

REPORT



Global expression profiling and pathway analysis of mouse mammary tumor reveals strain and stage specific dysregulated pathways in breast cancer progression

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ABSTRACT

It is believed that the alteration of tissue microenvironment would affect cancer initiation and progression. However, little is known in terms of the underlying molecular mechanisms that would affect the initiation and progression of breast cancer. In the present study, we use two murine mammary tumor models with different speeds of tumor initiation and progression for whole genome expression profiling to reveal the involved genes and signaling pathways. The pathways regulating PI3K-Akt signaling and Ras signaling were activated in Fvb mice and promoted tumor progression. Contrastingly, the pathways regulating apoptosis and cellular senescence were activated in Fvb.B6 mice and suppressed tumor progression. We identified distinct patterns of oncogenic pathways activation at different stages of breast cancer, and uncovered five oncogenic pathways that were activated in both human and mouse breast cancers. The genes and pathways discovered in our study would be useful information for other researchers and drug development.

Abbreviations: B6: C57BL/6J; FVB: FVB/NJ; DEGs: differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: gene ontology; MF: molecular function; H&E: haematoxylin and eosin stain; ECM: extracellular matrix; TCGA: The Cancer Genome Atlas; FPKM: Reads Per Kilobase of exon model per Million mapped reads; PCA: principal component analysis; PI3K: phosphoinositide 3-kinase; PH: pleckstrin homology; IGF-1R: insulin-like growth factor-1 receptor

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Introduction



Breast cancer is the most prevalent cancer among women globally [1]. In 2012, the cancer mortality rate was ranked 5th among cancers due to significant advances in the clinical treatment and basic research of breast cancer, but the number of deaths was still very large (522,000 deaths) [1]. In China, there were 278,800 new cases in 2013 and 64,600 attributable deaths during the same year [2,3]. Therefore, it is important to investigate the molecular mechanisms involved in the progression of breast cancer and to identify novel prognostic biomarkers of breast cancer. Accordingly, these investigations will contribute to a better prognosis and provide potential therapeutic targets for breast cancer.

Overactivation of PI3K-Akt pathway has been reported in many types of cancers including breast cancer [4,5], bladder cancer [6], and ovarian cancer [7]. PI3K-Akt pathway is a signaling cascade which operates downstream to the receptor tyrosine kinases such as insulin-like growth factor-1 receptor (IGF-1R) [8], the relay of signals from the aforementioned insulin-like growth factor-1 receptor leads to the activation of

Phosphoinositide 3-kinase (PI3K) which catalyses the generation of phosphatidylinositol-3,4,5-triphosphate from phosphatidylinositol-4,5-biphosphate [9]. In turn, PIP3 interacts with pleckstrin homology (PH) domains of phosphoinositide-dependent kinase (PDK) and Akt and activates them [10]. After phosphorylation by PDK, Akt was activated as phospho-Akt (p-Akt) to stimulate downstream targets which are involved in regulation of apoptosis, cell cycle, transcription and translation [11,12]. Therefore, the PI3K/Akt/mTOR pathway plays a central role in regulation of the cell proliferation, survival, migration and extensively contributes to oncogenesis [13,14]. So the potential for cancer treatment with agents inhibiting this pathway is attractive and useful [10,15,16].

Extracellular matrix (ECM) is the non-cellular component present within all tissues and organs, and contains fibrous proteins and polysaccharides such as collagen, laminin and fibronectin [17]. Cell-ECM adhesion is mediated by the ECM receptors, including integrins [18], CD44 [19], and proteoglycans [20]. ECM- receptor interaction regulates many pathways

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in cancer cells, including PI3K-Akt [11], Wnt [21], Src-FAK [22], and Rho-GTPases [23], and activation of these pathways promote breast cancer development and progression [24]. ECM-receptor interaction also involves in therapy resistance, Hyaluronan is a component of the ECM that promotes resistance to a variety of chemotherapeutic drugs, by activation of PI3K signaling that supports survival via AKT [25]. A number of ECM molecules also participate in the preparation of the pre-metastatic niche [26,27], LOX accumulates in the pre-metastatic niche and promotes recruitment of MMP-2 producing myeloid cells [28], and fibronectin is upregulated at the distant site as well as the remodeling enzymes MMP-2 and MMP-9 [26].

By sharing both morphological and molecular biomarkers with the human breast cancer [29] and resembling the human luminal B subtype of breast cancer based on transcriptome profiling [30], MMTV-PyMT mouse model has been widely used to study breast cancer [31,32]. By activating multiple signaling pathways, including Shc [33], PI3-kinase [34] and phospholipase C_γ1 (PL_γ1), the membrane scaffold protein PyMT activates the MAPK, PI3K and DAG/PKC [35] pathways and promotes breast cancer progression. Previous studies have shown that the host genetic background has a significant impact on the biological behavior of subsequent tumors induced by PyMT and that tumor latency, the tumor growth rate and the metastatic index varies among different F1 strain combinations, which were generated by breeding MMTV-PyMT mice with 27 different inbred strains [36]. Global expression profiling of four F1 strain combinations identified a gene signature of tumor virulence in MMTV-PyMT mice [37]; the gene signature had been confirmed in human breast cancer [38]. Compared with MMTV-PyMT mice in the FVB/NJ background (simplified as Fvb in this article), tumor initiation is significantly delayed in the C57BL/6J (simplified as B6 in this article) background [39], and the molecular mechanisms involved remain unknown. We have hypothesized that a mechanism inside the human body can postpone cancer onset [40] and that B6 mice have strain-specific genes and pathways that delay tumor initiation and suppress tumor progression.

We determined the gene expression profiles of the MMTV-PyMT mammary tumors that developed in the Fvb strain and in the F1 strain combination Fvb.B6 at the adenoma stage and late carcinoma stage and compared these profiles to those of homologous normal tissues. Although the overall expression profiles of the PyMT tumors emerging in the Fvb and Fvb.B6 mice were similar, 148 to 417 strain- and stage-specific dysregulated genes were identified. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that PI3K-Akt and Ras signaling were specifically enriched in the Fvb background at the adenoma stage via up-regulated genes. Contrastingly, the pathways regulating apoptosis and cellular senescence were activated in Fvb.B6 mice. *Cdkn1a* (a cell cycle-related gene) was specifically down-regulated in the Fvb.B6 mice but up-regulated in the Fvb mice, and the survival analysis showed that high expression of *Cdkn1a* in human breast cancer was associated with poor prognosis by using The Cancer Genome Atlas (TCGA) database. These results suggest that Fvb.B6 mice exhibit modulated expression of genes involved in

PI3K-Akt and Ras signaling to suppress mammary tumor initiation and progression.

Results

Proper tissue clustering

Principal component analysis (PCA) depicts the expression values as points on a two-dimensional scale (Figure 1(A)). Each dot in the figure represents the normalized expression data of one unique sample. A total of 20 dots are visible in Figure 1(A), corresponding to the 20 samples used in this study. Based on this analysis, tumor samples were clearly clustered and separated from the homologous normal tissues, indicating proper tissue processing with high quality sequencing results.

Differentially expressed genes and strain-specific dysregulated pathways

DEGs were screened and generated by comparing the tumor samples with homologous normal tissues. The analysis identified 2,364 and 2,736 DEGs in the Fvb mice at the adenoma stage and late carcinoma stage, respectively, and the numbers of DEGs in the Fvb.B6 mice were 2,916 and 2558, respectively (fold change > 2, FDR < 0.05, Table S1). We also found that the number of down-regulated DEGs was higher than the numbers of up-regulated DEGs at each stage in the Fvb and Fvb.B6 mice (Table S1), which was consistent with a previous study [41]. The numbers of up- and down-regulated DEGs at the adenoma stage were higher in the Fvb.B6 mice than in the Fvb mice, but the opposite result was apparent at the late carcinoma stage. Among the down-regulated DEGs, a significant proportion of DEGs (1129 genes) appeared at all tumor stages in both the Fvb and Fvb.B6 mice (Figure 1(B)), and the up-regulated DEGs showed the same trend (Figure 1(C)).

We then performed KEGG pathway analysis of the DEGs identified at the adenoma and late carcinoma stages in the two genotypes of mice. At the adenoma stage, the pathways regulating PI3K-Akt signaling (KEGG:04151) and Ras signaling (KEGG: 04014) were activated in the Fvb tumor but not in the Fvb.B6 tumor (Figure 2(C) and Table 1). Activation of the PI3K-Akt signaling pathway plays a pivotal role in fundamental cellular functions, such as cell proliferation and survival, via phosphorylation of a variety of substrates [42], and activated Ras regulates a variety of endpoints relevant to breast cancer progression, including anchorage-dependent growth, anchorage-independent growth, steroid sensitivity and cell invasion [43]. Conversely, the pathways regulating cellular senescence (KEGG: 04218) and apoptosis (KEGG: 04210) were only activated in the Fvb.B6 tumors (Figure 2(C) and Table 1), and an activated apoptosis pathway leads to programmed cell death [44]. At the late carcinoma stage of Fvb mice, the pathways regulating the cell cycle (KEGG: 04110) were enriched in the up-regulated DEGs, suggesting a high proliferative rate for the tumors at this stage (Figure 2(A)). We determined and analyzed the significantly enriched pathways ($p < 0.05$) and found that five pathways were enriched in the up-regulated DEGs at the adenoma and late carcinoma stages in the Fvb.B6 mice and that another five pathways were enriched in the Fvb mice

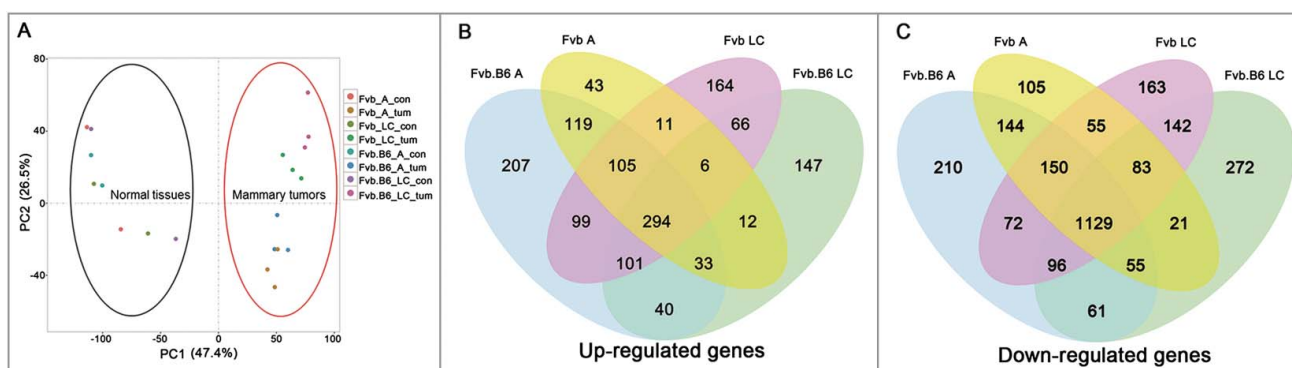


Figure 1. Differentially expressed genes along tumor progression. (A) Principle component analysis (PCA) of the mRNA expression data in the tumor and homologous normal tissue samples. A total of 20 samples were analyzed, and the separation between the tumor and control samples was shown. (B-C) Venn diagram showing the numbers of the up-regulated (B) and down-regulated (C) genes with significant changes ($p < 0.05$) in expression at the adenoma and late carcinoma stages in the two types of mice. A, adenoma; LC, late carcinoma.

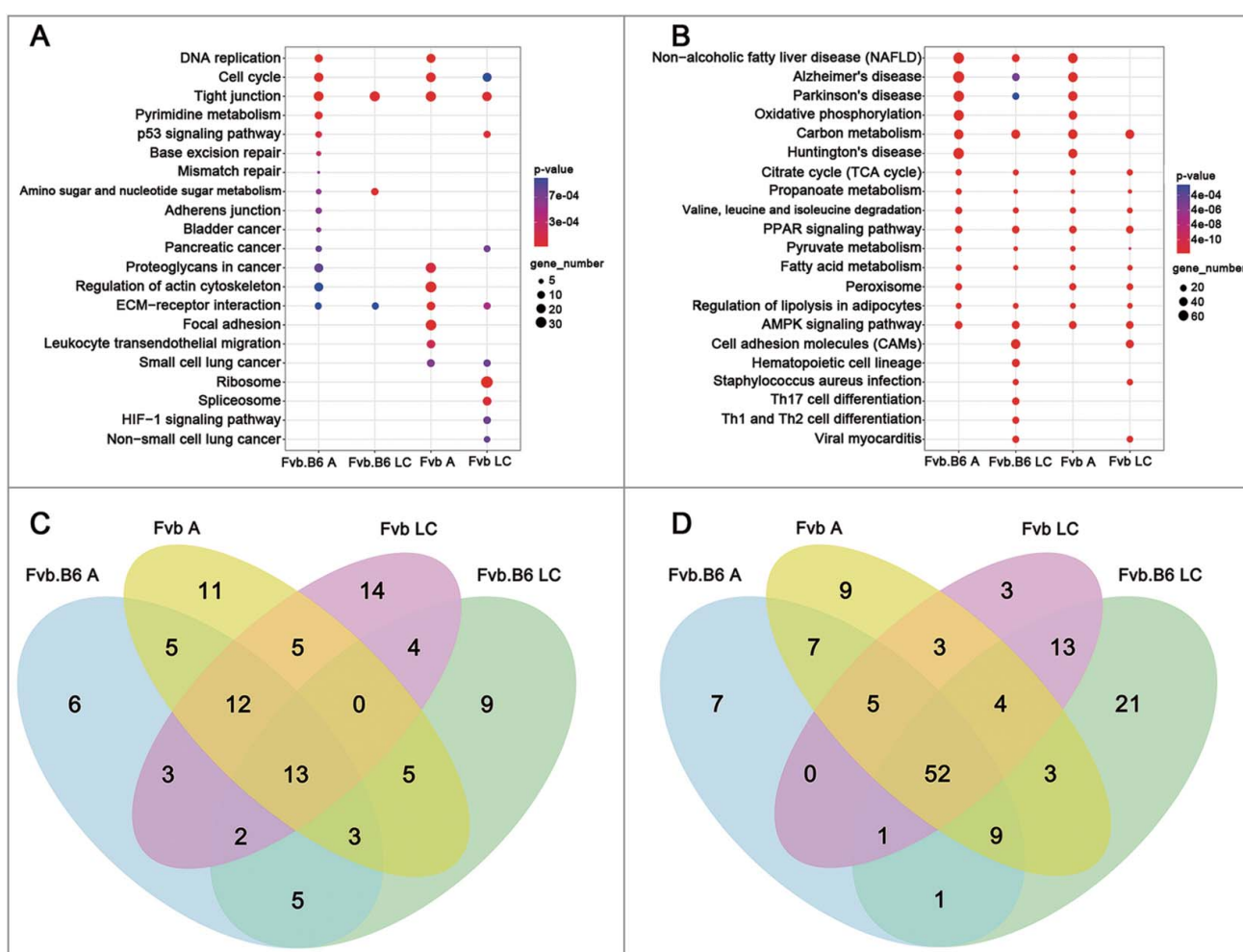


Figure 2. Pathway enrichment analysis. (A-B) KEGG enrichment of the up-regulated (A) and down-regulated (B) DEGs at the adenoma and late carcinoma stages in the two types of mice. The pathways with the most significant p-values were plotted. (C-D) Venn diagram showing the number of significant KEGG pathways (p -value < 0.05) by using the up-regulated (C) and down-regulated (D) DEGs at the adenoma and late carcinoma stages in the two types of mice. Expression levels are depicted according to the color bar beside the heat map. A, adenoma; LC, late carcinoma.

(Figure 2(C) and Table 1). One and three pathways were enriched in the down-regulated DEGs at the adenoma and late carcinoma stages in the Fvb.B6 and Fvb mice, respectively (Figure 2(D)).

Strain-specific up-regulated DEGs and their molecular functions

We examined strain specific up-regulated genes at the adenoma and late carcinoma stages and found that 447 and 232 up-

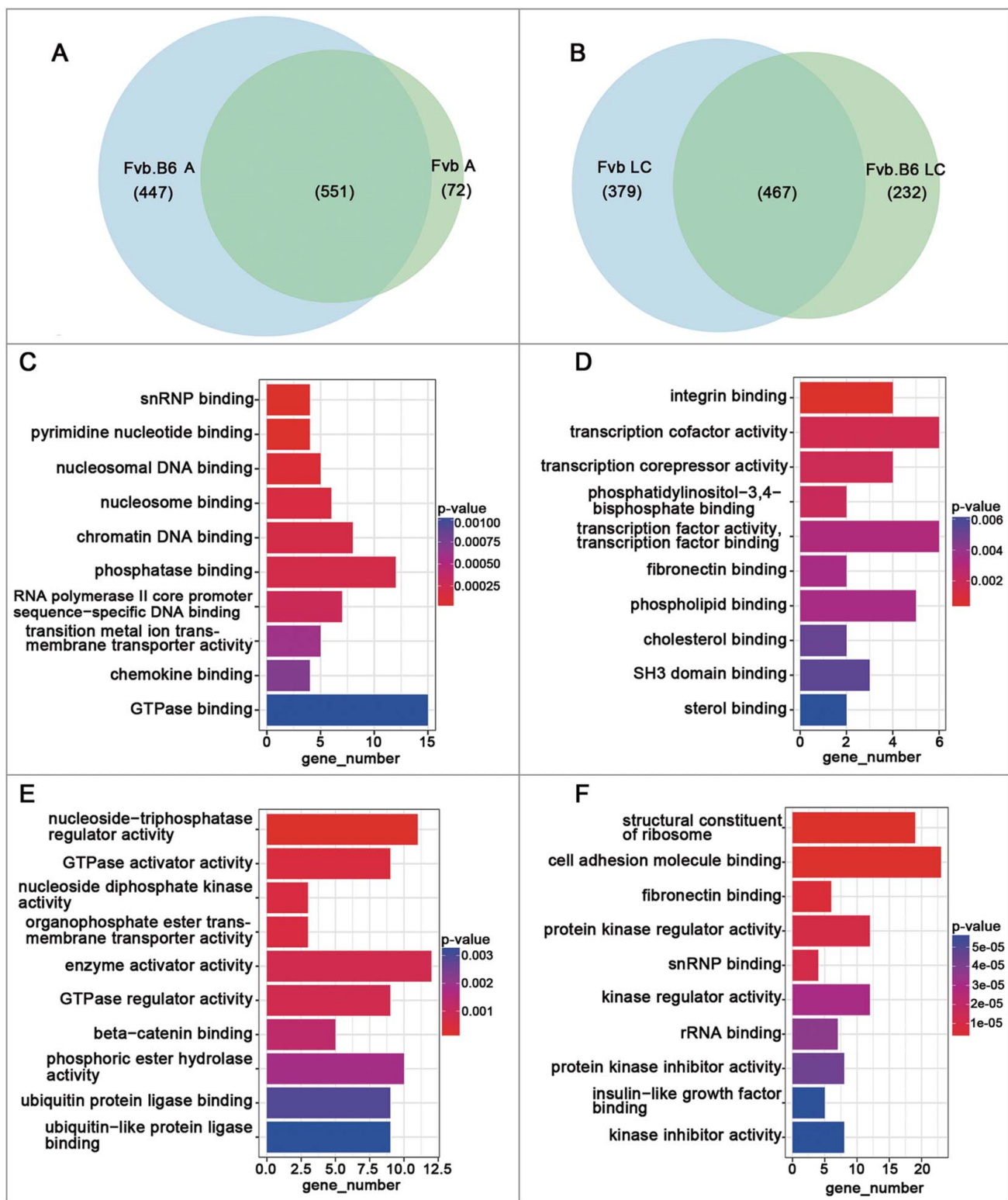


Figure 3. Strain-specific up-regulated DEGs and pathways at the adenoma and late carcinoma stages. (A-B) Venn diagram showing the numbers of strain-specific up-regulated genes with significant changes ($p < 0.05$) in expression at the adenoma (A) and late carcinoma (B) stages in the two types of mice. (C-D) Molecular Function Gene Ontology (MF-GO terms) analysis of the strain-specific up-regulated genes at the adenoma stage in the Fvb.B6 mice (C) and Fvb mice (D). (E-F) Molecular Function Gene Ontology (MF-GO terms) analysis of the strain-specific up-regulated genes at the late carcinoma stage in the Fvb.B6 mice (E) and Fvb mice (F). The color gradient from red to blue corresponds to an increase in the p-value.

regulated genes were specifically expressed in the Fvb.B6 mice at the adenoma and late carcinoma stages, respectively (Figure 3(A)), and that 72 and 379 up-regulated genes were specifically expressed in the Fvb mice at the adenoma and late carcinoma

stages, respectively (Figure 3(B)). We then performed Gene Ontology Molecular Function (GOMF) analysis of the strain-specific up-regulated genes. Ubiquitin protein ligase binding (GO:0031625) and ubiquitin-like protein ligase binding

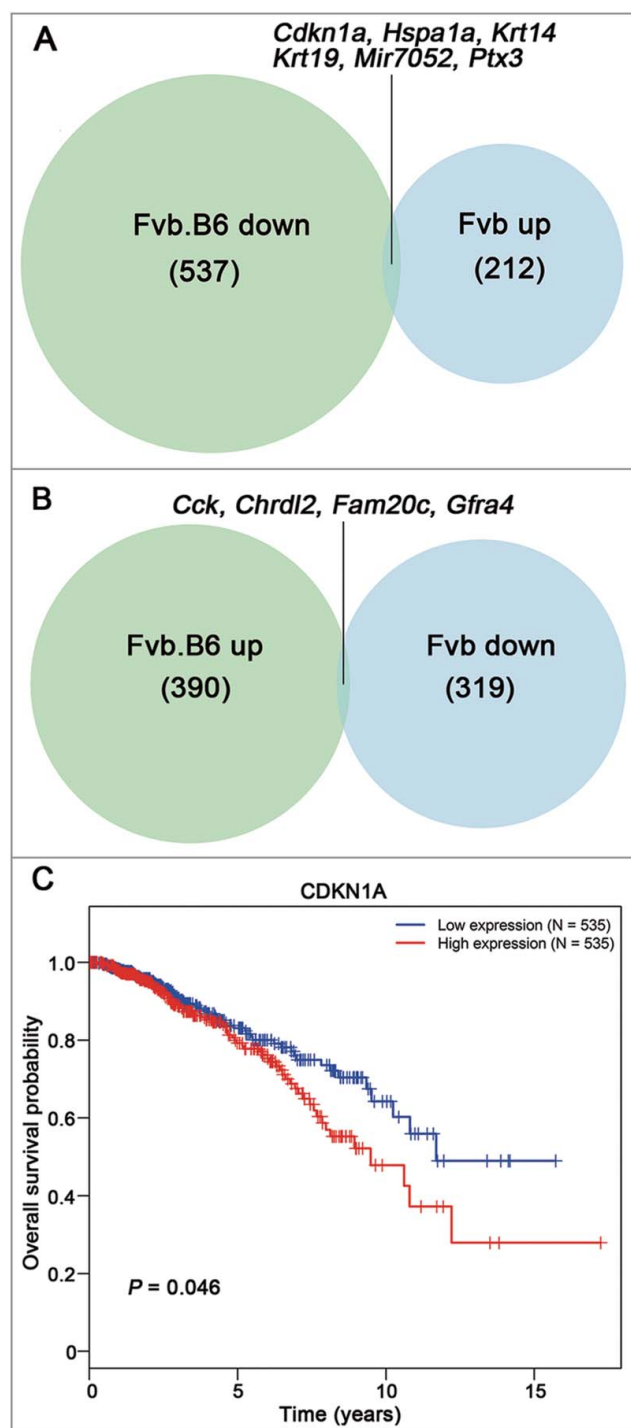


Figure 4. Venn diagram showing the inversely expressed genes between the Fvb. B6 mice and Fvb mice. (A-B) Venn diagram showing the numbers of the DEGs at both the adenoma and late carcinoma stages in the two types of mice. Six genes were down-regulated in the Fvb.B6 mice but up-regulated in the Fvb mice (A), and four genes were up-regulated in the Fvb.B6 mice but down-regulated in the Fvb mice (B). (C) Correlation of CDKN1A expression with overall survival for BRCA patients. The data were retrieved from The Cancer Genome Atlas (TCGA) database. The survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test.

(GO:0044389) were enriched in the Fvb.B6 mice at the late carcinoma stage (Figure 3(E)), and were involved in ubiquitin-mediated proteolysis and played important roles in cell cycle control [45]. Integrin binding (GO:0005178) and fibronectin binding (GO:0001968) were enriched in the Fvb mice at the

adenoma and late carcinoma stages (Figure 3(D–F)). Interestingly, integrin is a transmembrane receptor that facilitate ECM adhesion [46], and fibronectin is a high-molecular-weight (~440 kDa) glycoprotein of the ECM that binds to a membrane spanning receptor protein known as an integrin [47]. Therefore, integrin binding (GO:0005178) and fibronectin binding (GO:0001968) were all involved in the ECM-receptor interaction (KEGG:04512).

We examined all strain-specific DEGs at the adenoma and late carcinoma stages and found that 390 up-regulated genes and 537 down-regulated genes in total were specifically expressed in Fvb.B6 mice and that the number was 212 and 319 in the Fvb mice (Figure 5(A,B)). Interestingly, six genes (*Cdkn1a*, *Hspa1a*, *Krt14*, *Krt19*, *Mir7052*, *Ptx3*) were down-regulated in the Fvb.B6 mice but up-regulated in the Fvb mice, and four genes (*Cck*, *Chrdl2*, *Fam20c*, *Gfra4*) were up-regulated in the Fvb.B6 mice but down-regulated in the Fvb mice (Figure 5(A,B)). To verify our RNA-sequencing results, we performed qPCR on six independent tumor samples from the Fvb.B6 and Fvb mice. The expression patterns of six genes (*Cdkn1a*, *Hspa1a*, *Cck*, *Chrdl2*, *Fam20c*, *Gfra4*) were confirmed in the two genotypes of mice (p-value < 0.05, Fig. S2C). A previous study had showed that high expression of CDKN1A was closely correlated with poor prognosis in breast cancer [48] and the result confirmed in human breast cancer by using TCGA database (Figure 4(C)).

Commonly dysregulated pathways in the two genotypes of mice

We collected and analyzed the significantly enriched pathways (p < 0.05) and found that 13 pathways were enriched in the up-regulated DEGs at the adenoma and late carcinoma stages in the two genotypes of mice, and one of these pathways was the pathway regulating ECM receptor interaction (KEGG:04512). DNA replication (KEGG: 03030) and the cell cycle (KEGG: 04110) were enriched in the up-regulated DEGs at the adenoma stage in the two genotypes of mice (Figure 2(A)), suggesting a high proliferative rate for the tumors at this stage. We also found that 52 pathways were enriched in the down-regulated DEGs at the adenoma and late carcinoma stages in the two genotypes of mice (Figure 2(B)), including the TCA cycle (KEGG:00020); PPAR (peroxisome proliferator-activated receptor) signaling (KEGG:03320); AMPK (AMP-activated protein kinase) signaling (KEGG:04152); carbon metabolism (KEGG:01200); propanoate metabolism (KEGG:00640); valine, leucine and isoleucine degradation (KEGG:00280); pyruvate metabolism (KEGG:00620); and fatty acid metabolism (KEGG:01212). Notably, metabolic dysregulation is widely recognized in cancer [49].

As described above, the ECM-receptor interaction regulates many pathways in cancer cells and plays important roles in breast cancer progression. We examined all genes involved in the pathway and found a total of 16 up-regulated and 22 down-regulated genes. Of the 38 genes, the functions and roles of 14 genes in breast cancer progression have been studied, according to the PubMed database search (<https://www.ncbi.nlm.nih.gov/pubmed/>). We found that the up-regulated genes had tumor-promoting effects and that the down-regulated genes had tumor-suppressive effects on breast cancer, however, the

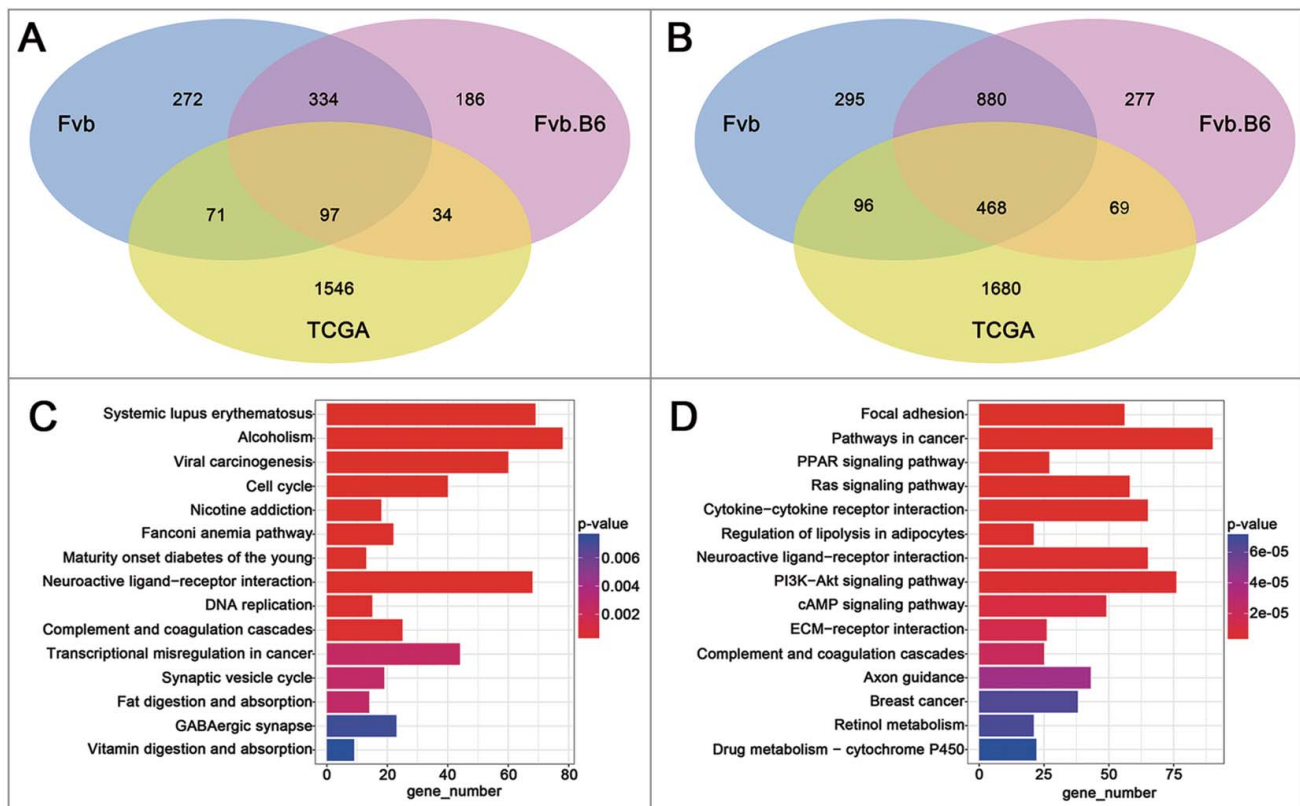


Figure 5. Analysis of TCGA data. (A-B) Venn diagram showing the numbers of the up-regulated (A) and down-regulated (B) genes with significant changes ($p < 0.05$) in expression in the TCGA human breast carcinoma samples and late carcinoma stage of the mice. (C-D) KEGG enrichment of the up-regulated (C) and down-regulated (D) DEGs in TCGA human breast carcinoma samples. The pathways with the most significant p-values were plotted, and the expression levels are depicted according to the color bar beside the heat map.

Table 1. The genes involved in ECM-receptor interaction and their functions in cancer progression.

| Gene | Log ₂ FC | Fuction | Cancer | Reference (PUBMED ID) |
|---------------|---------------------|---|------------------------------|-----------------------|
| <i>Npnt</i> | 3.853332471 | Promote tumor progression | Melanoma | 18271919 |
| <i>Spp1</i> | 3.146711848 | Promote tumor growth | Breast cancer | 25600648 |
| <i>Lamb3</i> | 2.311440893 | Supress tumor cell apoptosis | Breast cancer | 22673183 |
| <i>Tnc</i> | 2.278986035 | Promote tumor metastasis | Breast cancer | 21706029 |
| <i>Itgb3</i> | 2.2043643 | Promote tumor invasion | Brease cancer | 21935711 |
| <i>Col4a5</i> | 1.796828833 | Promote tumor progression | Lung cancer | 25992553 |
| <i>Cd44</i> | 1.75555128 | Promote tumor invasion | Breast cancer | 22621373 |
| <i>Thbs1</i> | 1.713801646 | Promote tumor metastasis | Breast cancer | 23614916 |
| <i>Hmmer</i> | 1.662370598 | Promote tumor invasion and metastasis | Breast cancer | 24367099 |
| <i>Sdc4</i> | 1.398242958 | Promote tumor metastasis | Breast cancer | 22513363 |
| <i>Itgb4</i> | 1.394540634 | Promote tumor metastasis | Breast cancer | 11320086 |
| <i>Itga6</i> | 1.257482721 | Promote tumor invasion and tumor-initiating cell activities | Breast cancer | 27001172 |
| <i>Itga3</i> | 1.245195681 | Promote tumor invasion | Oral squamous cell carcinoma | 19130304 |
| <i>Lama4</i> | -1.270026569 | Promote tumor invasion and metastasis | Liver cancer | 18084776 |
| <i>Thbs2</i> | -1.336701509 | Supress tumor growth | Breast cancer | 20714802 |
| <i>Col1a2</i> | -1.414277191 | Promote tumor proliferation and migration | Bladder cancer | 19424577 |
| <i>Col4a2</i> | -1.607119544 | Supress tumor growth | Oral squamous cell carcinoma | 19838634 |
| <i>Itga11</i> | -1.692400314 | Promote tumor growth | Prostate cancer | 26990302 |
| <i>Lamc1</i> | -1.876286991 | Regulate tumor progression | Uterine carcinoma | 26343160 |
| <i>Itga5</i> | -1.979636346 | Supress tumor growth | Breast cancer | 20564209 |
| <i>Hspg2</i> | -2.154008804 | Regulate cancer cell chemotherapy resistance | Breast cancer | 23436656 |
| <i>Itgb7</i> | -3.117291092 | Promote antitumor activity | Lung cancer | 17325197 |
| <i>Thbs3</i> | -3.433549457 | Promote tumor progression | Osteosarcoma | 17022822 |
| <i>Lama2</i> | -3.810378201 | Supress cancer cell growth | Liver cancer | 25159915 |
| <i>Vwf</i> | -3.939227195 | Promote tumor progression | Melanoma | 25977583 |
| <i>Itga7</i> | -4.093037687 | Supress tumor growth | Prostate | 20460506 |
| <i>Tnxb</i> | -6.200235271 | Supress tumor initiation | Neurofibromas | 17202312 |
| <i>Cd36</i> | -7.73946806 | Regulate cancer cell chemotherapy resistance | Breast cancer | 15050739 |

functions of the other 14 genes had been investigated in other cancers, and the remaining 10 genes (*Col9a2*, *Col9a3*, *Aggrn*, *Lamb2*, *Col4a1*, *Col6a1*, *Col6a2*, *Col6a3*, *Lamb1*, *Lamc3*) had no reported roles in any cancer (Table 1).

Validated pathways and genes in human patient cohorts

To validate the pathways and genes identified from the Fvb and Fvb.B6 mice, we used publicly available RNA-sequencing data of human breast invasive carcinoma (BRCA) from TCGA database (<http://cancergenome.nih.gov/>). We downloaded data for 397 luminal B subtype BRCA and 44 adjacent tissues (classified by the PAM50 model [50]), which most closely resembled the MMTV-PyMT mouse model [30]. The Level3 TCGA BRCA-aligned RNA-seq data were processed the same way as our sequencing data, 1748 up-regulated DEGs and 2313 down-regulated DEGs were identified (fold change > 2, FDR < 0.05). The expression patterns of 97 up-regulated and 468 down-regulated DEGs were validated in both humans and mice (Figure 5 (A,B)), and the human homolog gene list was downloaded from MGI (Mouse Genome Informatics, <http://www.informatics.jax.org/>). KEGG pathway analysis of the DEGs from the TCGA samples showed that the pathways regulating the cell cycle and DNA replication were enriched in the up-regulated DEGs (Figure 5(C)) and that the two pathways were also enriched in the up-regulated DEGs at the adenoma stage in the two genotypes of mice (Figure 2(A)). The pathways regulating focal adhesions (KEGG: 04510), the ECM-receptor interaction (KEGG: 04512), and cell adhesion molecules (KEGG: 04514) were enriched in the down-regulated DEGs in both humans (Figure 5(D) and Table S2) and mice (Figure 2(D)).

Discussion

By 5 weeks of age, all female mice in the Fvb background had developed palpable mammary tumors [31], and the Fvb.B6 mice had a median tumor latency of 64.5 days [37]. The Fvb mice also had increased tumor growth and metastasis rates compared with the Fvb.B6 mice [37]. To better decipher the molecular mechanisms involved in the influence of the genetic background on tumor behavior, we analyzed the global gene expression patterns of mammary tumors at the adenoma and late carcinoma stages in two genotypes of mice.

KEGG pathway analysis showed that the PI3K-Akt signaling pathway (KEGG:04151) was the pathway that was specifically enriched in the up-regulated DEGs in the Fvb mice. Previous studies have shown that the middle T antigen oncogene interacts with phosphatidylinositol 3-kinase (PI3K) and activates the PI3K-Akt signaling pathway by phosphorylated tyrosine residue Y315 [51,52]. PI3K-Akt signaling plays an important role in the regulation of apoptosis, the cell cycle, transcription and translation [11], and activation of this pathway promotes breast cancer progression [53,54]. Ras proteins are well-known small GTPases that mediate MAPK cascades and regulate cell proliferation, survival, growth, and migration [55]. The Ras signaling pathway was also specifically enriched in the up-regulated DEGs at the adenoma stage in the Fvb mice.

Apoptosis is typically considered an anti-oncogenic process since caspase activation can promote the elimination of

Table 2. Strain specific pathways enriched by up-regulated DEGs at the adenoma and late carcinoma stage in the two types of mice.

| KEGG(ID) | Description | p-value |
|-------------------------------------|--|-----------|
| Adenoma (Fvb mice) | | |
| mmu05412 | Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 0.0081085 |
| mmu04392 | Hippo signaling pathway – multiple species | 0.0089201 |
| mmu05231 | Choline metabolism in cancer | 0.0140725 |
| mmu05410 | Hypertrophic cardiomyopathy (HCM) | 0.0180511 |
| mmu05211 | Renal cell carcinoma | 0.0188743 |
| mmu05416 | Viral myocarditis | 0.0227298 |
| mmu05414 | Dilated cardiomyopathy (DCM) | 0.024019 |
| mmu04151 | PI3K-Akt signaling pathway | 0.0296419 |
| mmu04919 | Thyroid hormone signaling pathway | 0.031504 |
| mmu04014 | Ras signaling pathway | 0.0320875 |
| mmu04114 | Oocyte meiosis | 0.0329362 |
| mmu00564 | Glycerophospholipid metabolism | 0.0361768 |
| mmu05166 | HTLV-I infection | 0.0380264 |
| mmu00534 | Glycosaminoglycan biosynthesis – heparan sulfate / heparin | 0.0403475 |
| mmu00561 | Glycerolipid metabolism | 0.0455971 |
| mmu04072 | Phospholipase D signaling pathway | 0.0470431 |
| Late carcinoma (Fvb mice) | | |
| mmu03010 | Ribosome | 0.000136 |
| mmu03040 | Spliceosome | 0.0002356 |
| mmu05223 | Non-small cell lung cancer | 0.0022882 |
| mmu05214 | Glioma | 0.0037957 |
| mmu03018 | RNA degradation | 0.009785 |
| mmu04141 | Protein processing in endoplasmic reticulum | 0.0155598 |
| mmu04915 | Estrogen signaling pathway | 0.0253697 |
| mmu04392 | Hippo signaling pathway – multiple species | 0.0258285 |
| mmu05410 | Hypertrophic cardiomyopathy (HCM) | 0.0313479 |
| mmu05164 | Influenza A | 0.0344478 |
| mmu05412 | Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 0.0387488 |
| mmu05134 | Legionellosis | 0.0416162 |
| mmu05414 | Dilated cardiomyopathy (DCM) | 0.0421162 |
| mmu04971 | Gastric acid secretion | 0.043973 |
| mmu05145 | Toxoplasmosis | 0.0485932 |
| mmu00220 | Arginine biosynthesis | 0.0488507 |
| mmu00670 | One carbon pool by folate | 0.0488507 |
| mmu05218 | Melanoma | 0.0496314 |
| mmu04151 | PI3K-Akt signaling pathway | 0.0532724 |
| Adenoma (Fvb.B6 mice) | | |
| mmu00052 | Galactose metabolism | 0.001631 |
| mmu05161 | Hepatitis B | 0.0025484 |
| mmu00230 | Purine metabolism | 0.0049687 |
| mmu05323 | Rheumatoid arthritis | 0.0062735 |
| mmu05230 | Central carbon metabolism in cancer | 0.0088227 |
| mmu04210 | Apoptosis | 0.0088848 |
| mmu00100 | Steroid biosynthesis | 0.0192054 |
| mmu05133 | Pertussis | 0.0234864 |
| mmu05202 | Transcriptional misregulation in cancer | 0.0285217 |
| mmu04218 | Cellular senescence | 0.0312273 |
| mmu04978 | Mineral absorption | 0.0380667 |
| Late carcinoma (Fvb.B6 mice) | | |
| mmu00052 | Galactose metabolism | 0.0007166 |
| mmu05202 | Transcriptional misregulation in cancer | 0.0018766 |
| mmu04974 | Protein digestion and absorption | 0.0129149 |
| mmu04973 | Carbohydrate digestion and absorption | 0.0161787 |
| mmu00565 | Ether lipid metabolism | 0.0194157 |
| mmu04978 | Mineral absorption | 0.0194157 |
| mmu01521 | EGFR tyrosine kinase inhibitor resistance | 0.0210883 |
| mmu04550 | Signaling pathways regulating pluripotency of stem cells | 0.021165 |
| mmu04144 | Endocytosis | 0.0219855 |
| mmu05230 | Central carbon metabolism in cancer | 0.0234871 |
| mmu05323 | Rheumatoid arthritis | 0.0252586 |
| mmu05203 | Viral carcinogenesis | 0.0310027 |
| mmu04666 | Fc gamma R-mediated phagocytosis | 0.0316522 |
| mmu04931 | Insulin resistance | 0.0361468 |

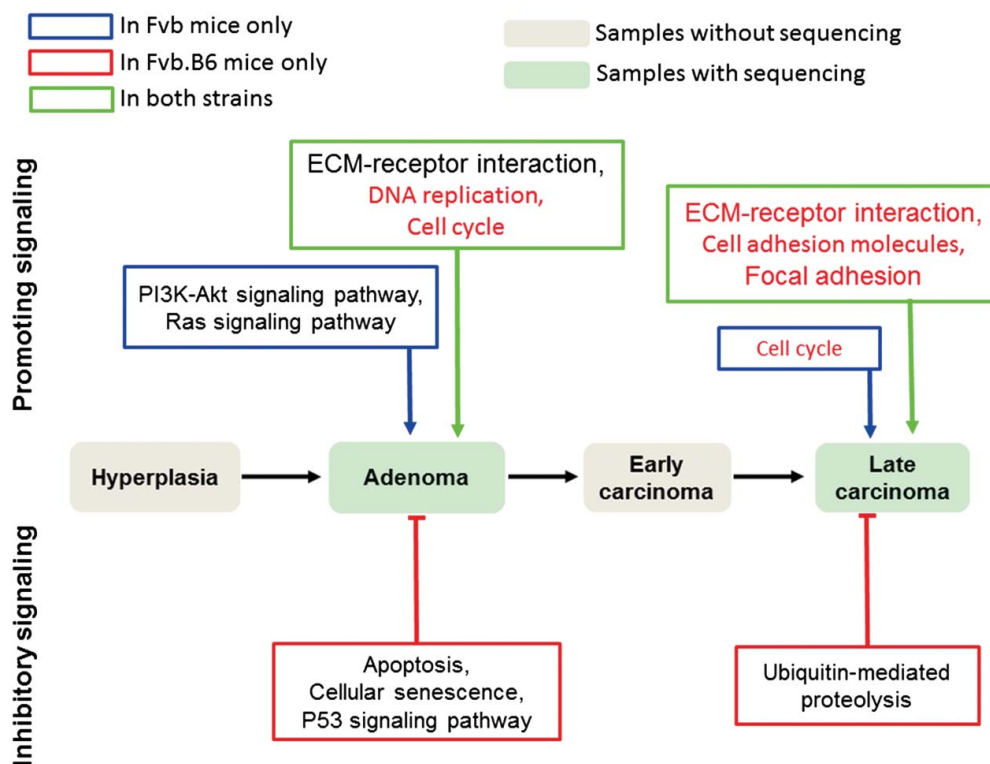


Figure 6. Schematic of the findings in this study. The PI3K-Akt signaling pathway and Ras signaling pathway were activated in the Fvb mice and promoted breast cancer progression. The pathways regulating cellular senescence and apoptosis were enriched in the up-regulated DEGs in the Fvb.B6 mice and suppressed breast cancer progression. The oncogenic pathways validated by analyzing available human breast invasive carcinoma samples in TCGA database are indicated in red.

genetically unstable or damaged cells [56]. We observed that the promotion of apoptosis existed in the Fvb.B6 mice as apoptosis-related pathways were enriched in strain-specific up-regulated genes (Table 2). Therefore, the suppression of breast cancer progression in the Fvb.B6 mice was partly due to the activation of the apoptotic process.

The pathways regulating the cell cycle and DNA replication were activated in both the human and mouse breast cancers, suggesting their fundamental roles in breast cancer progression. Actually, inhibiting the expression of DNA replication-initiating genes induces apoptosis in human cancer cells [57], and uncontrolled cell proliferation is a hallmark of cancer [58]. The pathways regulating focal adhesion, ECM-receptor interactions and cell adhesion molecules were enriched in the down-regulated DEGs in both the human and mouse breast cancers. Interesting, all three pathways were involved in the interaction between the cancer cells and extracellular matrix. It is well known that the interaction between tumor cells and their microenvironment is important for establishing metastatic colonies and for defining the balance between dormancy and malignant growth [59,60]. We collected and analyzed the genes involved in the ECM-receptor interaction pathway in two genotypes of mice and found that a small subset of up-regulated genes and down-regulated genes had tumor-promoting and tumor-suppressing effects, respectively, in breast cancer, and that more than half of the DEGs had no reported roles in breast cancer progression.

In conclusion, we have identified five oncogenic pathways that regulate breast cancer progression in both humans and mice and found strain-specific pro-oncogenic and anti-oncogenic pathways that affect the progression of breast cancer. Breast cancer progression in the Fvb mice was promoted by the activation of the PI3K-Akt signaling pathway and Ras signaling pathway, and breast cancer progression was suppressed in the Fvb.B6 mice by the activation of the apoptotic process (Figure 6). The upstream molecular mechanisms involved in the pro-oncogenic PI3K-Akt and Ras signaling pathways, which are activated in the Fvb mice but not the Fvb.B6 mice, and the anti-oncogenic apoptosis-related pathways, which are activated in the Fvb.B6 mice but not the Fvb mice, remain relatively unknown and warrant further study. Our results have great clinical implications. For example, the PI3K-Akt signaling pathway is activated in many types of human cancers [4–6], and PI3K-Akt inhibitors have many side effects [61,62]. Therefore, inactivation of the PI3K-Akt pathway by targeting upstream molecules is a novel and promising approach.

Materials and methods

Mice and animal care

FVB/N-Tg(MMTV-PyMT)634Mul/J mice were purchased from Jackson Laboratory (Stock No:002374, <https://www.jax.org/strain/002374>) and B6 mice were purchased from Guangdong Medical Laboratory Animal Center. Mice were housed under specific pathogen-free conditions in Animal Center of

Guangdong Pharmaceutical University. All animal experiments were performed in accordance with institutional guidelines and were approved by the Sun Yat-sen University Cancer Center Institutional Animal Care and Usage Committee, with the ethical approval number: L102012017000G.

Fvb.B6 F1 strain combinations were generated by crossing FVB/N-Tg(MMTV-PyMT)634Mul/J mice to B6 mice. FVB/N-Tg(MMTV-PyMT)634Mul/J male animals were bred to B6 female animals to produce transgene-positive Fvb.B6 F1 hybrid female progeny. These virgin F1 strain combinations and FVB/N-Tg(MMTV-PyMT)634Mul/J mice were euthanized for tissue harvesting. Mammary tumors were harvested when the diameter of largest tumor per mouse reached approximately 2 millimeters and 10 millimeters, which correspond to the Adenoma/MIN and late carcinoma stage (Fig. S1A-B).

RNA-seq and analysis

Six tumor samples and four homologous normal tissues from the FVB/N-Tg(MMTV-PyMT)634Mul/J and Fvb.B6 F1 strain combinations were stored at RNASTore Reagent (Cat.# DP408, TIANGEN) for RNA isolation, respectively. Total RNA was isolated from the 20 samples, which three tumor samples in each group, and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA was sheared and reverse transcribed using random primers to obtain cDNA which was used for library construction. The library was subsequently sequenced through BGISEQ-500 platform at Beijing Genomic Institution (www.genomics.org.cn, BGI).

All statistical analyses were carried out with R v3.3.1, after removing transcriptionally inactive genes (read count per million < 1) from raw RNA sequencing gene counts, we got high confident gene counts. For gene expression analysis, the matched reads were calculated and then normalized to Reads Per Kilobase of exon model per Million mapped reads (FPKM) using RESM software [63]. The R package edgeR v3.18.1 was used to perform statistical analysis on gene counts and to detect differentially expressed genes (DEGs). DEGs at each stage or site were used for further analyses of GO (gene ontology) Molecular Function and KEGG pathways using the R package clusterProfiler v3.4.4 [64].

Analyses of TCGA data

RNA-seq data were downloaded from TCGA database (<http://cancergenome.nih.gov/>), and the aligned RNA-seq data (FPKM format) of all available invasive breast carcinoma datasets were downloaded in August 2016. Survival analysis was conducted using the survival package in R. Kaplan-Meier plots of overall patient survival were stratified by the median values of the individual mRNA expression in the TCGA datasets.

RT-PCR

The qPCR was performed using the $2^{-\Delta\Delta Ct}$ method. The RNA was converted to cDNA using PrimeScriptTM II 1st Strand cDNA Synthesis Kit (Cat.# 2690A, Takara), followed by reactions using Roche LightCycler 480 System (Roche). Each 10 μ l reaction included 5 μ l ChamQ SYBR qPCR Master Mix (Cat.#

Q311-02, Vazyme), 0.8 μ l RNase-free water, 4 μ l cDNA (5 nM) and 0.2 μ l primer. Actb was used as the internal control gene to give genes tested a relative fold change using the $2^{-\Delta\Delta Ct}$ method. The qPCR primers were from PrimerBank [65].

Histology

Mice mammary tumor tissues were embedded in paraffin, and 3- μ m sections were cut and stained with hematoxylin and eosin (H&E).

Acknowledgments

The authenticity of this data has been validated by uploading the critical raw data onto the Research Data Deposit (RDD) public platform (www.research-data.org.cn), with the approval RDD number as RDDB2018000303.


Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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