Published in final edited form as:

Genesis. 2015 March ; 53(0): 294–298. doi:10.1002/dvg.22849.

Derivation of Chondrocyte and Osteoblast Reporter Mouse Embryonic Stem Cell Lines

Yu Fu and Peter Maye*

Department of Reconstructive Sciences, School of Dental Medicine, University of Connecticut Health Center

Abstract

With the establishment of methods that provide evidence for the generation of chondrocyte and osteoblast cell types from ESCs, there is a need for reagents that will enable their further characterization. Here we report on the derivation of chondrocyte and osteoblast reporter ESCs from previously generated and characterized transgenic mouse lines, *Collagen type 2 alpha 1(Col2a1)-ECFP*, *Bone Sialoprotein (BSP)-Topaz*, and *BSP-Topaz/Dentin Matrix Protein 1 (DMP1)-Cherry* dual reporter mice. *Col2a1-ECFP* is highly expressed in chondrocytes, while *BSP-Topaz* and *DMP1-Cherry* are highly expressed in osteoblasts and osteocytes, respectively. These new skeletal reporter mouse ESC lines will serve as valuable reagents to investigate the functionality of ESC derived chondrocyte and osteoblast cell types.

Keywords

bone; cartilage; GFP; fluorescent reporter; skeletal

Results and Discussion

Within the field of skeletal biology, there is a great interest to use embryonic stem cells (ESCs) to study the molecular basis of different skeletal diseases and as a source from which to derive progenitor cells that could be used in applications of tissue regeneration. Several groups have reported different methodologies to generate chondrocytes and osteoblasts from ESCs (Cao et al., 2005, Ahn et al., 2006, Tong et al., 2007, Tanaka et al., 2009, Craft et al., 2013, Zhao et al., 2014). However, the efficiency by which different protocols work remains unclear. Additionally, the characterization of ESC derived skeletal cell types particularly as it pertains to their *in vivo* performance in models of tissue repair has been limited.

The genetic engineering of ESC lines with fluorescent protein reporter genes can be a valuable tool to assist in research efforts. When under the control of a tissue specific promoter, reporter expression provides a functional readout that can be monitored in living

*Corresponding Author: Peter Maye, Department of Reconstructive Sciences, MC3705, L7007, School of Dental Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, Phone: 860-679-7347, Fax: 860-679-2910 pmaye@neuron.uhc.edu.

Website: <http://regenerativemedicine.uhc.edu/faculty/bios/maye.html>

Disclaimers: None

cells without sacrificing the culture. This is quite advantageous for differentiation studies where changes in reporter expression can provide a rapid way to screen small molecules and growth factors or to determine optimal dosages or test different combinations of factors. Fluorescent reporter expression also connects gene expression to specific cell types, thus providing a quantitative determination of cell yields in cell types that would otherwise be difficult to identify. With this in mind, fluorescent reporter expression not only enables the isolation of specific cell types by FACS, but can also be leveraged to discover cell surface markers that could then be used to enrich for the same cell population in non-transgenic ESC lines. Finally, fluorescent reporters also provide a way to distinguish donor cells from host cells in transplantation studies and demonstrate their continued functionality in an *in vivo* environment.

For mouse ESCs, there are two possible ways to generate transgenic reporter lines. One way is to take already established ESC lines and introduce reporter gene DNA constructs into them by transfection or nucleofection. The other way is to derive ESC lines from existing transgenic mouse lines containing reporter genes. In this latter approach, there is the added benefit of having confidence in the fidelity of reporter gene expression based upon its *in vivo* gene expression. Past work by us and others have led to the generation and characterization of different skeletal reporter mice including *Collagen type 2 alpha 1 (Col2a1)*-ECFP, *Bone Sialoprotein (BSP)*-Topaz, and *BSP*-Topaz/*Dentin Matrix Protein 1 (DMP1)* Cherry dual reporter mice (Maye et al., 2011, Maye et al., 2009, Strecker et al., 2012, Chokalingam et al., 2009). The *Col2a1*-ECFP mouse line was engineered with a defined promoter/enhancer transgene, while *BSP*-Topaz and *BSP*-Topaz/*DMP1*-Cherry mice were engineered with BAC transgenes (Fig.1). Previous characterization of reporter expression from these animal models has demonstrated their selective expression in different skeletal cell types, with *Col2a1*-ECFP being highly expressed in chondrocytes, whereas *BSP*-Topaz and *DMP1*-Cherry being highly expressed in osteoblasts and osteocytes, respectively. Therefore, we have utilized these mouse lines to derive skeletal reporter mouse ESC lines.

Historically, only 129 substrains of mice gave rise to germline competent ESC lines. Therefore, *Col2a1*-ECFP and *BSP*-Topaz mice CD1 mice were bred into the 129SvJ background for six generations. Afterwards *Col2a1*-ECFP and *BSP*-Topaz mice were intercrossed and ESC lines were derived from these intercrosses. In total, twenty-nine ESC lines were derived from these crosses and genotyping for reporter genes identified single reporter *Col2a1*-ECFP and *BSP*-Topaz lines in addition to *Col2a1*-ECFP/*BSP*-Topaz dual reporter ESC lines. Three individual single reporter *Col2a1*-ECFP and *BSP*-Topaz ESC lines were expanded and stored away. Four dual reporter *Col2a1*-ECFP/*BSP*-Topaz ESC reporter lines were not only expanded and cryopreserved, but also examined for their genetic stability and pluripotency via chimeric animal studies (Fig.2). All four dual reporter *Col2a1*-ECFP (blue) /*BSP*-Topaz (green) ESC lines efficiently contributed to animal chimerism with clone 25 showing less contribution than clones 7, 18, and 28 (Fig.2).

Breakthroughs in identifying the mechanisms that control the “ground-state” of mouse ESCs have made the derivation of ESCs from essentially any mouse strain possible (Ying et al., 2008, Nichols et al., 2009, Silva et al., 2009, Wray et al., 2010, Buehr et al., 2008). Therefore, we used the 2i+ LIF strategy (Buehr et al., 2008) and derived new ESC lines

from *BSP*-Topaz/*DMP1*-Cherry dual reporter mice, which were maintained in a CD1 background. Utilizing this strategy, several ESC lines were generated and two were expanded and cryopreserved for future studies. To provide evidence for the functionality of these ESC lines, teratoma studies were carried out where 1×10^6 cells were injected into the thigh muscle of four different mice. Forty days later, the four teratomas were harvested and viewed under a fluorescent dissecting microscope for reporter expression. Given the diverse range in cell types that exist within teratomas, the vast majority of cells displayed no reporter expression. However, a few small regions did contain reporter expression and these regions were dissected out and processed for histological examination. In tissue section, *BSP* (Fig.3A, green) and *DMP1* (Fig.3B, red) reporter expression were seen in cells that resembled osteoblasts and osteocytes, respectively. The merged image (Fig.3C) and regions of interest (Fig.3A'-D') show how reporter expression was expressed in cells lining and embedded in bone tissue identified by von Kossa staining (Fig.3D, brown).

Few, if any, chondrocyte or osteoblast reporter ESC lines currently exist that would enable a better understanding of how to direct the differentiation of ESCs into different skeletal cell types. In this study, we reported on the derivation of 12 new reporter ESC lines (Table 1) containing single or dual reporter genes for *Col2a1*, *BSP*, and *DMP1*. Because these new ESC lines have been derived from previously characterized transgenic mouse lines, there should be no concerns with the fidelity of reporter gene accuracy and the mouse model of origin may provide a valuable reference for research studies. Additionally, embryonic chimeras and teratoma formation assays were used to validate the skeletal specific reporter expression of these new ESC lines. These newly generated reporter lines will be made available to the scientific community with the hope that they will assist in the development and functional validation of chondrocytes and osteoblasts generated from ESCs.

Materials & Methods

Animals

Col2a1-ECFP mice were generously provided by Dr. Wenstrup (currently at Myriad Genetics) (Chokalingam et al., 2009). *BSP*-Topaz and *BSP*-Topaz/*DMP1*-Cherry reporter mice were previously generated by us (Maye et al., 2009) and are available at Jackson Laboratories and the MMRRC, respectively (*BSP*-Topaz: Stock Number: 017472) (*BSP*-Topaz/*DMP1*-Cherry: Stock Number: 031695-UNC). CD1 and NIH-III mice were obtained from Charles River. 129SvJ mice were obtained from Jackson Laboratories. *Col2a1*-ECFP and *BSP*-Topaz reporter mice were bred into the 129SvJ background for six generations followed by intercrossing to generate single and dual reporter ESCs lines. *BSP*-Topaz/*DMP1*-Cherry reporter mice were maintained on a CD1 background. Generation of chimeric mice was carried out by the University of Connecticut Health Center Gene Targeting and Transgenic Facility. All mice were maintained in a pathogen free barrier facility and all experiments were carried out in a humane manner after receiving approval from our institutional animal care committee (University of Connecticut Health Center).

Timed Pregnancies and Blastocyst Harvest

For timed pregnancies, six week old female mice were injected I.P. with 5.0 IU of pregnant mares serum followed 48 hours later with 5.0 IU of human chorionic gonadotropin. Immediately after the second injection, females were placed into the male's cage for breeding. Blastocysts were harvested as previously described (Nagy, 2003). Blastocysts were collected and transferred individually to wells of a 24-well feeder plate. Blastocysts derived from *Col2a1*-ECFP and *BSP*-Topaz intercrosses were grown in standard ESC media containing LIF. Blastocysts derived from *BSP*-Topaz/*DMP1*-Cherry dual reporter mice were grown in 2i medium plus LIF as previously described (Nichols et al., 2009). Blastocysts were allowed to attach and grow for 2 days. Thereafter, medium was changed daily for the next 6-7 days to allow for expansion of the inner cell mass. Individual blastocysts were picked, dropped into a small volume of 0.25% trypsin, incubated at 37°C and 5% CO₂ for 3-5 minutes and broken up by pipetting up and down. Medium was then added and cells were transferred to one well of a 24-well feeder plate.

Cell Culture

ESC lines derived from *Col2a1*-ECFP and *BSP*-Topaz intercrosses were maintained on a feeder layer of irradiated mouse embryonic fibroblasts and grown in high glucose DMEM (Life Technologies) containing 4mM glutamine, 15% fetal calf serum (lot selected), 100μM non-essential amino acids, 1mM sodium pyruvate, 50μg/ml penicillin, 50μg/ml streptomycin, 10⁻⁴M β-mercaptoethanol, and 1000U/ml LIF. ESC lines derived from *BSP*-Topaz/*DMP1*-Cherry mice were grown on a feeder layer of irradiated mouse embryonic fibroblasts in a 1:1 mixture of DMEM/F12 and Neurobasal medium containing N2, B27, 0.05% BSA, 50μg/ml penicillin, 50μg/ml streptomycin, 1.5×10⁻⁴ M monothioglycerol, 1μM PD0325901, 3μM CHIR99021, and 1000U/ml LIF.

Genotyping of Skeletal Reporter ESC Lines

To determine which transgenes were present in different ESC lines genotyping was carried out for individual reporter genes. For this genomic DNA was prepared from newly derived ESC lines and PCR was carried using the following primer sets: *Col2a1* reporter (sense) 5'-GGGAGCATATAACTGGAGCCTCTGAAG-3', *CT* Genotype (antisense) 5'-GTTTACGTCGCCGTCCAGCTCGACCAGGAT-3'; *BSP* reporter (sense) 5'-GTCTGATACCTCCGAAGAGCTCAC-3', and *CT* Genotype, *DMP1* Reporter (sense) 5'-GTTAGGTTGCTGTGTAATACTGGC-3', and Cherry Genotype (antisense) 5'-GCACCTTGAAGCGCATGAACTCCTTGATGA-3'.

Imaging

Whole mount images of chimeric embryonic animals were taken using a Zeiss SteREO Lumar V.12 fluorescent microscope using ECFP (ET436/20x, ET480/40m) and EYFP (ET500/20 Ex, ET535/30 Em) filter sets (Chroma Technologies) and photographed with an Axiocam MRm digital camera (Zeiss) and Axiovision software. Terotoma tissue sections were imaged on a Zeiss Observer Z.1 microscope using Cherry (HQ577/20 Ex, HQ640/40 Em, Q595lp beam splitter) and EYFP (HQ500/20 Ex; HQ 535/30 Em, Q515lp beam splitter) filter sets (Chroma Technologies). The same tissue sections were then stained with Mayer's

hematoxylin (Poly Scientific) and photographed using an Axiocam MRc digital camera (Zeiss).

Teratoma Studies

Teratomas were created by injecting 1×10^6 ESCs per mouse into the thigh muscle of four male NIH-III immunodeficient mice. Forty days later injected mice were sacrificed and the teratoma was dissected out and viewed under a Zeiss SteREO Lumar for fluorescent protein reporter expression. Reporter expressing regions were cut out of the teratoma, fixed in 10% formalin, frozen embedded, and sectioned for imaging reporter expression. Tissue sections were then stained for bone mineral by the von Kossa method. In brief, tissue sections were incubated with 5% silver nitrate solution while crosslinking for 2 cycles at 1200 μ joules $\times$ 100 in a UV Stratalinker (Stratagene, La Jolla, CA). Mineralized nodules were seen as dark brown or black spots.

Acknowledgements

We would like to thank Dr. Wenstrup for providing the *Col2a1*-ECFP mice and Xiaonan Xin for assisting in teratoma formation studies. We also would like to thank Chris Stoddard from the University of Connecticut Gene Targeting and Transgenic Facility for assisting in the derivation of embryonic stem cell lines and chimeric animal studies. This work was supported by a grant from the NIAMS 5R21AR56391-2.

References

- AHN SE, KIM S, PARK KH, MOON SH, LEE HJ, KIM GJ, LEE YJ, PARK KH, CHA KY, CHUNG HM. Primary bone-derived cells induce osteogenic differentiation without exogenous factors in human embryonic stem cells. *Biochem Biophys Res Commun*. 2006; 340:403–8. [PubMed: 16389066]
- BUEHR M, MEEK S, BLAIR K, YANG J, URE J, SILVA J, MCLAY R, HALL J, YING QL, SMITH A. Capture of authentic embryonic stem cells from rat blastocysts. *Cell*. 2008; 135:1287–98. [PubMed: 19109897]
- CAO T, HENG BC, YE CP, LIU H, TOH WS, ROBSON P, LI P, HONG YH, STANTON LW. Osteogenic differentiation within intact human embryoid bodies result in a marked increase in osteocalcin secretion after 12 days of in vitro culture, and formation of morphologically distinct nodule-like structures. *Tissue Cell*. 2005; 37:325–34. [PubMed: 15979113]
- CHOKALINGAM K, HUNTER S, GOOCH C, FREDE C, FLORER J, WENSTRUP R, BUTLER D. Three-dimensional in vitro effects of compression and time in culture on aggregate modulus and on gene expression and protein content of collagen type II in murine chondrocytes. *Tissue Eng Part A*. 2009; 15:2807–16. [PubMed: 19231914]
- CRAFT AM, AHMED N, ROCKEL JS, BAHT GS, ALMAN BA, KANDEL RA, GRIGORIADIS AE, KELLER GM. Specification of chondrocytes and cartilage tissues from embryonic stem cells. *Development*. 2013; 140:2597–610. [PubMed: 23715552]
- MAYE P, FU Y, BUTLER DL, CHOKALINGAM K, LIU Y, FLORET J, STOVER ML, WENSTRUP R, JIANG X, GOOCH C, ROWE D. Generation and characterization of Col10a1-mcherry reporter mice. *Genesis*. 2011; 49:410–8. [PubMed: 21328521]
- 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 Biotechnol*. 2009; 9:20. [PubMed: 19284652]
- NAGY, A. Manipulating the mouse embryo : a laboratory manual. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 2003.
- NICHOLS J, SILVA J, ROODE M, SMITH A. Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development*. 2009; 136:3215–22. [PubMed: 19710168]

- SILVA J, NICHOLS J, THEUNISSEN TW, GUO G, VAN OOSTEN AL, BARRANDON O, WRAY J, YAMANAKA S, CHAMBERS I, SMITH A. Nanog is the gateway to the pluripotent ground state. *Cell*. 2009; 138:722–37. [PubMed: 19703398]
- STRECKER S, FU Y, LIU Y, MAYE P. Generation and characterization of Osterix-Cherry reporter mice. *Genesis*. 2012 Epub, ahead of publication.
- TANAKA M, JOKUBAITIS V, WOOD C, WANG Y, BROUARD N, PERA M, HEARN M, SIMMONS P, NAKAYAMA N. BMP inhibition stimulates WNT-dependent generation of chondrogenic mesoderm from embryonic stem cells. *Stem Cell Res*. 2009; 3:126–41. [PubMed: 19700382]
- TONG W, BROWN SE, KREBSBACH PH. Human Embryonic Stem Cells Undergo Osteogenic Differentiation in Human Bone Marrow Stromal Cell Microenvironments. *J Stem Cells*. 2007; 2:139–147. [PubMed: 20671800]
- WRAY J, KALKAN T, SMITH AG. The ground state of pluripotency. *Biochem Soc Trans*. 2010; 38:1027–32. [PubMed: 20658998]
- YING QL, WRAY J, NICHOLS J, BATLLE-MORERA L, DOBLE B, WOODGETT J, COHEN P, SMITH A. The ground state of embryonic stem cell self-renewal. *Nature*. 2008; 453:519–23. [PubMed: 18497825]
- ZHAO J, LI S, TRILOK S, TANAKA M, JOKUBAITIS-JAMESON V, WANG B, NIWA H, NAKAYAMA N. Small molecule-directed specification of sclerotome-like chondroprogenitors and induction of a somitic chondrogenesis program from embryonic stem cells. *Development*. 2014; 141:3848–58. [PubMed: 25294938]

Figure 1.

Transgene Schematics Detailing the Design of *Col2a1*, *BSP*, and *DMP1* Reporter Genes. The *Col2a1*-ECFP transgene regulates ECFP expression through a 1034bp promoter element (chromosome 15: 98004568 to 98005602; GRCm38.p3 C57BL/6J) and a 641bp enhancer element (chromosome 15: 98001965 to 98002605; GRCm38.p3 C57BL/6J). *BSP* and *BSP/DMP1* reporter genes were created from a ~145KB piece of DNA containing both genes (Chromosome 5:104180109 to 104326758; GRCm38.p3 C57BL/6J). This piece of DNA is 26,635bp upstream of the *DMP1* translational start site and 15,596bp downstream of the *BSP* stop codon. Fluorescent proteins were introduced into this large DNA fragment using bacterial recombination approaches as previously described (Maye et al., 2009).

Figure 2.

Col2a1-ECFP/*BSP*-Topaz dual reporter ESCs contribute to animal chimerism. Whole animal (**A-D**) and whole skull (**E-G**) imaging of *Col2a1*-ECFP (blue) and *BSP*-Topaz (green) reporter expression derived from four new dual reporter ESC clones (**A,E**) clone 7, (**B**) clone 18, (**C,F**) Clone 25, and (**D,G**) clone 28.

Figure 3.

BSP-Topaz/DMPI-Cherry reporter expression in teratoma generated bone tissue. Imaging of *BSP-Topaz* (A, green) and *DMPI-Cherry* (B, red) reporter expression lining bone tissue (D) stained by von Kossa (brown) and hematoxylin (blue). Merged image (C) and regions of interest (A'-D') show how reporter expression is highly associated with the surface of bone mineral and present in cells embedded in bone mineral.

Table 1

Reporter Genotype	Number of Lines	Mouse Strain
<i>Col2a1-ECFP</i>	3	129SvJ/CD1
<i>SSP-Topaz</i>	3	129SvJ/CD1
<i>Col2a1-ECFP BSP-Topaz</i>	4	129SvJ/CD1
<i>BSP-Topaz DMP1-Cherry</i>	2	CD-1