the full extent of β-galactosidase activity or did not detect staining at all. The S-gal/TNBT assay, however, can lead to high background levels if allowed to proceed for too long. In our experiments, a 3-hour staining reaction was the longest we could conduct this assay in embryos at E5.5-E6.5 without obscuring the β-galactosidase staining pattern. This, however, was compensated for by the short time required to visualize the activity of β-galactosidase: optimally, a 20-minute staining reaction sufficed to detect β-galactosidase without sacrificing sensitivity and with negligible background staining. An alternative assay is S-gal combined with NBT; this combination produced less background staining than S-gal/TNBT, yet proved slightly less sensitive.

The addition of ferri- and ferro-cyanide to the traditional β-galactosidase reaction is known to lead to reduction in the activity of acidic traditional β-galactosidase (Lojda, 1970). Thus, it is possible that the background staining observed in the S-gal/TNBT assay is due to endogenous acidic β-galactosidase activity. This may be complicated by the fact that the reduction of tetrazolium salts to produce formazan compounds can proceed beyond the formazan stage, leading to break up of the molecule to produce other chromogenic compounds, such as anilin (Altman, 1976). Because of these possibilities, caution should be exerted when using tetrazolium salts in β-galactosidase detection assays and proper controls should be used. In our experiments, the utility of the S-gal/TNBT assay was dampened by darkly stained tissues such as the liver in whole-mount preparations. In these cases, the use of histological sections, as reported by Gugliotta and co-workers (Gugliotta et al., 1992), may provide a more convenient approach.

Using X-gal/FeCN assays, canonical Wnt activity has been reported in the extra-embryonic visceral endoderm of transgenic E5.5 embryos carrying the TCF/Lef-lacZ transgene (Mohamed et al., 2004). This transgene, like the BAT-Gal transgene, is also a reporter for the canonical Wnt signaling pathway. In our experiments, we noticed that when the X-gal/FeCN reaction was allowed to proceed for too long, the extra-embryonic visceral endoderm stained blue in some embryos. This extra-embryonic β-galactosidase activity, however, was not consistently observed in embryos of the same developmental stages and was sometimes observed in wild-type embryos. One explanation for these results is that long staining times can lead to acidification of the staining solution, which favors the detection of endogenous acidic β-galactosidase activity in the extra-embryonic visceral endoderm. Acidic β-galactosidase activity is present in several adult tissues (Lojda, 1970) and could also be present in this tissue. However, we cannot discard the possibility that this discrepancy is due to differences in the transgenic line used.

In recent years, there has been a resurgence of in vivo genetic lineage-tracing experiments aimed at determining the cellular origin of tissues and organs (Joyner and Zervas, 2006; Petit et al., 2005). These experiments typically use a tissue-specific promoter to drive expression of Cre recombinase in a particular group of cells and a *lacZ*-based reporter line, such as the ROSA-26 reporter (Soriano, 1999) to detect the descendants of the recombined precursors. This is a powerful tool that can provide invaluable data. Our data, however, suggest that caution should be exerted when using this strategy. As shown here, the traditional X-gal/FeCN assay can lead to false-positive or false-negative results that can lead to erroneous conclusions. The superior sensitivity of our S-gal/TNBT procedure, if used properly, can help avoid these problems and combined with its fast detection and combination with whole-mount in situ hybridization offers an attractive method for β-galactosidase staining in mouse embryological experiments. Our methodology also offers an alternative to detect low levels of β-galactosidase activity in *lacZ*-based knockout mice (Austin et al., 2004; Hansen et al., 2008; Skarnes et al., 2011; Testa et al., 2004; Valenzuela et al., 2003) available through the international knockout mouse consortium (http://www.knockoutmouse.org/).

Acknowledgements

We thank James Li for critically reading the manuscript and Kat Hadjantonakis for kindly providing *Ttr*^{cre} mice.

Funding

This work was supported by the National Institutes of Health grant [GM87130 to J.A.R.-P.]. R.B. was supported by the Ben F. Love Endowment and the Kleberg Foundation. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

References

Aguzzi, A. and Theuring, F. (1994). Improved in situ beta-galactosidase staining for histological analysis of transgenic mice. *Histochemistry* **102**, 477-481.

Altman, F. P. (1976). Tetrazolium salts and formazans. *Prog. Histochem. Cytochem.* **9**, 1-56.

Austin, C. P., Battey, J. F., Bradley, A., Bucan, M., Capecchi, M., Collins, F. S., Dove, W. F., Duyk, G., Dymecki, S., Eppig, J. T. et al. (2004). The knockout mouse project. *Nat. Genet.* **36**, 921-924.

Barrow, J. R., Thomas, K. R., Boussadia-Zahui, O., Moore, R., Kemler, R., Capecchi, M. R. and McMahon, A. P. (2003). Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev.* **17**, 394-409.

Bonnerot, C. and Nicolas, J. F. (1993). Application of LacZ gene fusions to postimplantation development. *Methods Enzymol.* **225**, 451-469.

Brunet, L. J., McMahon, J. A., McMahon, A. P. and Harland, R. M. (1998). Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* **280**, 1455-1457.

Cotson, S. and Holt, S. J. (1958). Studies in enzyme cytochemistry. IV. Kinetics of aerial oxidation of indoxyl and some of its halogen derivatives. *Proc. R. Soc. Lond. B Biol. Sci.* **148**, 506-519.

Goring, D. R., Rossant, J., Clapoff, S., Breitman, M. L. and Tsui, L. C. (1987). In situ detection of beta-galactosidase in lenses of transgenic mice with a gamma-crystallin/lacZ gene. *Science* **235**, 456-458.

Gugliotta, P., Pacchioni, D. and Bussolati, G. (1992). Staining reaction for beta-galactosidase in immunocytochemistry and in situ hybridization. *Eur. J. Histochem.* **36**, 143-148.

Hansen, G. M., Markesich, D. C., Burnett, M. B., Zhu, Q., Dionne, K. M., Richter, L. J., Finnell, R. H., Sands, A. T., Zambrowicz, B. P. and Abuin, A. (2008). Large-scale gene trapping in C57BL/6N mouse embryonic stem cells. *Genome Res.* **18**, 1670-1679.

Joyner, A. L. and Zervas, M. (2006). Genetic inducible fate mapping in mouse: establishing genetic lineages and defining genetic neuroanatomy in the nervous system. *Dev. Dyn.* **235**, 2376-2385.

Kemp, C. R., Willems, E., Wawrzak, D., Hendrickx, M., Agbor Agbor, T. and Leyns, L. (2007). Expression of Frizzled5, Frizzled7, and Frizzled10 during early mouse development and interactions with canonical Wnt signaling. *Dev. Dyn.* **236**, 2011-2019.

Kishigami, S., Komatsu, Y., Takeda, H., Nomura-Kitabayashi, A., Yamauchi, Y., Abe, K., Yamamura, K. and Mishina, Y. (2006). Optimized beta-galactosidase staining method for simultaneous detection of endogenous gene expression in early mouse embryos. *Genesis* **44**, 57-65.

Kwon, G. S. and Hadjantonakis, A. K. (2009). Transthyretin mouse transgenes direct RFP expression or Cre-mediated recombination throughout the visceral endoderm. *Genesis* **47**, 447-455.

Kwon, G. S., Viotti, M. and Hadjantonakis, A. K. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. *Dev. Cell* **15**, 509-520.

Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* **22**, 361-365.
Lojda, Z. (1970). Indigogenic methods for glycosidases. I. An improved method for beta-D-glucosidase and its application to localization studies on intestinal and renal enzymes. *Histochemie* **22**, 347-361.
Maretto, S., Cordenonsi, M., Dupont, S., Braghetta, P., Broccoli, V., Hassan, A. B., Volpin, D., Bressan, G. M. and Piccolo, S. (2003). Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. *Proc. Natl. Acad. Sci. USA* **100**, 3299-3304.
Mohamed, O. A., Clarke, H. J. and Dufort, D. (2004). Beta-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. *Dev. Dyn.* **231**, 416-424.
Pearson, B., Wolf, P. L. and Vazquez, J. (1963). A comparative study of a series of new indolyl compounds to localize beta-galactosidase in tissues. *Lab. Invest.* **12**, 1249-1259.
Petit, A. C., Legué, E. and Nicolas, J. F. (2005). Methods in clonal analysis and applications. *Reprod. Nutr. Dev.* **45**, 321-339.
Rivera-Pérez, J. A. and Magnuson, T. (2005). Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. *Dev. Biol.* **288**, 363-371.
Rivera-Pérez, J. A., Jones, V. and Tam, P. P. (2010). Culture of whole mouse embryos at early postimplantation to organogenesis stages: developmental staging and methods. *Methods Enzymol.* **476**, 185-203.
Roelink, H. and Nusse, R. (1991). Expression of two members of the Wnt family during mouse development – restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* **5**, 381-388.
Rosner, M. H., Vigano, M. A., Ozato, K., Timmons, P. M., Poirier, F., Rigby, P. W. and Staudt, L. M. (1990). A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686-692.
Salinas, P. C., Fletcher, C., Copeland, N. G., Jenkins, N. A. and Nusse, R. (1994). Maintenance of Wnt-3 expression in Purkinje cells of the mouse cerebellum depends on interactions with granule cells. *Development* **120**, 1277-1286.
Skarnes, W. C., Rosen, B., West, A. P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A. O., Thomas, M., Harrow, J., Cox, T. et al. (2011). A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* **474**, 337-342.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Testa, G., Schaft, J., van der Hoeven, F., Glaser, S., Anastassiadis, K., Zhang, Y., Hermann, T., Stremmel, W. and Stewart, A. F. (2004). A reliable lacZ expression reporter cassette for multipurpose, knockout-first alleles. *Genesis* **38**, 151-158.
Valenzuela, D. M., Murphy, A. J., Frendewey, D., Gale, N. W., Economides, A. N., Auerbach, W., Poueymirou, W. T., Adams, N. C., Rojas, J., Yasenchak, J. et al. (2003). High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. *Nat. Biotechnol.* **21**, 652-659.
Watson, C. M., Trainor, P. A., Radziewic, T., Pelka, G. J., Zhou, S. X., Parameswaran, M., Quinlan, G. A., Gordon, M., Sturm, K. and Tam, P. P. (2008). Application of lacZ transgenic mice to cell lineage studies. *Methods Mol. Biol.* **461**, 149-164.
Zhang, Y. Z., Naleway, J. J., Larison, K. D., Huang, Z. J. and Haugland, R. P. (1991). Detecting lacZ gene expression in living cells with new lipophilic, fluorogenic beta-galactosidase substrates. *FASEB J.* **5**, 3108-3113.