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Dynamic Expression of a LEF-EGFP WNT Reporter in Mouse Development and Cancer

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Summary

We have characterized a transgenic mouse line in which enhanced green fluorescent protein (EGFP) is expressed under the control of multimerized LEF-1 responsive elements. In embryos, EGFP was detected in known sites of Wnt activation, including the primitive streak, mesoderm, neural tube, somites, heart, limb buds, mammary placodes, and whisker follicles. In vitro cultured transgenic embryonic fibroblasts upregulated EGFP expression in response to activation of Wnt signaling by GSK3 β inhibition. Mammary tumor cell lines derived from female LEF-EGFP transgenic mice treated with the carcinogen 7, 12-dimethylbenz[a]anthracene (DMBA) also express EGFP. Thus, this transgenic line is useful for ex vivo and in vitro studies of Wnt signaling in development and cancer.

Keywords

Wnt reporter; EGFP; embryo; β -catenin; transgenic mouse; breast cancer

INTRODUCTION

The Wnt signaling pathway was originally identified in *Drosophila melanogaster* as an important signaling pathway in wing development (Sharma and Chopra, 1976; Siegfried *et al.*, 1994). Since then, Wnt signaling has been found to play a key role in cell fate regulation, organogenesis, and other fundamental developmental processes in many animal species. Wnts are a family of secreted glycoprotein ligands rich in cysteines that can signal through several pathways (Barker, 2008). Activation of the canonical Wnt/ β -catenin pathway results in the stabilization of β -catenin, a transcriptional coactivator (Gordon and Nusse, 2006; Huang and He, 2008). Stabilization of β -catenin is mediated by its release from a “destruction” complex composed of casein kinase 1 (CK1), glycogen synthase kinase 3 β (GSK3 β), axin, and adenomatous polyposis coli (APC), as well as further stabilization by CK2 phosphorylation (Dominguez *et al.*, 2004; Song *et al.*, 2000, 2003; Wu *et al.*, 2009). Stabilized β -catenin translocates into the nucleus, where it associates with DNA-binding factors of the lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) family. β -catenin/TCF complexes promote transactivation of developmental genes such as *siamois*

(Brannon *et al.*, 1997; Carnac *et al.*, 1996) and *brachyury* (Arnold *et al.*, 2000; Yamaguchi *et al.*, 1999).

Beginning with the first reports of mammalian Wnt-1 inducing tumors in mice (Nusse and Varmus, 1982; Rijsewijk *et al.*, 1987), research has shown that dysregulated canonical Wnt signaling plays a major role in tumorigenesis (Chen *et al.*, 2008; Matushansky *et al.*, 2008). Recent data suggest that Wnt signaling could be mediating some of the effects of carcinogen exposure in breast cancer, as elevated levels of β -catenin and CK2 α are found in mouse mammary tumors from DMBA fed rats and mice (Landesman-Bollag *et al.*, 2001; Currier *et al.*, 2005). In addition, DMBA-derived tumors display increased levels of *cyclin D1* (Currier *et al.*, 2005), a Wnt/ β -catenin target gene that is required for intestinal tumor formation in *Apc*^{Min} mice (Ilyas, 2005). However, studies in mice suggest that cyclin D1 may not be required for breast tumor formation induced by Wnt signaling (Yu *et al.*, 2001).

The lack of tissue-specific target genes to analyze endogenous activation of canonical Wnt signaling has prompted the development of Wnt reporters in different species (recently reviewed in Barolo, 2006) to determine if canonical Wnt signaling is involved in a particular function in cells or in vivo. In mice, several transgenic Wnt reporters with different promoters and numbers of TCF/LEF binding sites have been created (Table 1), either driving the expression of *LacZ*, the gene encoding β -galactosidase (DasGupta and Fuchs, 1999: p 31; Lustig *et al.*, 2002: p 86; Maretto *et al.*, 2003: p 9; Mohamed *et al.*, 2004: p 35; Nakaya *et al.*, 2005: p 68; Moriyama *et al.*, 2007: p 10), or driving the expression of *EGFP* (Jho *et al.*, 2002; Moriyama *et al.*, 2007). Here we describe a LEF-EGFP reporter transgenic mouse that utilizes seven multimerized LEF-1 binding sites to drive expression of *EGFP*. The *EGFP* reporter allows us to directly visualize activation of the transgene during early embryonic development by fluorescence microscopy. In addition, this model allows us to test activation of β -catenin/TCF signaling in tumors derived from DMBA-fed mice and in vitro in cell lines analyzed by cell sorting.

RESULTS AND DISCUSSION

Generation of LEF-EGFP Reporter Mice

To identify tissues and cells in development and cancer in which β -catenin/TCF signaling is activated, we generated transgenic mice expressing EGFP under control of seven copies of a LEF-1-binding site derived from the pLEF7-fos-luciferase reporter construct (Hsu *et al.*, 1998; Roth *et al.*, 2004) (Fig. 1a). This reporter construct is a faithful Wnt-responsive reporter in vitro (Hsu *et al.*, 1998; Sussman, 2002). The transgene construct contained a minimal c-fos promoter. Two LEF-EGFP transgenic FVB/N lines were created. The lines exhibited similar developmental expression patterns; thus, only one line was expanded for further detailed study. This line was bred to homozygosity and embryos were evaluated for EGFP expression at several stages of development. The presence of the transgene in embryos and adult mice was confirmed by PCR analysis of yolk sac or tail DNA, respectively. Embryos that were positive for the LEF-EGFP transgene DNA were confirmed to express EGFP protein by immunoblot (Fig. 1b).

EGFP Reporter Gene Expression in Embryonic Tissues

Wnts are essential for axis formation and organogenesis during embryonic development. To evaluate how faithfully the LEF-EGFP is expressed in tissues known to respond to Wnt/ β -catenin signaling during mouse embryonic development, we analyzed the pattern of expression of EGFP protein by fluorescence microscopy in isolated embryos at different stages, and we compared EGFP expression with the pattern of activated β -catenin and with

other Wnt reporters (Table 1). In parallel, embryos isolated from wild type (WT) FVB mice were used as controls for tissue autofluorescence.

We found that EGFP was expressed in the posterior region of LEF-EGFP transgenic embryos from E6 to E8 (Fig. 2 and Supporting Information Fig. S1; embryos were staged according to Downs and Davies, 1993). EGFP was detected in the extraembryonic visceral endoderm (Supporting Information Fig. S1) in prestreak embryos, where nonphosphorylated β -catenin has also been found between E5.5–E6 (Mohamed *et al.*, 2004). EGFP expression detected in the primitive streak and the mesoderm at streak and neural plate stages (Fig. 2 and Supporting Information Fig. S1) is similar to other Wnt-reporters at this stage (BAT-gal; Maretto *et al.*, 2003, Axin2-lacZ; ten Berge *et al.*, 2008, TCF/Lef-LacZ; Mohamed *et al.*, 2004), and more extended than β -galactosidase activity described in TOPGal embryos (Merrill *et al.*, 2004). EGFP fluorescence in the posterior of the streak embryo coincides with the location where non-phosphorylated β -catenin, a marker of active Wnt-signaling, is first detected in the embryo proper (Mohamed *et al.*, 2004). Mesodermal EGFP fluorescence in embryos at early and late head fold stages extended as anteriorly in expression as in BATlacZ (Nakaya *et al.*, 2005) and TCF/Lef-LacZ embryos (Mohamed *et al.*, 2004) and more than in TOPGal embryos (Merrill *et al.*, 2004). No fluorescence was detected in control embryos at these stages (Fig. 2 and Supporting Information Fig. S1, and data not shown).

Expression of EGFP changed dynamically during the early stages of somite formation (E8.0). At three somites, the strongest EGFP fluorescence was observed in the somites and presomitic mesoderm, followed by the lateral mesoderm; none was detected in control embryos (Fig. 3d). As embryos developed from three to seven somites (Supporting Information Fig. S2), faint EGFP fluorescence in the midbrain region intensified (Supporting Information Fig. S2) and EGFP fluorescence underneath the surface ectoderm close to the neural folds (Supporting Information Fig. S2a,b) expanded ventrally reaching the developing first pharyngeal arch (Supporting Information Fig. S2b,d,f,h). This pattern of EGFP expression is similar to that of migratory cranial neural crest cells reaching the first pharyngeal arch (Yoshida *et al.*, 2008) which begins around the four to five somite stage with neural crest cells migrating from the midbrain (Serbedzija *et al.*, 1992). EGFP fluorescence in E8 embryos was similar to the expression pattern in BATlacZ embryos (Dunty *et al.*, 2008), although the EGFP expression is stronger in the somites than in presomitic mesoderm. EGFP fluorescence is not detected in the node (BAT-gal; Maretto *et al.*, 2003, BATlacZ; Dunty *et al.*, 2008) and it is less extensive than other reporters; for example TCF/Lef-LacZ, which is positive in the anterior portion of the embryo earlier (Lin *et al.*, 2007; Mohamed *et al.*, 2004), and Ax2/d2EGFP, which is expressed in the dorsal neural tube (Jho *et al.*, 2002). A dynamic increase in EGFP fluorescence was also detected in the cardiac region as the heart tube forms (Supporting Information Fig. S2). EGFP fluorescence was detected in the heart tube and in a region dorsal and medial to the heart tube (Supporting Information Fig. S2). By comparison to staining in TCF/Lef-LacZ embryos (Lin *et al.*, 2007), this medial region may correspond to the splanchnic mesoderm, where a portion of the cardiac progenitors reside (Cai *et al.*, 2003).

At E9.0, the strongest EGFP fluorescence was observed in the somites, septum transversum, and unsegmented paraxial mesoderm (Fig. 4a). EGFP fluorescence was detected in the anterior part of the embryo consistent with the midbrain, the hindbrain, mesenchymal cells in the anterior part of the face, and the first and second pharyngeal arches. At 18 somites, staining around the ventral side of the optic vesicle was observed (Fig. 4 and Supporting Information Fig. S3). No fluorescence was detected in control embryos (Fig. 4a insert). The pattern of EGFP fluorescence was similar to the pattern of expression of other Wnt reporters such as BATgal and TOPgal (Brugmann *et al.*, 2007),

although less extended than that of TCF/Lef-LacZ (Lin *et al.*, 2007; Mohamed *et al.*, 2004). EGFP is detected throughout the somites and not exclusively in the dorsal portion as in (Maretto *et al.*, 2003). At E9.5, strong EGFP fluorescence was observed in the somites, forelimb bud and unsegmented presomitic mesoderm (see Fig. 4). No fluorescence was detected in control embryos (Fig. 4b, insert). Changes in EGFP fluorescence were seen as somite number increased. EGFP fluorescence was detected in the forebrain (particularly in the diencephalon), midbrain (more strongly in the ventral region), midbrain/hindbrain boundary, and in cells in the hindbrain region (Fig. 4 and Supporting Information Fig. S3). EGFP fluorescent cells were observed in the anterior part of the face, around the optic vesicle, in the dorsal part of the otic vesicle and in the first, second, and third pharyngeal arches (Supporting Information Fig. S3). Dynamic EGFP fluorescence was also detected in the cardiac region as the heart tube formed (Supporting Information Fig. S3). EGFP positive cells became visible in the primitive ventricle and outflow tract at 16 somites, in the presumptive atria at 18 somites, and in a region dorsal and medial to the heart tube consistent with the splanchnic mesoderm at 19 somites. At 21 somites, additional fluorescence appeared in the conus region and in a region consistent with the proepicardium and epicardial migratory cells.

At E10.5 and 11.5, EGFP was detected in the dorsal diencephalon, mesencephalon, mesencephalon/metencephalon boundary, and anterior metencephalon (Fig. 5 and not shown). Cranial expression also included the dorsal part of the otic and optic vesicles, the mandibular and maxillary components of the first pharyngeal arch and the second pharyngeal arch. EGFP was visible in the somites as well as the apical ectodermal ridge (AER) of both forelimb and hindlimb buds at E10.5 and 11.5 (Fig. 5 and not shown). No fluorescence was observed in tissues of control embryos (E10.5 shown in Fig. 5). At E10.5, otic vesicle, mandibular, maxillary, and AER expression is similar to that found in other TCF/ β -catenin reporters. Areas with divergent expression include the brain, where the EGFP expression pattern is less extended than that of some Wnt reporters (e.g., midbrain and hindbrain region in BAT-gal embryos; Brugmann *et al.*, 2007; Maretto *et al.*, 2003), and dorsal neural tube in Ax2/d2EGFP embryos (Jho *et al.*, 2002); the nasal folds, where EGFP is not detected compared to other reporters (TCF/Lef-LacZ mice; Mohamed *et al.*, 2004 and BAT-gal embryos; Maretto *et al.*, 2003) and the tail bud, where EGFP is less expressed than in other reporters including the ins-TOPEGFP (Moriyama *et al.*, 2007).

In E12.5 and E13.5 embryos, additional sites of EGFP expression were detected (Fig. 6 and Supporting Information Fig. S4), further demonstrating the dynamic expression of the transgene during development. EGFP was detected in the whisker follicles and in the mesencephalon, as observed in other reporters (DasGupta and Fuchs, 1999; Moriyama *et al.*, 2007); in the anterior and posterior of the pinna of the ear and in mesenchymal cells surrounding the forelimb bud at E13.5, similar to other reporters (Boras-Granic *et al.*, 2006; Chu *et al.*, 2004; Maretto *et al.*, 2003; Moriyama *et al.*, 2007); and in the five pairs of mammary placodes, as a ring of cells and in surrounding cells, as found by (Boras-Granic *et al.*, 2006; Chu *et al.*, 2004; Maretto *et al.*, 2003; Moriyama *et al.*, 2007). EGFP was also detected along the back of the embryo in a pattern we have not seen described elsewhere. EGFP expression in the AER of the fore and hindlimb buds, and in the tail bud (Supporting Information Fig. S4) is similar to other reporters. In these two tissues, some autofluorescent cells were observed in control embryos in regions overlapping the anterior and posterior portion of the limb buds and the ventral side of the tail bud (Supporting Information Fig. S4). In the coelomic cavity, EGFP positive cells were detected in two localized areas in the anterior part of the ovaries and in the dorsal side of the heart (Supporting Information Fig. S4). Around this stage, other Wnt reporters show expression in the atrioventricular endocardial cushions and the out-flow tract (Gitler *et al.*, 2003). A tubular-like structure in the mesonephros in both males and females appear to be autofluorescent (Supporting

Information Fig. S4). Hepatic fluorescence was limited to an unidentified subpopulation of cells present throughout the liver (Supporting Information Fig. S4), which was not reported in other Wnt reporter models. However, adult hepatocytes around the central veins are positive with the insTOP-reporters (Moriyama *et al.*, 2007). A limited cell population in the lungs is EGFP positive and this bronchial pattern is consistent with cells of the nervous system (Supporting Information Fig. S4); this expression pattern was different from other Wnt reporters (Maretto *et al.*, 2003).

The LEF-EGFP reporter is a faithful Wnt reporter as EGFP fluorescence coincides with regions in the embryo where nonphosphorylated β -catenin is detected, such as the primitive streak (Mohamed *et al.*, 2004). In addition, the expression pattern of LEF-EGFP closely overlaps that reported for TCF-1, TCF-4, and LEF-1 transcripts (Korinek *et al.*, 1998; Oosterwegel *et al.*, 1991). During organogenesis, the LEF-EGFP transgene is expressed in tissues shown to express Wnts such as the neural tube and heart (Joyner, 1996; Oosterwegel *et al.*, 1991; van Genderen *et al.*, 1994); developing somites (Ikeya and Takada, 1998); the AER (Hill *et al.*, 2006; Kengaku *et al.*, 1998; Oosterwegel *et al.*, 1991); neural crest cells (Bastidas *et al.*, 2004; Wu *et al.*, 2005; Yanfeng *et al.*, 2003); otic vesicle (Lovicu and McAvoy, 2005; Riccomagno *et al.*, 2005); mammary placodes; and whiskers (Boras-Granic *et al.*, 2006; Chu *et al.*, 2004; Korinek *et al.*, 1998).

The pattern of LEF-EGFP expression was similar to expression of other Wnt reporters although some divergences can be observed at E13.5. Differences in the extent of the expression of Wnt-reporters have been previously described (Brugmann *et al.*, 2007; Nakaya *et al.*, 2005), perhaps due to different staining times (Lin *et al.*, 2007; Mohamed *et al.*, 2004) and imaging techniques. Our model provides a well characterized additional reporter system that corroborates other observations. This model provides some particular advantages in terms of direct visualization of Wnt activation, including the high dynamic range of fluorescent detection, allowing visualization of both high and low expressing cells in a single embryo preparation, and the ability to isolate positive cells by FACS.

FACS Analysis of E13.5 and E9.5 Embryos

Consistent with the highly restricted EGFP expression pattern observed at E13.5 by fluorescence microscopy, the proportion of cells expressing EGFP as determined by flow cytometry comprised <0.3% of cells in single cell suspensions prepared from whole embryos (see Fig. 7), highlighting the extremely cell-specific activation of Wnt signaling at this stage. In contrast, the proportion of cells expressing EGFP as determined by flow cytometry in E9.5 embryos was ~ 37% (Supporting Information Fig. S5). This percentage corresponds with the broader extension of EGFP detected in fluorescence microscopy (Figure 4). Fluorescent microscopy or flow cytometry analysis allows for *ex vivo* studies, however, disadvantages of fluorescence may include attenuation of signal in larger organs or thicker tissue, or quenching by lipids. Autofluorescence of tissues can also be a disadvantage, in embryos and in adult tissues, for example in the mammary gland, where both transgenic and nontransgenic control mice exhibited autofluorescence during pregnancy, presumably due to lipid content of the glands (data not shown). These disadvantages can be overcome in part by examination of tissue sections by IHC or the use of *in situ* hybridization techniques, and by careful examination of nontransgenic controls.

EGFP Expression is Upregulated in Response to GSK-3 β Inhibition and Correlates With β -Catenin Expression in Cultured Mouse Embryonic Fibroblasts (MEFs)

To determine whether the LEF-EGFP reporter responds to signals that upregulate the Wnt pathway, we generated primary mouse embryonic fibroblast (MEF) cell lines for *in vitro* studies. One way to activate the Wnt pathway in cells is by inhibition of the kinase GSK-3 β

using pharmacological agents. We have previously used the drug SB-216763 and shown that it is an efficient activator of the Wnt pathway (Farago *et al.*, 2005). While DMSO-treated LEF-EGFP transgenic MEFs exhibited very little endogenous fluorescence (Fig. 8a), more than 80% of MEFs treated with SB-216763 were fluorescent. Control MEFs exhibited no fluorescence after treatment with either SB-216763 or DMSO (not shown). In parallel with the increased fluorescence, β -catenin and EGFP proteins assessed by immunoblot were increased with SB-216763 treatment compared to DMSO (Fig. 8b,c) corroborating activation of the Wnt pathway. Thus, cultured LEF-EGFP embryonic fibroblasts may be useful for understanding activation of Wnt signaling in vitro.

LEF-EGFP is Expressed in DMBA-Induced Mammary Tumor Cell Lines

We have previously reported that carcinogen-induced mammary tumors frequently have high levels of β -catenin and cyclin D1, suggesting that canonical Wnt signaling is activated in these tumors (Currier *et al.*, 2005). To corroborate this, we treated LEF-EGFP transgenic mice with DMBA as in (Currier *et al.*, 2005). Here, we were able to analyze fluorescence in mammary tumor cell lines derived from the transgenic mice. Two adenosquamous tumors were isolated and showed increased β -catenin and CK2 proteins by immunoblot (Fig. 9a) as in previous experiments in FVB/N mice (Currier *et al.*, 2005). Cell lines were established from these tumors, and designated EGFP-D1.1 and EGFP-D1.2. The fluorescence of the cells derived from these tumors was assayed by flow cytometry. A nontransgenic mammary tumor cell line generated after exposure to DMBA, designated 3983, was used to control for autofluorescence. Both of the transgenic lines exhibited significantly more fluorescence than the control cell lines, consistent with constitutive Wnt pathway activation (Fig. 9b,c). Thus, the LEF-EGFP mice provide further evidence that Wnt signaling is activated in carcinogen-induced mouse mammary tumors. Our experiments in epithelial cell lines show that β -catenin and cyclin D1 activation depends upon CK2 activity (Song *et al.*, 2000, 2003; Wu *et al.*, 2009). Thus, it is plausible that the elevated levels of β -catenin and cyclin D1 in carcinogen-induced tumors are also CK2-dependent. The EGFP-D1.1 and EGFP-D1.2 (Lou *et al.*, 2008) tumor lines together with conditional ablated CK2 mice will be useful for further studies of Wnt regulation in mammary tumorigenesis.

In summary, the LEF-EGFP Wnt reporter is a useful model for embryonic and tumorigenesis studies concerning Wnt signaling.

METHODS

Breeding and Genotyping of LEF-EGFP Transgenic Mice

The pLEF-EGFP transgene construct was generated by inserting a 400 bp Eco RI/Bam HI fragment containing seven multimerized wild-type LEF-1 binding sites and a minimal c-fos promoter (derived from a Xba I/Kpn I fragment of pLEF-fos-luciferase, generously provided by Dr. Grosschedl (University of California, San Francisco; Hsu *et al.*, 1998; Roth *et al.*, 2004) into the Eco RI/Bam HI sites of pEGFP-1 (Clontech). The LEF-EGFP transgene was prepared for injection by digesting pLEF-EGFP with Afl I/Xho I, isolating the 1.45-kb fragment by electrophoresis in a 1% low melting point agarose gel, and purifying the fragment using GENECLEAN II (Bio101) according to the manufacturers protocol. Transgenic mice were generated in the core facility at the University of Maryland; two FVB lines were bred. Genotyping was confirmed by PCR analysis by amplifying 659-bp fragment of the transgene EGFP sequence (sense 5'-ATG GTG AGC AAG GGC GAG GAG -3' and antisense 5'-GAC CAT GTG ATC GCG CTT CTC -3') was performed in an iCycler (Bio-Rad, Hercules, CA) with 35 cycles $\times$ 95°C for 40 s, 55°C for 50 s, and 72°C for 1 min 20 s.

Embryo Collection and Fluorescence Microscopy

LEF-EGFP mice were housed as described (Currier *et al.*, 2005). LEF-EGFP mice pairs were mated and females examined daily for vaginal plugs (presence of the plug was considered as embryonic day E0.5). Isolated embryos were placed in cold 1× PBS; presomitic embryos were staged according to (Downs and Davies, 1993) and somitic embryos according to the number of somites. The number of transgenic embryos examined included 3 at ~ 6.5, 19 at ~ E7.5, 11 at ~ E8.5, 24 at ~ E9.5, 36 at ~ E10.5, 8 at ~ E11.5, 7 at ~ E12.5, and 22 at ~ E13.5. For controls, we examined 6 at ~ E7.5, 7 at ~ E8.5, 14 at ~ E9.5, 24 at E10.5, 12 at ~ E11.5, 9 at ~ E12.5, 12 at ~ E13.5. Representative embryos at different stages of development were photographed using a fluorescent stereomicroscope (Olympus SZX16) fitted with a digital camera (QImaging RETRIGA-2000RV, Fast1394) and QCapture software (QCaptureSuite 2.95.0 2007). Exposure times for whole embryo photographs were 3 s at E7.5, 5 s at E8.5, 4 s at E9.5, 1.5 s at E10.5, and 13.6 s at E13.5. Photographs were all pseudo-colored green. Only magnified photos were adjusted by increasing brightness and contrast to enhance EGFP visibility. Some embryos were snap-frozen on dry-ice and stored at −80°C for further analysis.

Embryonic Fibroblast Cell Culture

E13.5 embryos were placed in sterile cold 1× PBS, head and visceral organs removed, and embryos mechanically minced with a sterile razor blade, digested by incubating in 0.25% trypsin (Mediatech, Herndon, VA) for 5 min at 37°C, then homogenized to create a single cell suspension. The suspension was taken up in DMEM (Mediatech), 12.5% fetal bovine serum (SeraCare Life Sciences, Milford, MA), 50 U ml^{−1} penicillin 50 mg ml^{−1} streptomycin (Mediatech), and 4 mM L-glutamine (Mediatech), and filtered through a 70-μm filter (B.D, Lexington, KY) and grown in the same media at 37°C.

DMBA Treatment and Tumor Cell Culture

Ten virgin female LEF-EGFP mice were each given 6 weekly 1.0-mg dose of DMBA in 0.2 ml of sesame oil by oral gavage, beginning at 5 weeks of age (Currier *et al.*, 2005; Trombino *et al.*, 2000). Mice were then followed until either tumors developed or the mice died. Mice bearing tumors >0.5 cm in diameter were euthanized; mammary tumors and grossly normal mammary glands were excised, and portions of the tissues were prepared for histology. To establish cell lines derived from the tumors, a portion of the tumors was isolated, fragmented with a sterile razor blade under sterile conditions and digested by incubating in 0.25% trypsin/EDTA for 5 min at 37°C. The digested tumors were then added to DMEM, 10% fetal bovine serum, 50 U ml^{−1} penicillin 50 mg ml^{−1} streptomycin, and 4 mM L-glutamine, and filtered through a 70-μm cell filter, and cells were grown in the same media at 37°C. Two tumor cell lines, “EGFP-D1.1” and “EGFP-D1.2” (derived from Tumor 1 and 2, respectively) were generated. The remaining tissue was frozen on dry ice and stored at −80°C for molecular and biochemical studies.

Cell Culture and Treatment of Cell Lines

Mouse embryonic fibroblast (MEF) cell lines were grown in DMEM, 12.5% fetal bovine serum, 50 U ml^{−1} penicillin, 50 mg ml^{−1} streptomycin, and 4 mM L-glutamine. EGFP-D1.1 cell line was grown in DMEM/F12 (Gibco Invitrogen, Carlsbad, CA), 5% horse serum (Gibco Invitrogen), 50 U ml^{−1} penicillin 50 mg ml^{−1} streptomycin, 5 mg insulin (Sigma-Aldrich, St. Louis, MO), 0.25 mg hydrocortisone (Sigma-Aldrich), and 0.01 mg EGF. EGFP-D1.2 cell line was grown in DMEM, 10% fetal bovine serum, 50 U ml^{−1} penicillin 50 mg ml^{−1} streptomycin, and 4 mM L-glutamine. To control for autofluorescence and to reduce the number of mice used, a preexisting cell line was used, rel-3983D (Shin *et al.*, 2006).

The GSK3 inhibitor, SB-216763 (Sigma-Aldrich) was dissolved in DMSO (Sigma-Aldrich) to a concentration of 10 mM, and stored at -20°C . MEFs, EGFP-D1.1, EGFP-D1.2, and rel-3983D cells were cultured on six-well plates for 24 h before SB-216763 treatment, and then cell media was supplemented with 10 mM SB-216763 or DMSO at 1:1,000 dilution to achieve a final drug concentration of 10 μM . Cells were treated for the indicated time, and photographed on a Nikon fluorescence microscope (Melville, NY) fitted with a digital camera (Hamamatsu Diagnostic Instruments, Sterling Heights, MI). The software used was OpenLab (Improvision, Waltham, MA).

Flow Cytometry

Cultured embryonic fibroblasts or mammary tumor cell lines were detached using either 0.25% trypsin EDTA solution (Mediatech) or Hyqtase (Hyclone, Logan, UT), respectively for 5 min at 37 or 20°C , respectively. Cells were then placed in complete media and centrifuged at 1,200 rpm for 5 min at 4°C . Supernatant was removed and cells were reconstituted in $1\times$ PBS, 2% fetal bovine serum, and incubated for 30 min in $20\text{ }\mu\text{g ml}^{-1}$ propidium iodide (PI, Sigma-Aldrich). Cells from digested embryos were washed in $1\times$ PBS, incubated for 30 min in $20\text{ }\mu\text{g ml}^{-1}$ propidium iodide in PBS (PI, Sigma-Aldrich), diluted with twice the volume of PBS and filtered through a $70\text{-}\mu\text{m}$ filter. Cells were analyzed on a Becton/Dickinson FACscan (BD Biosciences, Franklin Lakes, NJ). Live cells were identified by exclusion of propidium iodide and their EGFP signal intensity was examined. The software used was CellQuest Pro (BD Biosciences).

Immunoblot Analysis

Protein extracts prepared by homogenizing frozen embryos, cells, or tumors in lysis buffer containing a cocktail of protease inhibitors, gel electrophoresis, transfer, immunoblotting, band visualization, and quantitative analysis performed as previously described (Currier *et al.*, 2005). Primary antibodies were: anti- β -catenin (B.D.), anti- β -actin (Sigma-Aldrich), anti-GFP (Santa Cruz), and CK2 α (B.D.).

Statistics

P-values were obtained with the Student's *t*-test using the statistical analysis package in Excel.

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FIG. 1. LEF-EGFP reporter transgene. **(a)** Representation of the transgene construct with seven murine LEF-1 response elements inserted into the pEGFP-1 expression vector. **(b)** E12.5 embryo genotyping PCR for the LEF-EGFP transgene (top panel) and immunoblot analysis with a GFP antibody (middle panel) and β -actin, used as a loading control, (lower panel).

FIG. 2.

LEF-eGFP reporter transgene expression in presomitic embryos. Bright-field next to corresponding UV images. (a) Early streak (ES, E6.75–7.25) lateral view; (b) late streak (LS, E7.25–7.75), lateral view; and (c–e) E8 embryo: (c) anterior, (d) posterior and (e) lateral views. (f) Control wild-type embryos at early head fold (EHF) stage. A: anterior; P: posterior; al: allantois; fg: foregut pocket; nf: neural fold.

FIG. 3.

LEF-eGFP reporter gene expression in E8 embryos. Bright-field next to corresponding UV images. (a) 3-somite embryo, ventral view; (b,c) 5-somite embryo, ventral and lateral view; (d) control embryo, ventral view at 7-somites. A: anterior; P: posterior; al: allantois; fg: foregut; ht: heart tube; lm: lateral mesoderm; mg: midgut; n: node; nf: neural folds; ps: primitive streak; psm: presomitic mesoderm; s: somite.

FIG. 4.

LEF-EGFP reporter gene expression in E9.0 and E9.5 embryos. **(a)** 16-somite (E9.0) embryo in bright-field and UV, with (i) magnified head, (ii) anterior somites, and (iii) tail bud. **(b)** 22-somite (E9.5) embryo in bright-field and UV, with (i) magnified head, (ii) anterior somites, and (iii) tail bud. di: diencephalon; fb: forebrain; fl: forelimb bud; hb: hindbrain; ht: heart tube; mb: midbrain; otp: otic pit; otv: otic vesicle; ov: optic vesicle; p1: first pharyngeal arch; p2: second pharyngeal arch; p3: third pharyngeal arch; ps: primitive streak; psm: presomitic mesoderm; s: somite; st: septum transversum; tel: telencephalon.

FIG. 5.

LEF-EGFP reporter gene expression in E10.5 embryos. Bright-field next to corresponding UV images. (a) Lateral views of 34-somite embryos. Bright-field next to corresponding UV image. Magnified images of (b) the head, (c) the tail bud and (d) the fore and hindlimb. (e) Control 34-somite embryos. Bright-field next to corresponding UV image; di: diencephalon; fl: forelimb bud; hb: hindbrain; hl: hindlimb bud; mes: mesencephalon; otv: otic vesicle; ov: optic vesicle; p1: first pharyngeal arch; p2: second pharyngeal arch; s: somite; tel: telencephalon.

FIG. 6.

LEF-eGFP reporter gene expression in E13.5 embryos. Magnified images of (a) the snout and whisker primordia, (b) the head, (c) outer ear (d) the forelimb region: white arrowhead: mammary placodes, black arrowhead: mesenchyme surrounding forelimb bud. (e) Magnified image of the mesenchyme surrounding forelimb bud. (f) Magnified image of the mammary placodes. (g) Punctate staining along the back of the embryo. (h) Control E13.5 embryos, lateral view. Bright-field next to corresponding UV image. mes: mesencephalon; tel: telencephalon; wf: whisker follicles.

FIG. 7.

Flow cytometry analysis of cells from E13.5 LEF-EGFP embryo (left panel) and wild-type control (right panel). EGFP-positive cells appeared within the gate. In three transgenic embryos, $0.29\% \pm 0.04\%$ of cells were EGFP positive, while $<0.05\%$ of cells from two littermate wild-type controls were detected in the gated region.

FIG. 8.

SB-216763-induced EGFP expression in cultured transgenic MEFs. MEFs generated from E13.5 LEF-EGFP embryos were exposed to 10 μ M SB-216763 or DMSO vehicle control. (a) EGFP fluorescence is observed in SB-216763-treated cells but not DMSO-treated controls (left); corresponding brightfield images (right). (b) EGFP and β -catenin proteins are upregulated in SB-216763-treated MEFs by immunoblot; β -actin loading control is shown. (c) Relative amounts of induced EGFP (left) and β -catenin protein (right). Gray bars, DMSO-treated cells, Black bars, SB-216763-treated cells.

FIG. 9. Expression of EGFP in mammary tumor cell lines from LEF-EGFP transgenic mice. DMBA-induced mammary tumors in LEF-EGFP mice have elevated expression of canonical Wnt signaling components. **(a)** Whole cell extracts from DMBA-induced LEF-EGFP mammary tumors (Tumors 1 and 2) and normal LEF-EGFP mammary gland (Normal) were subjected to immunoblotting for CK2 α , β -catenin, and β -actin as loading control. **(b)** Flow cytometry analysis of cell lines EGFP-D1.1 (gray line) and EGFP-D1.2 (black line) established from tumors induced in LEF-EGFP transgenic mice by treatment with DMBA, compared to a non-EGFP-transgenic rel-3983D mouse mammary tumor cell

line (filled gray). (c) Average EGFP fluorescence of three replicates of non-EGFP-transgenic rel-3983D control cell line and EGP-D1.1 and 1.2. P values shown in histogram.

Table 1**Mouse Transgenic WNT Reporters**

Driving the expression of a <i>LacZ</i> reporter		
TOPGal	Three LEF/TCF consensus binding sites based on the pTOPFLASH reporter (Molenaar <i>et al.</i> , 1996) with a <i>c-fos</i> minimal promoter	(DasGupta and Fuchs, 1999)
Axin2 ^{LacZ}	A knock-in of the <i>LacZ</i> gene with a nuclear localization signal in the <i>Axin2</i> gene locus	(Lustig <i>et al.</i> , 2002)
BAT-gal	Seven TCF/LEF binding sites upstream of a 0.13-kb fragment containing the TATA box of <i>siamois</i>	(Maretto <i>et al.</i> , 2003)
TCF/LEF-lacZ	Six TCF/LEF response elements with an hsp68 minimal promoter	(Mohamed <i>et al.</i> , 2004)
BATlacZ	A TCF/LEF binding sequence upstream of a 0.13 kb <i>Xenopus siamois</i> minimal promoter; nuclear <i>LacZ</i>	(Nakaya <i>et al.</i> , 2005)
ins-TOPGAL	Six LEF/TCF consensus binding sites and a minimal thymidine kinase promoter derived from pTOPFLASH; nuclear <i>LacZ</i>	(Moriyama <i>et al.</i> , 2007)
Driving the expression of an <i>EGFP</i> reporter		
Ax2/d2EGFP	A 5.6-kb DNA fragment including the <i>Axin2</i> promoter, exon 1, and intron 1 sequences that contain several TCF/LEF binding sites; <i>d2EGFP</i>	(Jho <i>et al.</i> , 2002)
ins-TOPEGFP	Six LEF/TCF consensus binding sites and a minimal thymidine kinase promoter derived from pTOPFLASH	(Moriyama <i>et al.</i> , 2007)
LEF-EGFP	Seven multimerized LEF-1 binding sites with a <i>c-fos</i> minimal promoter	(current report)