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Characterization of WWOX Inactivation in Murine Mammary Gland Development

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The WW domain-containing oxidoreductase (WWOX) is commonly inactivated in multiple human cancers, including breast cancer. *Wwox* null mice die prematurely precluding adult tumor analysis. Nevertheless, aging *Wwox*-heterozygous mice at C3H genetic background develop higher incidence of mammary tumors. We recently generated a *Wwox* conditional knockout mouse in which loxp sites flank exon 1 in the *Wwox* allele and showed that total ablation of WWOX in these mice resembles that of conventional targeting of *Wwox*. Here, we report the characterization of WWOX ablation in mouse mammary gland using *MMTV-Cre* transgenic line. We demonstrated that WWOX ablation leads to impaired mammary ductal growth. Moreover, targeted deletion of WWOX is associated with increased levels of fibronectin, a component of the extracellular matrix. In addition, we showed that shRNA knockdown of WWOX in MCF10A breast epithelial cells dramatically increased fibronectin and is associated with enhanced cell survival and impaired growth in three-dimensional culture Matrigel assay. Taken together our results are consistent with a critical role for WWOX in normal breast development and tumorigenesis.

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The WW domain-containing oxidoreductase (WWOX) was identified as a potential tumor suppressor in breast cancer (Bednarek et al., 2000). Several lines of evidence support this fact: (1) loss or reduced expression of WWOX occurs in approximately two-thirds of breast cancers due to frequent loss of heterozgosity and/or hypermethylation of its regulatory element (Guler et al., 2005; Nunez et al., 2005; Aqeilan et al., 2007a). WWOX loss is associated with more aggressive breast cancers including basal and triple negative subtypes (Aqeilan et al., 2007a); (2) WWOX overexpression in WWOX-negative breast cancer cells attenuates cell growth in vitro and inhibits tumor growth in immunocompromised mice (Bednarek et al., 2001; Iliopoulos et al., 2007); (3) Aging *Wwox*-heterozygous C3H mice strain spontaneously develop higher incidence of mammary tumors (Abdeen et al., 2011); (4) at the molecular level WWOX inhibits the transactivation function of well known oncoproteins implicated in breast carcinogenesis including AP2γ (Aqeilan et al., 2004) and ErbB4 (Aqeilan et al., 2007a, 2005). These findings led to hypothesize that WWOX acts as a tumor suppressor in breast cancer. Since *Wwox*-knockout mice die at or before weaning age (Aqeilan et al., 2008, 2007b; Ludes-Meyers et al., 2009), detailed analysis of mammary development and tumorigenesis was not possible so far.

The WWOX protein encodes a 46-kDa that contains two WW domains and a short-chain dehydrogenase/reductase domain (SDR) (Bednarek et al., 2000; Ried et al., 2000). Through its first WW domain, WWOX binds and regulates localization, stability, and transactivation function of PPxY-containing proteins [reviewed in (Del Mare et al., 2009; Salah et al., 2010)]. The SDR domain was shown to bind non-PPxY proteins (Chang et al., 2007), though no substrates were identified.

To better explore the tumor suppressor function of WWOX in vivo, we recently set a strategy to generate a conditional knockout mouse model for the *Wwox* gene ($Wwox^{fl/fl}$) hoping this would enable careful adult tumor analysis. $Wwox^{fl/fl}$ mice are viable and fertile (Abdeen et al., 2012). Breeding these mice with a general deleter (*EIIa-Cre*) transgenic strain resulted in phenotypes resembling conventional *Wwox* knockout mice, that is, sever metabolic defect, bone growth defects and death by 3 weeks of age (Abdeen et al., 2012). These findings were also consistent with data obtained from (Ludes-Meyers et al., 2009).

To examine the possible role of WWOX in mammary development and adult mammary tumorigenesis, we generated mice with a targeted deletion of *Wwox* in the mammary gland epithelium ($Wwox^{MGE-/-}$). Analysis of mammary gland development in these mice revealed a transient defect in branching morphogenesis and ductal outgrowth. Monitoring these mice for more than a year did not associate with spontaneous mammary tumor formation. Nevertheless, mechanistic insight into mammary gland biology revealed impaired expression of fibronectin. Additionally, WWOX knockdown in immortalized mammary epithelial MCF10A cells was associated with increased fibronectin levels and enhanced survival and spheroid formation. These results suggest an important role of WWOX in mammary gland biology and tumorigenesis.

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Materials and Methods

Animals

Wwox$^{fl/fl}$ mice (Abdeen et al., 2012) were crossed with *MMTV-Cre* (obtained from JAX laboratory, Bar Harbor, ME; #003553). Genotyping of Cre was performed using the following primers: Forward, 5′-ATG TCC AAT TTA CTG ACC GTA CAC C-3′; 5′-Reverse, CGC CTG AAG ATA TAG AAG ATA ATC G-3′. All experiments involving mice were approved by the Hebrew University Institutional Animal Care and Use Committee.

Whole-mount mammary gland analysis

Excised mammary fat pad (MFP) were spread onto glass slides and fixed with Tellyesniczky's fixative overnight. MFPs were then soaked in three changes of 100% acetone each for 6 h, followed by 2 h-treatments with each 100%, 95%, and 70% ethanol, and stained with 0.2% carmine red overnight. After rinsing in water, MFPs were dehydrated sequentially in 50%, 70%, 95%, and 100% ethanol each for 2 h, cleared with xylene overnight and examined under a dissecting microscope. Mammary gland whole mounts were evaluated by extent of ductal outgrowth: maximal outgrowth beyond MFP lymph node measured in millimeters. A minimum of 3–5 mice per age-category and genotype were analyzed.

Immunohistochemistry

Tissues were fixed in 4% formalin. Paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in 10 mM sodium citrate buffer pH 6.0 (for fibronectin, ER, CK5, SMA, and CK18) or EDTA buffer pH 8.0 (for β-catenin, Ki67, and WWOX) using pressurized chamber for 2.5 min. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min. The sections were then incubated with blocking solution (CAS Block, Invitrogen, Grand Island, NY) for 30 min to reduce non-specific binding followed by incubation with the primary antibody: Polyclonal anti-WWOX antibody (Guler et al., 2005) [dilution of 1:15,000], or rabbit monoclonal anti CK5 antibody (ab52635, 1:350 from Abcam, Cambridge Science Park, Cambridge, UK), or mouse monoclonal anti smooth muscle actin (SMA) (MA1-37027, 1:600 from Thermo Scientific, Waltham, MA), or goat polyclonal anti-cytokeratine 18 (sc-31700, 1:300 from Santa Cruz, Santa Cruz, CA), or rabbit monoclonal anti Ki67 (RM-9106-S, 1:100 from Thermo Scientific), or rabbit polyclonal anti fibronectin (ab2413, 1:300 from Abcam), or mouse monoclonal anti β-catenin (610154, 1:200 from BD Transduction, San Jose, CA) in humidity chamber for overnight incubation. Slides were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse or anti-goat immunoglobulin antibody for 30 min. The enzymatic reaction was detected in a freshly prepared 3,3 diamminobenzidine tetrahydrochloride using DAKO Liquid DAB Substrate-Chromogen (Carpinteria, CA) Solution for several minutes at room temperature. The sections were then counterstained with hematoxylin.

Isolation of primary mouse epithelial cells (MECs)

Mammary glands were isolated and minced from the indicated mice. For each gram tissue, 5 ml digestion mix [DMEM media, 5% fetal bovine serum, 1% penstrep, 1:100 collagenase A (from stock 1.5 mg/ml) and 1:1,000 DNase-I (from 10 mg/ml stock)] was added and left for 1.5 h at 37°C under moderate shaking (50 rpm). MECs were separated from fat by differential centrifugation (three times) at 700*g* for 30″. MECs were washed twice with PBS.

RNA extraction and RT-PCR

Total RNA was isolated using Tri reagent (Sigma, St Louis, MO). RNA (1 μg) was reversed transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was done using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The real-time PCR was performed using *Ubc* primers: Forward 5′-CAG CCG TAT ATC TTC CCA GAC T-3′, Reverse 5′-CTC AGA GGG ATG CCA GTA ATC TA-3′ and *Wwox* primers: Forward 5′-TCA CAC TGA GGA GAA GAC CCA-3′, Reverse 5′-CCT ATT CCC GAA TTT GCT CCA-3′.

Immunoblotting

Whole cell lysates were prepared using lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors (1:100). Lysates were resolved on SDS/PAGE. Antibodies used were Rabbit polyclonal anti-GST-WWOX (1:10,000) (Guler et al., 2005) and mouse monoclonal anti-GAPDH (1:10,000) (Calbiochem, San Diego, CA) and rabbit polyclonal anti-fibronectin (1:1,000; Sigma–Aldrich, St. Louis, MO; cat # F3648).

Cell culture assays

Immortal MCF10A cells were cultured as in (Debnath et al., 2003) For colony formation assay, MCF10A cells were plated at a density of 300 cells/well in a six wells plate in triplicate. After 1–2 weeks the cells were fixed with 70% Ethanol, stained with Giemsa and counted. The test was performed in triplicates. For three-dimensional (3D) culture assay, 3,000 cells were seeded on a solidified layer of growth factor reduced Matrigel measuring approximately 1–2 mm in thickness. The cells are grown in an assay medium containing 5 ng/ml EGF and 2% Matrigel. To test for cell survival, the growth medium was depleted of growth factors.

Statistical analysis

Results were expressed as mean ± SD or ± SEM. The Student's *t*-test was used to compare values of test and control samples. $P < 0.05$ indicated significant difference.

Results

Characterization of WWOX expression in the developing mammary gland

To characterize the role of WWOX in murine mammary gland development, we determined the WWOX protein expression levels during the different stages. Mammary gland epithelium from virgin (3, 5, and 8 weeks), pregnant (P18.5) and lactating females from control (*Wwox*$^{fl/fl}$) mice were isolated, paraffin embedded and immunostained with anti-WWOX antibodies (Fig. 1). At 3 weeks of age, very low levels of WWOX were detected. Of note, WWOX levels were induced in 5 and 8 weeks virgin mammary gland epithelium. WWOX levels continue to be high at pregnancy, although there was a tendency of decreased levels, while it reached peak levels in lactating glands (Fig. 1). These results suggest that WWOX might be important for proper mammary gland epithelium function, particularly at early stages of puberty (4 weeks).

Specific WWOX ablation in mammary gland epithelium (*Wwox*$^{MGE-/-}$) mice

To investigate the role of WWOX in pubertal mammary gland development, *Wwox*$^{fl/fl}$ mice were bred with an *MMTV-Cre* strain. Proper mice genotypes were next used to examine WWOX expression and its ablation. Histological sectioning of mammary glands and immunohistochemistry using anti-WWOX antibodies revealed absence of WWOX in *Wwox*$^{MGE-/-}$ mice as compared to *Wwox*$^{fl/fl}$ mice (Fig. 2A). Absent WWOX was correlated with expression of CRE as indicated in Figure 2B. This was further confirmed by quantitative real-time PCR of *Wwox* mRNA expression in P18.5 females (Fig. 2C).

WWOX knockout at puberty induces transient delay of ductal growth

To further learn about WWOX significance during mammary gland maturation, carmine red-stained mammary fat pad (MFP) from 3-, 5- and 12-week-old, virgin *Wwox*$^{fl/fl}$ and *Wwox*$^{MGE-/-}$ mice were analyzed. At 3 and 5 weeks of age, mammary glands

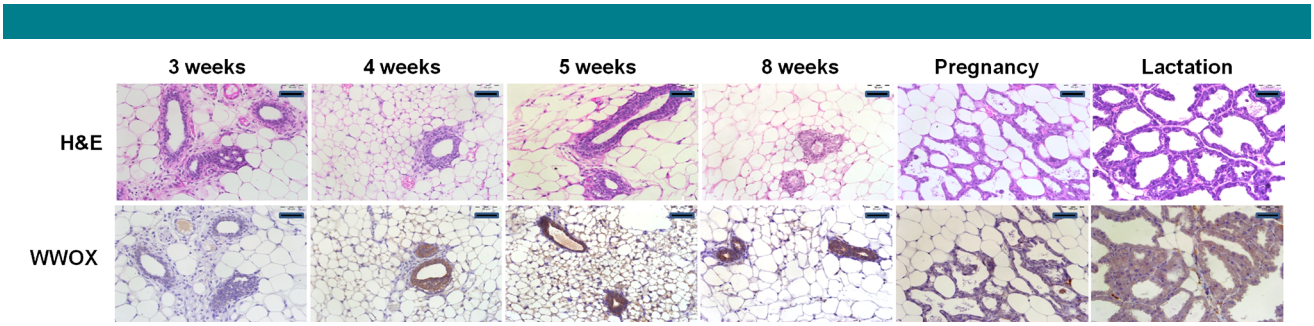

Fig. 1. WWOX expression during mammary gland development. H&E (upper) and immunohistochemical (IHC) staining using anti WWOX antibody (lower) were performed on mammary glands excised from wild type mice at different ages as indicated. Magnification bar represents 50 μm.

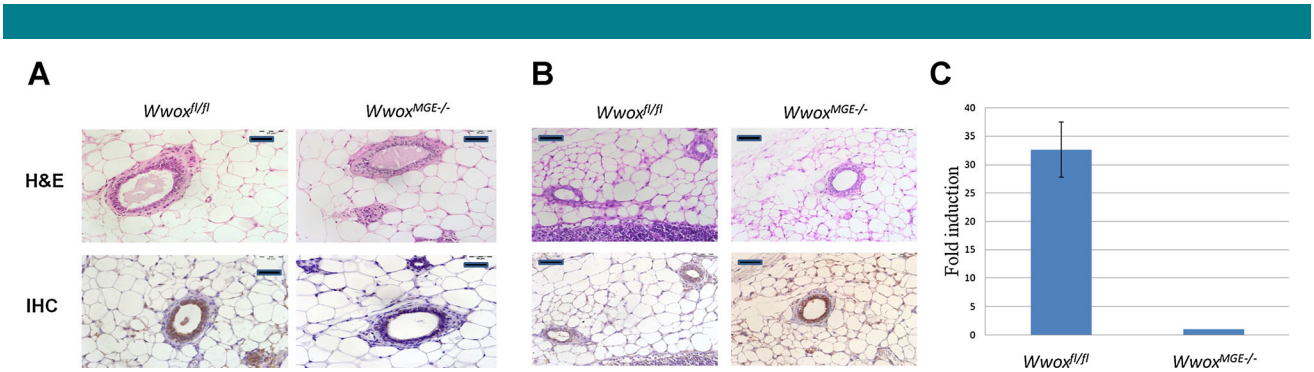

Fig. 2. Conditional knockout of WWOX in mammary gland epithelium using *MMTV-cre* transgenic mice. A: Immunohistochemical staining of mammary gland epithelium isolated from 2-month wild type (*Wwox*$^{fl/fl}$) versus conditional knockout (*Wwox*$^{MGE-/-}$) using anti-WWOX antibody. B: Immunohistochemical staining of mammary gland epithelium isolated from 2-month-old in wild type (*Wwox*$^{fl/fl}$) versus conditional knockout (*Wwox*$^{MGE-/-}$) using anti-Cre antibody. Scale bar represents 50 μm. C: Real-time PCR of WWOX using total RNA isolated from mammary epithelial cells (MECs) isolated from pregnant mice.

of *Wwox*$^{MGE-/-}$ mice exhibited a statistically significant retardation in ductal outgrowth relative to *Wwox*$^{fl/fl}$ (Fig. 3A). At 12 weeks of age, mammary glands of *Wwox*$^{MGE-/-}$ mice displayed variable growth of ducts and branching as compared with *Wwox*$^{fl/fl}$ mice (Fig. 3A,B); that is, in ~50% of mice we observed higher number of branching though this could be due to the mosaic effect of *MMTV-cre* recombinase (Wagner et al., 1997). This later effect could also resemble a compensatory effect due to the early ductal growth retardation.

Quantification of ductal growth in both mice genotypes is shown in Figure 3C.

Previous analysis of *Wwox*-heterozygous mice at the susceptible mammary tumor C3H genetic background, revealed increased incidence of mammary tumors as compared with wild type littermates (Abdeen et al., 2011). Therefore, we set to determine whether conditional *Wwox* ablation in mammary gland epithelium is sufficient to induce spontaneous mammary tumors. Examining of *Wwox*$^{MGE-/-}$ and *Wwox*$^{fl/fl}$

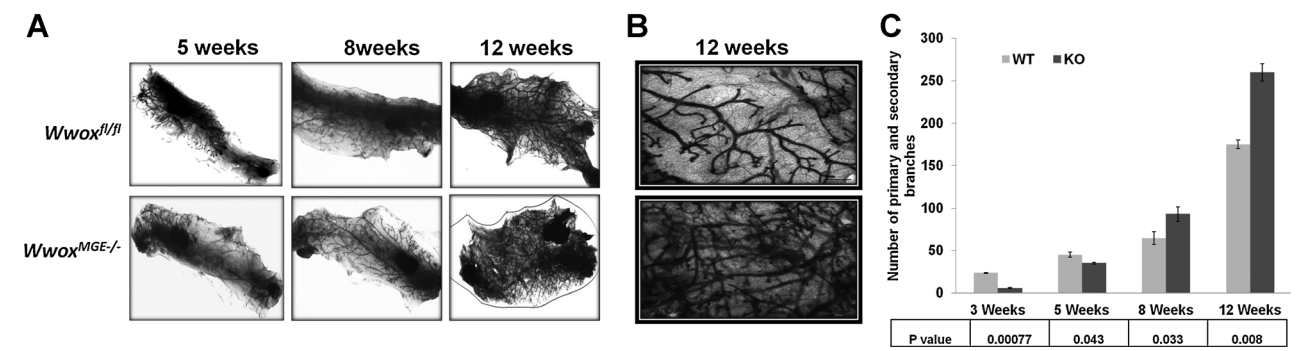

Fig. 3. Targeted WWOX deletion results in delayed ductal growth. A: Carmine red staining of whole mount mammary fat pad of the indicated virgin ages. Magnification of 0.7× (A) and 4× (B). C: Quantification of ductal outgrowth beyond the MFP lymph node at different ages of virgin females. Error bars indicate SEM.

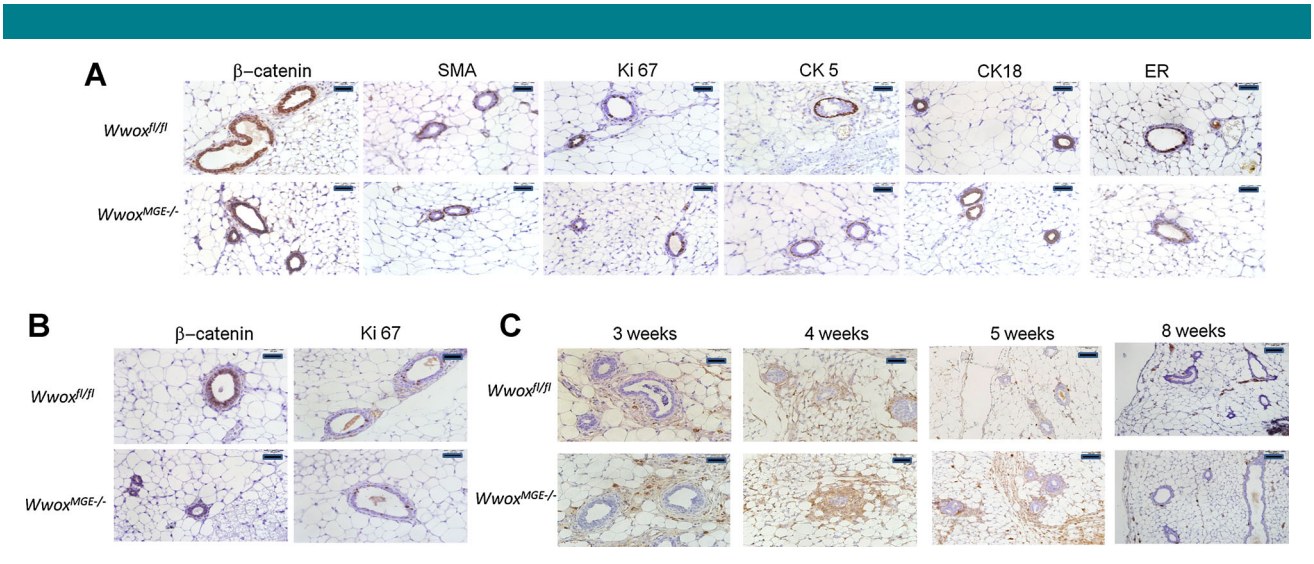

Fig. 4. Immunohistochemical staining of mammary epithelial markers during mammary development. A: Immunohistochemical staining of the different indicated markers of virgin 5-week old mice (A), 8 weeks old mice (B). C: Immunohistochemical of fibronectin of the indicated virgin mice. Magnification bar represents 50 μm.

mice in mixed B6-129 genetic background up to 16-month-old did not reveal a tumor phenotype (data not shown).

WWOX loss is associated with upregulation of fibronectin in the developing mammary gland

To further learn on the phenotype obtained in *Wwox*$^{MGE-/-}$ mice, we examined several key markers for the mammary gland epithelium. Immunohistochemical analysis of luminal cytokeratin-18 (K18), basal (K-5) showed very mild differences between *Wwox*$^{MGE-/-}$ mice and their counter control mice at 5 weeks, respectively (Fig. 4A). We also examined whether proliferation (Ki67) is affected and found no significance differences between WwoxMGE/mice and their counter control mice at 5 and 8 weeks (Fig. 4A,B). Staining of β-catenin also did not reveal major differences among the different genotypes, though a slight increase in nuclear β-catenin was observed in 4 weeks old virgin mammary glands (Fig. 4A,B). Interestingly, we observed a clear and significant difference in fibronectin staining specifically at 4–5 weeks old mice. *Wwox*$^{MGE-/-}$ mice displayed higher levels of fibronectin as compared to control littermates (Fig. 4C).

Knockdown of WWOX in MCF10A cells is associated with impaired growth and acinar morphogenesis

To further investigate whether WWOX loss in normal human breast epithelial cells would have an impact on the ability of these cells to form acini, we utilized non-transformed, immortalized MCF10A human breast epithelial cells. MCF10A immortalized breast epithelial cells serve as an in vitro model system to understand normal epithelial function and development. MCF10A cells express low-moderate levels of WWOX (Fig. 5A). WWOX knockdown effectively lowered WWOX protein levels up to 90% in two cell lines (Fig. 5A). Interestingly, fibronectin levels became higher in MCF10A-shWWOX cells (Fig. 5A) consistent with our in vivo data (Fig. 4C). Analysis of cell survival revealed that WWOX knockdown led to enhanced cell survival as compared to control, approximately fourfold greater in MCF10A-shWWOX cells (Fig. 5B). Next, we conducted a three-dimensional culture Matrigel assay. An equal number of cells were plated onto Matrigel in the absence or presence of EGF, and acinar formation was followed at different times after culturing the cells. MCF10A-shWWOX cells formed acini in the presence or absence of EGF as early as 3–4 days, whereas visible acini were only seen in MCF10A control cells in the presence of EGF (Fig. 5C). Furthermore, the acini morphology of MCF10A-shWWOX cells was less organized. Collectively, these data show that WWOX loss in vitro is associated with impaired growth of normal mammary epithelial cells.

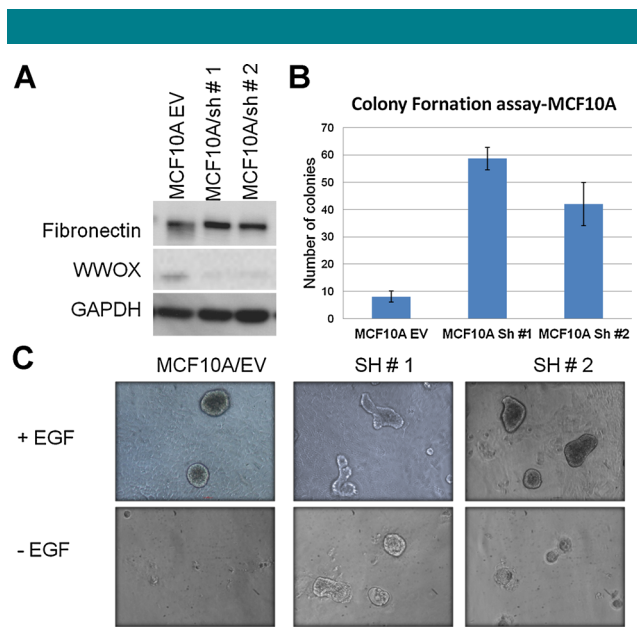

Fig. 5. Effect of WWOX knockdown on MCF10A cells. A: Immunoblot analysis of MCF10A cells transduced with empty vector (EV) or shRNAs against WWOX. Whole cell lysates were probed with antibodies against fibronectin and WWOX. GAPDH was used as a loading control. B: Clonogenic assay of MCF10A cells. The number of surviving colonies was counted after 2 weeks. Data are presented as mean ± SD. C: Effect of WWOX knockdown in MCF10A cells on spheroid formation in three-dimensional Matrigel culture. Spheroid formation progression is shown on Day 14.

Discussion

Previous characterization of WWOX function in breast cancer exposed its tumor suppressor function (Chang et al., 2007; Lewandowska et al., 2009; Salah et al., 2010). In fact, WWOX is deleted or altered in high percentage of breast cancer cases and its overexpression in breast cancer cells suppresses tumorigenecity (Guler et al., 2005; Iliopoulos et al., 2007; Aqeilan et al., 2007a). However, the outcome of WWOX ablation on murine mammary development could not be revealed so far due to the postnatal lethality of conventional *Wwox*$^{-/-}$ mice (Aqeilan et al., 2007b, 2008). In the current study, we examined the phenotype of specific *Wwox* inactivation in mammary gland epithelium. Our results demonstrate that *Wwox* ablation is associated with impaired ductal growth and increased fibronectin levels. Furthermore, WWOX knockdown in immortalized MCF10A cells revealed increased cell survival and impaired 3D cell culture spheres. Together, these findings argue for an important function of WWOX in normal mammary biology.

Our data demonstrate that WWOX expression in mammary gland epithelium is induced at 3–4 weeks of age. It is also at this age that we observed a delayed ductal growth in *Wwox*$^{MGE-/-}$ mice suggesting that WWOX expression at this time is playing a critical role. These findings are in agreement with recent observations of Ferguson et al. (2012). Analysis of several mammary lineage markers revealed no major differences between knockout and control mice. Of note, we also observed some inconsistencies in mammary ductal branching and density in 2–3 months old *Wwox*$^{MGE-/-}$ mice. This could be well related to the known mosaic MMTV-Cre expression in ductal mammary epithelium (Wagner et al., 1997). Importantly, depletion of WWOX expression in the normal MCF10A mammary cell demonstrates increase survival and impaired 3D culture further highlighting WWOX role in normal breast development.

A novel WWOX effector that is revealed from our study is fibronectin. Fibronectin is a component of the extracellular matrix that is known to play a critical role as a mitogen and in invasion/migration (Williams et al., 2008; Jinka et al., 2012). The major fibronectin transducer is integrin $\alpha5\beta1$ through which it signals to induce its biological functions including proliferation, migration and invasion. Fibronectin levels are increased in mammary glands of *Wwox*$^{MGE-/-}$ mice and in WWOX-depleted MCF10A cells. In a recent report, (Gourley et al., 2009) demonstrate that WWOX overexpression in ovarian cancer cells results in reduced attachment and migration on fibronectin. How does WWOX lead to increased levels of fibronectin is to be determined. Intriguingly, specific ablation of fibronectin in mammary epithelium is also associated with transient retardation in outgrowth and branching of the ductal tree (Liu et al., 2010). It is thus possible that the observed increased in fibronectin levels could stem from a compensatory effect due to the impaired ductal growth. Nevertheless, it is very likely that ablation of WWOX in mammary gland epithelium affects other signaling pathways that remains to be revealed.

The fact that *Wwox*$^{MGE-/-}$ mice did not develop mammary tumors further confirm our previous observations in convectional *Wwox*-heterozygous mice at the B6-129 mixed genetic background which did not form mammary tumors as well. By contrast, *Wwox*-heterozygous mice at the mammary tumor susceptible C3H genetic background exhibited significantly higher incidence of mammary tumors as compared with litter-matched control mice (Abdeen et al., 2011). It is thus necessary to examine the incidence of mammary tumor formation of *Wwox*$^{MGE-/-}$ mice in C3H or other mammary tumor susceptible genetic backgrounds. Nonetheless, there are several examples in the literature showing that mice deficient for classical tumor suppressors do not develop tumors. For example, p53 null mice rarely develop mammary tumors (Donehower, 1996). It is only when transferring the p53-targeted alleles into the *balb c* genetic background that some aging p53-heterozygous mice (42%) develop mammary tumors of different kinds (Kuperwasser et al., 2000). Additionally, specific ablation of p53 alleles (*Trp53*$^{\Delta 2\text{-}10}$) in mammary gland epithelium at 129/FVB mixed background does not associate with mammary tumors (Jonkers et al., 2001). However, cooperative inactivation of different tumor suppressors, such as BRCA2 and BRCA1, with p53 results in mammary tumor development (Jonkers et al., 2001; Liu et al., 2007). It is also possible that WWOX plays a role in mammary tumor progression and that other earlier event are necessary to lead to tumor formation. Taken together, the notion that *Wwox*$^{MGE-/-}$ mice at the B6-129 mixed genetic background did not develop mammary tumors does not question WWOX tumor suppressor role in breast cancer. Further research work would decipher the specific contribution of WWOX toward breast carcinogenesis.

In conclusion, the present study demonstrates that expression of WWOX in normal breast epithelial cells is critical for proper ductal growth and survival.

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