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Characterization of Bacterial Artificial Chromosome Transgenic Mice Expressing mCherry Fluorescent Protein Substituted for The Murine Smooth Muscle Alpha-Actin Gene

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Abstract

Smooth muscle α actin (SMA) is a cytoskeletal protein expressed by mesenchymal and smooth muscle cell types, including mural cells (vascular smooth muscle cells and pericytes). Using Bacterial Artificial Chromosome (BAC) recombineering technology, we generated transgenic reporter mice that express a membrane localized cherry red fluorescent protein (mCherry), driven by the full-length SMA promoter and intronic sequences. We determined that the founders and F1 progeny of five independent lines contain 1-3 copies of the mCherry substituted BAC vector. Furthermore, we characterized the expression of SMA-mCherry in relation to endogenous SMA in the embryo and in adult tissues, and found that the transgenic reporter in each line recapitulated endogenous SMA expression at all time points. We were also able to isolate SMA expressing cells from embryonic tissues using fluorescence activated cell sorting (FACS). We demonstrated that this marker can be combined with other vital fluorescent reporters and it can be used for live imaging of embryonic cardiodynamics. Therefore, these transgenic mice will be useful for isolating live SMA-expressing cells via FACS and for studying the emergence, behavior and regulation of SMA-expressing cells, including vascular smooth muscle cells and pericytes throughout embryonic and postnatal development.

Keywords

BAC transgenesis; In vivo imaging; Cardiac function; Vascular development; Mesenchymal cell

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SMA is a cytoskeletal protein first expressed during embryonic cardiovascular development, and subsequently expressed in developing somites and gut. Postnatally, SMA is replaced with other actin isoforms in the heart, but remains expressed in smooth muscle cells of the cardiovascular and digestive systems (Black *et al.*, 1991). Studies of SMA function in the cardiovascular system reveal a role in maintaining blood pressure homeostasis (Schlidmeyer *et al.*, 2000). During embryonic development and postnatally, SMA expression is widely used to identify mural cells (vascular smooth muscle cells and pericytes) and their mesenchymal cell precursors. SMA is also commonly used as a marker of mesenteric smooth muscle and embryonic myocardium (Mack and Owens, 1999).

Previous characterization of murine SMA expression and regulatory elements *in vivo* utilized a LacZ reporter, driven by the endogenous SMA promoter (Mack and Owens, 1999). Although SMA-expressing cells can be visualized *in situ* with these mice, the use of the LacZ reporter system does not allow monitoring of cell behavior in real time *in vivo* or efficient isolation of live cells for *in vitro* studies. In these studies, we generated transgenic mice that faithfully express a fluorescent reporter in a pattern reflective of endogenous SMA expression during embryonic development and postnatally.

To most effectively recapitulate endogenous expression of SMA in our reporter mice, we employed a BAC transgenic approach using recombineering technology, which allows seamless modification of DNA independent of the presence of restriction enzyme sites (Lee *et al.*, 2001). BAC recombineering is a versatile technology allowing investigators to physically link multiple reporters or tag proteins of interest for functional studies (Maye *et al.*, 2009, Poser *et al.*, 2008). BAC transgenic clones contain a large component of genomic DNA and increase the probability of including important transcriptional control elements for genes of interest. For our studies, we obtained BAC clone RP23-132C11 from CHORI that contained the murine SMA gene locus. To express the myristoylated mCherry under the control of SMA regulatory elements, we modified the BAC by inserting the myristoylation ATG in place of SMA ATG (Fig. 1a). We modified the SMA locus by replacing exon 2, the first coding exon, along with 50bp 3' of exon 2 with the mCherry reporter-SV40pA construct. The resultant vector would express the membrane localized mCherry under the control of the complete SMA regulatory apparatus, including intragenic sequences of the first intron (Mack and Owens, 1999), while interrupting splicing from the accompanying SMA gene sequences. This was achieved by generating a linear target vector by PCR from a template that contained the reporter upstream of an FRT-PGKneo-FRT cassette. The resultant linear DNA targeting construct was used in a standard recombineering strategy (Lee *et al.*, 2001). Although published recombineering protocols produced potential substituted BAC clones, we found an increased number of recombined clones were obtained by allowing the reaction to continue overnight at 30° C instead of the recommended 1 hour incubation. Following identification of correctly targeted clones by Southern Blot, FRT recombinase was induced to remove the PGKneo cassette. The BAC was linearized with *PI-SceI* and used to microinject fertilized mouse eggs that yielded 5 founder lines.

We determined the SMA gene copy number for each mouse line using a qRT-PCR based method (Chandler *et al.*, 2007). We were able to generate a standard curve using modified BAC DNA spiked into genomic DNA (Fig. 1b). We found that all five transgenic lines contained between 1 and 3 copies of the SMA gene (Fig. 1c). An analysis of F1 progeny copy number matched that of the founders, indicating we have generated mice with a single insertion site and did not yield lines with multiple insertions, a phenomenon that has been previously reported (Chandler *et al.*, 2007).

We examined embryonic SMA-mCherry expression from E7.5-18.5. No SMA-mCherry or endogenous SMA expression was observed at E7.5 by fluorescent stereomicroscopy or

confocal microscopy (Fig. 2a). At E8.5 and E9.5, we observed SMA-mCherry expression in the developing heart (Fig. 2b-c). Additional analysis at E9.5 (not shown) and E10.5 (Fig. 2d) revealed mCherry expression in the heart and aorta (Fig. 2e-g), yolk sac vascular smooth muscle (Fig. 2h-j) and somites (Fig. 2k-m); this expression pattern also recapitulated endogenous SMA expression (Mack and Owens, 1999). At E13.5, reporter expression was detected in the heart (Fig. 2n-p), aorta and esophagus (Fig. 2q-s), and intestinal and gastric smooth muscle (Fig. 2t-v), as expected based on previous studies (McHugh, 1995). SMA-mCherry reporter expression was also coincident with endogenous SMA expression in the E18.5 heart (Fig. 2w-y) lung (Fig. 2z-bb) and esophagus (Fig. 2 cc-ee). All reporter expression in the transgenic lines co-localized with endogenous SMA expression.

We also examined SMA-mCherry expression in adult tissues along side WT controls. We observed reporter expression in the coronary arteries of the heart but not cardiac myocytes (Fig. 3a-d). We observed reporter expression around mesenteric vessels (Fig. 3e-h) and in smooth muscle of the stomach (Fig. 3i-l). SMA-mCherry expression was also detected in the vasculature of the lung (Fig. 3m-n) and kidney (Fig. 3q-t), but not other cell types within these organs. Reporter expression was detected in the smooth muscle of the ileum of the intestine (Fig. 3u-x), myoepithelial cells of the mammary gland (Fig. 3y-bb), and femoral artery (Fig. 3cc-ff) of the hindlimb. Thus, by the inclusion of DNA sequences within and surrounding the SMA locus, we have faithfully recapitulated expression of this gene in developing and adult tissues/cells; this includes expression within the vasculature of the skeletal muscle and not the skeletal myotubes (Fig. 3cc-ff), nor in cardiac myocytes (Fig. 3a-d). It is likely that by presence of DNA both 5' and 3' to the SMA gene locus in our recombiner BAC construct we have included regulatory sequences directing appropriate transcriptional capacity of the SMA promoter (Mack and Owens, 1999) focusing expression in smooth muscle cells and their mesenchymal precursors.

We isolated and cultured SMA positive cells from E15.5 yolk sac by flow cytometry. There was a distinct mCherry positive population (Fig. 4b) when compared to WT littermate controls (Fig. 4a). The isolated cells expressed mCherry in culture (Fig. 4c, red). Cultures cells were positive for endogenous cytoskeletal SMA that was coexpressed with reporter expression in the membrane (Fig. 4c, green).

One benefit of using mCherry fluorescent protein is the ability to combine this marker with other fluorescent proteins without spectral overlap. The mCherry has an emission peak at 610nm, therefore, it is easily separated from blue, green or yellow fluorescent proteins. We have crossed SMA-mCherry mice with mice from the Tg(ϵ -globin::GFP) line, expressing Green Fluorescent Protein (GFP) under the control of ϵ -globin promoter, which drives the expression in the embryonic erythrocytes (Dyer *et al.*, 2005). Crossing these markers produced embryos in which the myocardium is labeled with the mCherry and the blood is labeled with the GFP (Fig 5a-d).

Because the mCherry is highly expressed in the embryonic myocardium, we tested whether this marker can be used for dynamic visualization of the embryonic heartbeat in live embryo culture. Images of an E9.5 beating heart acquired at 16 fps using fast scanning confocal microscopy are shown in Fig 5a-c. Successive panels show different phases of the cardiac cycle. Even though the imaging plane was positioned at the depth of 100 μ m, the mCherry clearly outlines the myocardium. The changes of the relative fluorescence intensity for the mCherry (Fig 5d, red line) and the GFP (Fig 5d, green line) are shown for the region marked in (Fig 5a). These periodical changes are produced by the tissue dynamics and could be used to estimate the heart rate and characterize heart wall movements. These results demonstrate that the SMA-mCherry is suitable for dynamic imaging of the heart and could be a useful tool for studying early mammalian cardiac function during the time frame that it is possible

to maintain mouse embryos on the microscope stage (up to E10.5; see Jones et al 2002). Furthermore, such a marker could be used for a large variety of vital imaging experiments, provided that the cells or tissues of interest are accessible to confocal or two-photon microscopy.

In summary, we generated transgenic mouse lines that express membrane localized mCherry reporter driven by the SMA genetic apparatus included in the ~150 Kb BAC construct, and determined that reporter expression faithfully recapitulated endogenous SMA expression during embryonic development and in postnatal tissues. These mice will be useful for non-invasive real-time in vivo imaging, as well as FACS-mediated isolation of live SMA-expressing cells for in vitro studies of their cellular and molecular regulation.

Materials and Methods

BAC Recombineering

BAC clone RP23-132C11 was obtained from Children's Hospital Research Oakland Research Institute (CHORI) and electroporated into SW105. 25ml SW105-132C11 were induced by incubating at 42°C for 15min. Induced SW105-132C11 were washed 3x in 50ml 10% Glycerol in ddH₂O and used for electroporation of the targeting vector. Linear targeting vector was generated by PCR from a plasmid DNA constructed in our lab containing the coding sequence for myristoylated mCherry with SV40 pA upstream of FRT-PGKneo-FRT cassette. Forward and reverse primers used in linear target PCR reaction contained 75bp homology with targeted SMA locus 5' and 3', respectively, and 25bp homologous with targeting vector sequence. 1µg linear target was electroporated into SW105-132C11 with 1.8Kv 5.0µS pulse and the cells were resuspended in 1ml LB and placed at 30°C. After 1h, 100ul cells were plated on 15µg/ml Kanamycin plates and placed in 30°C incubator overnight. 4ml LB was added to the electroporated SW105-132C11 and incubated overnight at 30°C, after which 100ul cells were streaked onto 15µg/ml Kanamycin plates. The clones were confirmed by restriction mapping followed by Southern hybridization and positive clones were sequenced across recombination sites to confirm correct recombination. The Kanamycin selection cassette was removed by induction of FRT recombinase with 0.2% d-arabinose. BAC DNA for microinjection was prepared by the Mouse Embryo Manipulation Services at Baylor College of Medicine protocol. Mice were genotyped from tail DNA using primers: Forward 5'CCTGTCCCCTCAGTTCATGT3' Reverse 5'CTTCAGCTTCAGCCTCTGCT.

Copy number

Copy number of integrated SMA-mCherry BAC DNA was determined as previously described (Chandler, *et al.*, 2007). Mouse tail DNA was digested with Proteinase K overnight, extracted 2x by Phenol/ chloroform/ isoamyl alcohol (25:25:1), precipitated with ethanol and washed with 70% ethanol. Primers used in qRT-PCR are: Jun Mm 00495062_s1 (Applied Biosystems), Cherry Forward 5'GACCACCTACAAGGCCAAGAAG3' Reverse 5'AGGTGATGTCCAACCTTGATGTTGA3' Probe FAM5'CAGCTGCCCCGGCGCCTACA3'MGB. Reactions were performed using AmpTaq Gold with UNG erase (Applied Biosystems). Reactions were run at 50°C 2 min, 95°C 10 min, followed by 40 cycles of 95°C 15sec 60°C 30sec.

Immunohistochemistry

Embryos and yolk sac were dissected and fixed in 4%-paraformaldehyde at 4°C for 15min-1hr for E8.5-10.5 respectively. E13.5-18.5 embryos were fixed overnight at 4°C. Embryos and yolk sac were then washed with PBS, dehydrated with 10% sucrose followed by 20% sucrose solution in PBS, and embedded in OCT (tissue tek). 10µm sections were

obtained on a Shandon cryotome. Tissue was blocked in DSB₁₀ (10% Donkey Serum (Sigma), 1% BSA (Sigma) in PBST (PBS with 0.1% Tween 20)) for 1hr RT. SMA antibody (Genetex) was diluted in DSB₁₀ at 2ug/ml added to tissue and incubated overnight at 4°C. After extensive washing with PBST, secondary antibody, donkey anti rabbit 488 (Molecular Probes), was diluted in DSB₁₀ at 4ug/ml and incubated with the tissue for 1hr at RT. Slides were then rinsed and mounted for collecting images. Cells were fixed with 4% paraformaldehyde at 4°C for 10min and immunostained identically to tissue sections. Images were obtained on Zeiss Axiovert 200M with a Zeiss AxioCam MRm camera.

Live imaging of embryonic cardiodynamics

SMA-mCherry males were mated to the Tg(ϵ -globin::GFP) females expressing Green Fluorescent Protein (GFP) in the embryonic blood cells. The embryos were dissected with the yolk sac intact at E9.5 and cultured on the microscope stage according to previously reported protocols (Jones *et al.*, 2002). Fast confocal imaging was performed using ZEISS LSM 5LIVE line scanning microscope (Carl Zeiss Inc.) at 20X magnification. The mCherry was excited using 532-nm laser; GFP was excited using 488 nm laser. Time lapse images were acquired at a rate of 16 frames per second (fps).

Fluorescence Activated Cell Sorting

Yolk sac tissues were digested in 0.2% collagenase II HBSS solution at 37°C for 30min. Digest was filtered through 35um filter and cells were centrifuged at 1000x g at 4°C for 5min. Cells were resuspended in HBSS 2% FBS 1ug/ ml DAPI. Cells were sorted on BD AriaII cell sorter. The mCherry reporter was excited at 561nm and emission collected through a 610/20 bandpass filter into DMEM 10% FBS penstrep and grown at 37°C 5% CO₂. Images were obtained on Zeiss Axiovert 200M with a Zeiss AxioCam MRm camera.

Cell Culture

Cells were sorted into 0.1% gelatin coated 96 well plates at 5000 cells per well containing DMEM (high glucose), 10% Fetal Bovine Serum and penstrep. Cells were cultured at 37°C 5%CO₂ for 5 days after which they were examined for endogenous SMA expression by immunohistochemistry, as described above.

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References

- Black FA, Packer SE, Parker TG, Lloyd MH, Roberts R, Schwartz RJ, Schneider MD. The vascular smooth muscle α -actin gene is reactivated during cardiac hypertrophy provoked by load. *J Clin Invest.* 1991; 88:1581–1588. [PubMed: 1834699]
- Chandler KJ, Chandler RL, Broeckelmann EM, Hou Y, Southard-Smith M, Mortlock DP. Relevance of BAC transgene copy number in mice: transgene copy number variation across multiple transgenic lines and correlations with transgene integrity and expression. *Mamm Genome.* 2007; 18:693–708. [PubMed: 17882484]
- Dyer MA, Farrington SM, Mohn D, Munday JR, Baron MH. Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development.* 2001; 128:1717–30. [PubMed: 11311154]
- Jones EAV, Crotty D, Kulesa PM, Waters CW, Baron MH, Fraser SE, Dickinson ME. Dynamic in vivo imaging of postimplantation mammalian embryos using whole embryo culture. *Genesis.* 2002; 34:228–235. [PubMed: 12434332]

- Lee C, Daiguan Y, de Velasco J Martinez, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG. A Highly Efficient *Escherichia coli*-Based Chromosome Engineering System Adapted for Recombinogenic Targeting and Subcloning of BAC DNA. *Genomics*. 2001; 73:56–65. [PubMed: 11352566]
- Mack CP, Owens GK. Regulation of Smooth Muscle α -Actin Expression In Vivo Is Dependent on CArG Elements Within the 5' and First Intron Promoter Regions. *Circ Res*. 1999; 84:852–861. [PubMed: 10205154]
- Maye P, Stover ML, Liu Y, Rowe DW, Gong S, Lichtler AC. A BAC-bacterial recombination method to generate physically linked multiple gene reporter DNA constructs. *BMC Biotechnology*. 2009; 9:20. [PubMed: 19284652]
- McHugh KM. Molecular Analysis of Smooth Muscle Development in the Mouse. *Dev Dyn*. 1995; 204:278–290. [PubMed: 8573719]
- Poser I, Sarov M, Hutchins JRA, Heriche JK, Toyoda Y, Pozniakovsky A, Weigl D, Nitzsche A, Hegemann B, Bird AW, Pelletier A, Kittler R, Hua S, Naumann R, Augsburg M, Sykora MM, Hofemeister H, Zhang Y, Nasmyth K, White KP, Dietzel S, Mechtler K, Durbin R, Stewart AF, Peters JM, Buchholz F, Hyman AA. BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. *Nature Methods*. 2008; 5:409–415. [PubMed: 18391959]
- Schlidmeyer LA, Braun R, Taffet G, Debiase M, Burns AE, Bradley A, Schwartz RJ. Impaired vascular contractility and blood pressure homeostasis in the smooth muscle α -actin null mouse. *FASEB J*. 2000; 14:2213–2220. [PubMed: 11053242]
- Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Bio*. 2004; 12:1567–1572.

Figure 1. SMA-mCherry construct and characterization

(a) Diagram of BAC clone RP23-132C11 demonstrating the location of sequences substituted for SMA exon 2 creating the SMA-mCherry vector is shown. Exon 2 of the SMA locus was replaced with a myristoylated mCherry cassette at the AUG translational initiation codon to express membrane localized mCherry. (b) Standard curve to calculate copy number of SMA-mCherry lines. (c) Copy number of SMA-mCherry lines. Transgenic mice contained between 1 and 3 copies of the transgene.

Figure 2. Expression of SMA-mCherry in the developing mouse embryo

(a-d) Whole mount reporter expression in E7.5-10.5 embryos. (e,h,k,n,q,t,w,z,cc) Reporter expression (red) (f,i,l,o,r,u,x,aa,dd) SMA antibody staining (green) (g,j,m,p,s,v,y,bb,ee) and merged images of SMA-mCherry immunocytochemistry. Reporter expression in the developing heart and aorta at E10.5 (e-g) yolk sac (h-j) and somites (k-m) recapitulates endogenous SMA expression. Reporter expression in the E13.5 heart (n-p) aorta and esophagus (q-s) stomach and intestine smooth muscle (t-v) recapitulate endogenous SMA expression. Reporter expression in the E18.5 (w-y) heart (z-bb) lung (cc-ee) esophagus also recapitulates endogenous SMA expression. Scale bar (e-g, k-v) 100um (h-j, w-ee) scale bar 50um. A, Aorta; E, Esophagus; S, Stomach; I, Intestine.

Figure 3. SMA-mCherry expression in adult organs

(a-d) Heart (e-h) mesenteric vessels (i-l) stomach (m-p) lung (q-t) kidney (u-x) ileum of the intestine (y-bb) mammary gland (cc-ff) femoral artery. Expression was observed in (c-d, g-h, k-l, o-p, s-t, w-x, aa-bb, ee-ff) SMA-mCherry but not (a-b, e-f, i-j, m-n, q-r, u-v, y-z, cc-dd) WT controls. WT images for fluorescence were obtained with the same light intensity and exposure time as SMA-mCherry.

Figure 4. Isolation of SMA positive cells by flow cytometry

FACS profiles of (a) WT and (b) SMA-mCherry E15.5 yolk sac cells. Cultured cells expressed mCherry (c, red) and immunostained positive for SMA (c, green).

Figure 5. Live imaging of the beating embryonic heart at E9.5

The image series is acquired from a cross of the SMA-mCherry line to the Tg(ϵ -globin::GFP) line labeling embryonic erythrocytes at 16 frames per second (fps) at the depth of 100 μ m. (a-c) Representative frames from the image series showing different phases of the cardiac cycle. (d) Relative fluorescence intensity of the mCherry (red) and the GFP (green) as a function of time in the region marked by a circle in the panel (d).