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FoxD3 deficiency promotes breast cancer progression by induction of epithelial–mesenchymal transition

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ABSTRACT

The transcription factor forkhead box D3 (FOXD3) plays an important role in the development of neural crest and gastric cancer cells. However, the function and mechanisms of FOXD3 in the breast tumorigenesis and progression is still limited. Here, we report that FOXD3 is a tumor suppressor of breast cancer tumorigenicity and aggressiveness. We found that FOXD3 is down-regulated in breast cancer tissues. Patients with low FOXD3 expression have a poor outcome. Depletion of FOXD3 expression promotes breast cancer cell proliferation and invasion *in vitro*, whereas overexpression of FOXD3 inhibits breast cancer cell proliferation and invasion both *in vitro* and *in vivo*. In addition, depletion of FOXD3 is linked to epithelial–mesenchymal transition (EMT)-like phenotype. Our results indicate FOXD3 exhibits tumor suppressive activity and may be useful for breast therapy.

1. Introduction

Breast cancer is the leading cause of cancer death among females [1], and results from accumulated genetic and epigenetic alterations of various cancer genes, including oncogenes and tumor-suppressor genes [2]. Despite recent progress in breast cancer diagnosis and treatment, the survival rates for patients with metastatic breast cancer remain poor. Therefore, identification and determination of new genes/pathways involved in breast cancer carcinogenesis, especially metastasis, will help to develop safer and faster diagnosis and better disease prognosis predication following treatment of this disease.

FoxD3, a member of forkhead box (Fox) transcription factor family, has been shown to be expressed in embryonic stem cells in the late-stage gastrula inner cell mass [3] and is also required for extra embryonic tissue [4]. Importantly, FoxD3 is essential during normal murine development by maintaining pluripotent cells in the early mouse embryo and is required to establish murine embryonic stem cell lines *in vitro* [3,5,6]. The role of FoxD3 in later development has also been established, specifically in premigrating and migrating neural crest cells in avian embryo [7,8]. Furthermore, FoxD3 is an early molecular marker of neural crest cells and is responsible for the repression of melanogenesis in early migratory neural crest cells [7,9]. Recent evidence shows that ectopic expression of FoxD3 in melanoma cells induces a G1/S phase arrest and suppresses migration and invasion [9,10]. In addition, FOXD3 is down-regulated due to promoter hypermethylation, and overexpression of FOXD3 significantly inhibits gastric cancer cell proliferation and invasion [11]. Moreover, FOXD3 is down-regulated in human neuroblastoma, and FOXD3 exhibits tumor suppressor activity that affects the growth, invasion, metastasis, and angiogenesis of neuroblastoma cells through direct transcriptional regulation of NDRG1 [12]. However, the role of FOXD3 in breast cancer is still limited.

In the present study, we demonstrated that FOXD3 is down-regulated in breast cancer tissues and down-regulation of FOXD3 is associated with poor outcome in patients with breast cancer. We demonstrated that FOXD3 inhibits breast cancer cell proliferation and invasion both *in vitro* and *in vivo*. Furthermore, depletion FOXD3 is linked to the epithelial–mesenchymal transition (EMT)-like phenotype. These findings suggest that FOXD3 is a potential tumor suppressor in breast cancer development and progression, and may be useful for breast therapy.

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2. Material and methods

2.1. Cell lines and tumor samples

Breast cancer cell line MDA-MB-231 and MDA-MB-231-luc were maintained at 37 °C in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml of penicillin, and streptomycin.

160 primary breast cancer tissues and 20 adjacent normal breast tissues were analyzed using Real-time quantitative reverse transcription PCR (RT-PCR). This study was approved by the Human Ethics Review Committee of Tianjin Medical University Cancer Institute and Hospital. Documented informed consent was obtained from all patients in accordance with the Declaration of Helsinki and its later revision.

2.2. Plasmid, siRNA and transfection

The full-length FoxD3 cDNA was reverse transcriptase-polymerase chain reaction using total RNA from MDA-MB-231 cell line. The PCR products were cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen) (pcDNA3.1-FoxD3). The FoxD3 siRNAs was purchased from RiboBio (Shanghai, China). All constructs were fully sequenced. The cells were then transfected with the siRNA or plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. RT-PCR

RT-PCR was performed by using the Fast SYBR Green Master Mix System (Invitrogen) according to the manufacturer's instructions. Thermal-cycling reaction was performed in the 7500 Real-Time PCR system (Invitrogen) Melting-curve analysis and agarose gel electrophoresis of PCR products were further performed. Relative mRNA expression levels were standardized to β-actin levels.

2.4. Western blot analysis

For Western blot assay, cells were lysed in M-PER mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA). A total of 50 μg protein lysate was separated by SDS–PAGE and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in TBST, the membranes were incubated with FoxD3 (Thermo Scientific), Twist1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Snail (Santa Cruz Biotechnology), ZEB1 (Santa Cruz Biotechnology), Slug (Santa Cruz Biotechnology), or β-actin (Santa Cruz Biotechnology) at 4 °C overnight. Next day, the membranes were incubated with horseradish peroxidase conjugated-secondary antibody, and the bands were visualized using ECL detection reagents (Millipore).

2.5. MTT and colony formation assays

For MTT assay, cells were seeded in 96-well plates per well. Then 24, 48, 72, 96 h after transfection, the cells were incubated with 10 μl MTT (0.5 mg/ml; Sigma–Aldrich) at 37 °C for 4 h. The medium was then removed, and precipitated Formosan was dissolved in 100 μl DMSO. The absorbance at 490 nm was detected using a micro-plate auto-reader (Bio-Rad, Richmond, CA, USA).

For colony formation assay, 500 cells were seeded in 6-well plates per well after transfection. The plates were incubated at 37 °C and 5% CO_2 in a humidified incubator. Fresh culture medium was replaced every 3 days. After 3 weeks of culture, the cells were stained with crystal violet, and the numbers of colonies containing more than 50 cells were counted.

2.6. Transwell assay

Transwell assay was used to evaluate the ability of cell invasion. Cells in 0.2 ml DMEM without FBS were placed on the top chamber of each insert (BD, Biosciences, San Jose, CA, USA) with 40 μl of 1 mg/ml Matrigel. The lower chamber was filled with 500 μl of DMEM medium with 10% FBS to act as the nutritional attractant. After incubated with 16 h, the migrant cells were fixed with 4% paraformaldehyde in PBS for 20 min and stained for 20 min with crystal violet. The membranes were then carved and embedded under cover slips with the cells on the top. Cells in three different fields of view were counted.

2.7. Bioluminescence imaging and analysis

1×10^6 MDA-MB-231-luc-FOXD3 and control cells were washed and harvested in 0.1 ml PBS and injected into the left cardiac ventricle of 4-week-old, female BALB/c-nu/nu nude mice. Mice were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight) before injection. Anesthetized mice were injected with 100 mg/kg D-Luciferin in PBS. Bioluminescence images were obtained by using the Xenogen IVIS system 10 min after injection. Analysis was performed with LIVINGMAGE software (Xenogen, Alameda, CA, USA).

2.8. Statistical analysis

Statistical analyses were performed with SPSS version 19 (SPSS Inc., Chicago, IL, USA). Student *t* test (paired two tailed) was used to analyze the difference of FoxD3 expression between breast cancer tissues and adjacent normal breast tissues. The Kaplan–Meier method was used to estimate survival; log-rank test was used to test differences between the survival curves. Data are reported as means ± SD, and mean values were compared using the Student's *t* test. For all the tests, $P < 0.05$ was considered statistically significant.

3. Results

3.1. FoxD3 is frequently down-regulated in breast cancer

We first determined FOXD3 expression in 20 cases of primary breast cancer tissues and the paired adjacent normal breast tissues by RT-PCR. The result showed that FOXD3 was down-regulated in tumor tissues compared to the normal breast (Fig. 1A). Furthermore, we found that the FOXD3 expression was lower in advanced tumor stage III than tumor stage I/II in 160 cases of primary breast cancer tissues (Fig. 1B). The Kaplan–Meier analysis was used to evaluate the DFS of patients with breast cancer. The result showed that patients with reduced FOXD3 expression were likely to be with significantly shorter 5-year DFS (Fig. 1C). Together, these results indicate that FOXD3 is down-regulated in breast cancer and down-regulation of FOXD3 relates to poor outcome.

3.2. FOXD3 deficiency promotes breast cancer cell proliferation and invasion in vitro

To elucidate the function of FOXD3 in breast cancer, we examined the effect of FOXD3 knockdown on proliferation characteristics of breast cancers by colony formation and MTT assays. The RT-PCR and western blot assays showed that both mRNA and protein expressions of FOXD3 in FOXD3 siRNA-transfected MDA-MB-231 cells (siFOXD3-1 and siFOXD3-2) were significantly reduced than control cells (Fig. 2A and B). Colony formation analysis showed that cells transfected with FOXD3 siRNAs formed more colonies than

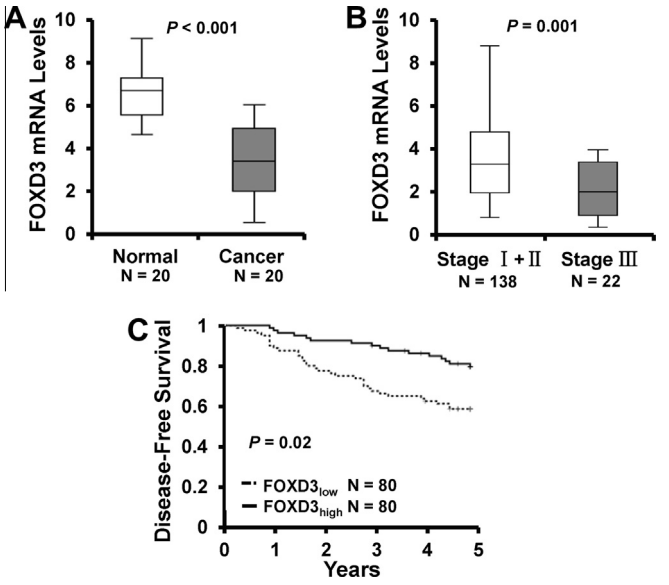

Fig. 1. Down-regulation of FOXD3 in breast cancer tissues associated with shorter DFS. (A) The FOXD3 mRNA expression in 20 cases of primary breast cancer tissues and the paired adjacent normal breast. (B) The FOXD3 mRNA expression in 160 breast cancer samples classified according tumor stage. (C) Kaplan–Meier survival analysis according to FOXD3 expression levels.

control cells (Fig. 2C). Furthermore, cells transfected with FOXD3 siRNAs grew much faster than the control cells by MTT analysis (Fig. 2D). Next, we performed transwell assay to evaluate the effects of FOXD3 on the invasive potential of MDA-MB-231 cells. As shown in Fig. 2E, cells transfected with FOXD3 siRNAs display higher ability of invasion than the control cells. Together, these results indicate that FOXD3 deficiency stimulates breast cancer cell proliferation and invasion *in vitro*.

3.3. Overexpression of FOXD3 inhibits tumor growth and metastasis in vivo

Next, we established a stable FOXD3-overexpressed MDA-MB-231-luc cell line. The RT-PCR and western blot analyses showed an increased level of FOXD3 expression in FOXD3-transfected cells (Fig. 3A and B). The Colony formation analysis showed that FOXD3-transfected cells formed fewer colonies than control cells (Fig. 3C). Furthermore, FOXD3-transfected cells grew much slower than the control cells by MTT analysis (Fig. 3D). Next, we performed transwell assay to evaluate the effects of FOXD3 on the invasive potential of MDA-MB-231-luc cells. As shown in Fig. 3E, FOXD3-transfected cells display lower ability of invasion than the control cells. The 231-FOXD3 and control cells were inoculated into the SCID mice (1×10^7 per mouse) to evaluate the effect of FOXD3 on breast tumor growth and metastasis *in vivo*. As determined by bioluminescence imaging of luciferase activity, 231-FOXD3 cells caused a decreased ability of metastasis than control cells (Fig. 3F). The tumor growth of FOXD3-transfected cells were significantly decreased in mice than control (Fig. 3G). These results indicate that FOXD3 inhibits breast cancer proliferation and metastasis *in vivo*.

3.4. Deletion of FOXD3 is linked to the EMT-like phenotype

As previous described, depletion of FOXD3 enhances the metastatic ability of breast cancer *in vitro*, whereas overexpression of FOXD3 inhibits metastatic ability *in vivo*. We therefore speculated that the contributions of FOXD3 to breast cancer metastasis might involve in the process of an EMT. To test this contention, we next investigated whether depletion of FOXD3 was capable inducing EMT. Compared with control cells, depletion of FOXD3 made MDA-MB-231 cells underwent a morphological change, which was more spindle-like and fibroblastic (Fig. 4A). EMT implicates loss of epithelial markers, concomitant acquisition of mesenchymal

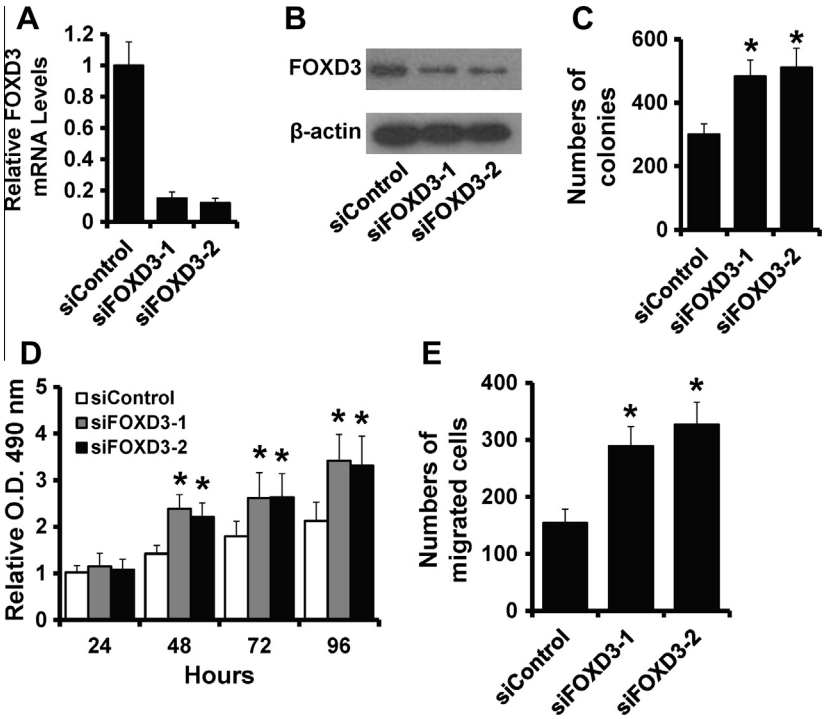

Fig. 2. FOXD3 deficiency promotes breast cancer proliferation and invasion *in vitro*. (A and B) FOXD3 expression in FOXD3 siRNAs-transfected MDA-MB-231 cells and control cells by RT-PCR (Aa) and western blot (B) assays. (C) Colony analysis of the cells as in (A). (D) MTT analysis of the cells as in (A). (E) Transwell analysis of the cells as in (A). *$P < 0.05$.

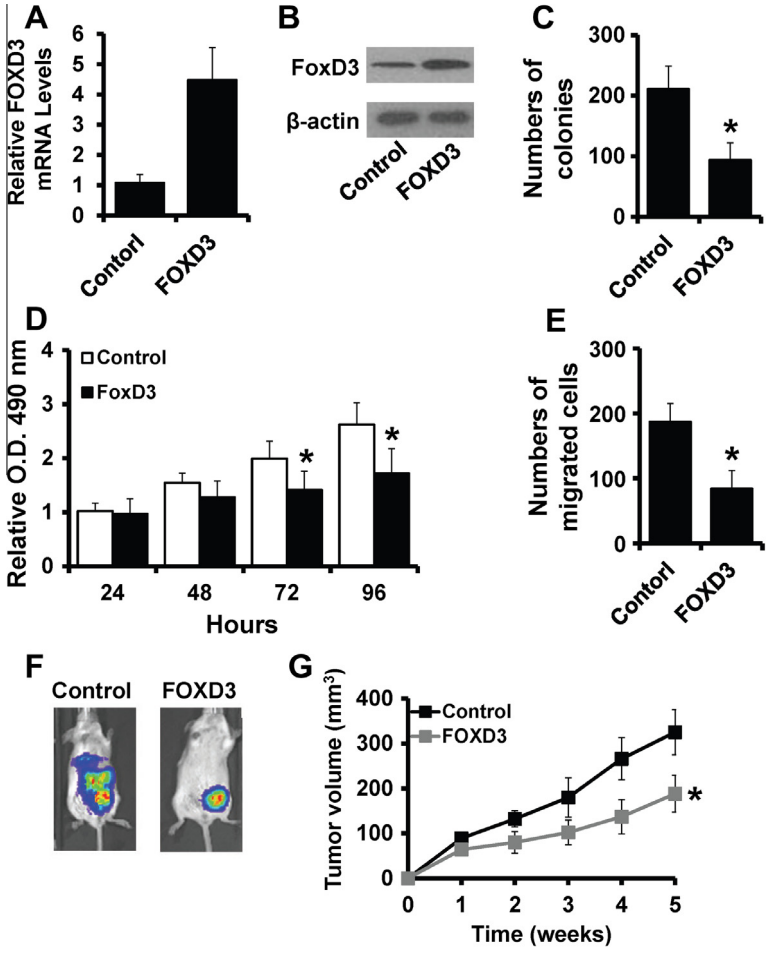

Fig. 3. FOXD3 inhibits breast cancer cell proliferation and metastasis *in vivo*. (A and B) FOXD3 expression in FoxD3-transfected MDA-MB-231-luc cells and control cells by RT-PCR (A) and western blot (B) assays. (C) Colony analysis of the cells as in (A). (D) MTT analysis of the cells as in (A). (E) Transwell analysis of the cells as in (A). (F) Representative photos of general physical condition of xenograft mice carrying MDA-MB-231-luc cells expressing either control or FOXD3 on 5 weeks after injection are shown. (G) The tumor volumes were recorded at indicated times. $^{*}P < 0.05$.

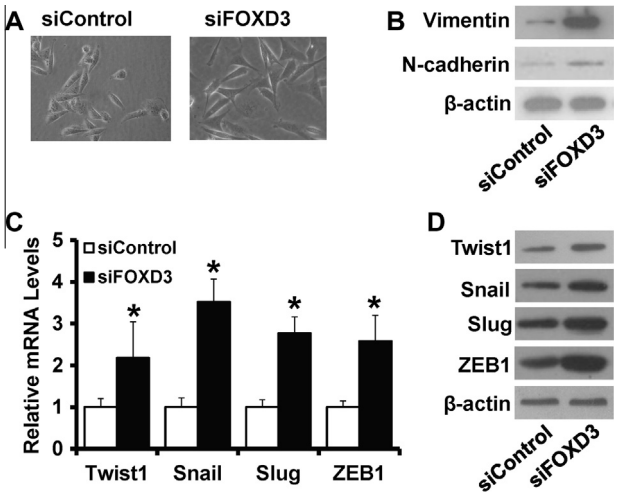

Fig. 4. Depletion of FOXD3 expression is linked to the EMT-like phenotype. (A) Phase contrast images of FOXD3-depleted and control MDA-MB-231cells are shown. (B) Western analysis for the indicated EMT markers in FOXD3-depleted and control MDA-MB-231 cells. (C) RT-PCR analysis of EMT-related transcription factors in FOXD3-depleted and control MDA-MB-231 cells. (D) Western analysis of EMT-related transcription factors in the FOXD3-depleted and control MDA-MB-231 cells. $^{*}P < 0.05$.

ones. Our results indicated that depletion of FOXD3 led to an increased expression of N-cadherin and Vimentin by Western blot assay (Fig. 4B). Furthermore, the EMT-related transcription factors, Twist1, Snail, Slug, and ZEB1 were significantly increased in FOXD3-transfected cells compared to control cells by RT-PCR (Fig. 4C) and Western blot assays (Fig. 4D). Collectively, these results indicate that depletion of FOXD3 promotes breast cancer progression through induction of EMT.

4. Discussion

Forkhead box (FOX) proteins, an evolutionarily conserved family of transcriptional factors, mediate a wide spectrum of biological processes, such as differentiation, metabolism, proliferation, migration, apoptosis and even tumorigenesis [13,14]. FoxD3 is a forkhead transcription factor with well-established roles in stem cell biology and lineage specification from the neural crest. FoxD3 inhibits cell proliferation of melanoma cells through up-regulation of the clyclin-dependent kinase inhibitor P21 [15]. FOXD3 have been demonstrated to be a tumor suppressor in gastric cancer [4]. FOXD3 inhibits melanoma cell migration and invasion, and serves as a B-RAF target and novel cell cycle repressor [11]. In the current study, we demonstrated that FOXD3 is down-regulated in breast cancer tissues and down-regulation of FOXD3 correlated to advanced stage, which is consistent with the study that down-regulation of FOXD3 is associated with lymph node metastases in

invasive ductal breast carcinoma [16]. Furthermore, our results revealed that depletion of FOXD3 expression in human breast cancer cells promotes cell proliferation and invasion *in vitro*. Moreover, we showed that ectopic overexpression of FOXD3 inhibits human breast cancer cells proliferation and invasion both *in vitro* and *in vivo*. These results indicated that FOXD3 as a potential tumor suppressor in breast carcinogenesis.

EMT occurs frequently during normal development in processes such as mesoderm and neural crest cell formation. During cancer progression, EMT is also crucial for loss of cell polarity of epithelial cells, thus facilitating migratory and invasive behavior [17,18]. Our results indicated that depletion of FOXD3 induces EMT programs of human breast cancer cells, including up-regulation of mesenchymal markers (N-cadherin and Vimentin) and EMT-related transcription factors, such as Twist1, Snail, Slug, and ZEB1, and a decrease in cell migration and invasion *in vitro*. These findings and the analysis of breast cancer cell lines demonstrated that FOXD3 is not only linked to cell proliferation but inhibits EMT by suppression of essential effectors of the process such as Twist1, Snail, Slug, and ZEB1. As a member of FOX family, FOXD3 is characterized by a monomeric DNA binding domain for nuclear localization and transcriptional regulation [4,6]. Thus, the future study should clarify its transcriptional targeted genes in carcinogenesis.

In summary, we demonstrated that FOXD3 is down-regulated in human breast cancer, and FOXD3 exhibits tumor suppressor activity that affects the breast cancer cell growth, invasion, and metastasis both *in vitro* and *in vivo* through induction of EMT. This study suggests that FOXD3 may be useful for breast therapy.

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Conflict of interest

The authors have no financial interests in or financial conflict with the subject matter discussed in this manuscript.

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References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, Global cancer statistics, CA Cancer J. Clin. 61 (2011) 69–90.
[2] A.P. Feinberg, B. Tycko, The history of cancer epigenetics, Nat. Rev. Cancer 4 (2004) 143–153.
[3] L.A. Hanna, R.K. Foreman, I.A. Tarasenko, D.S. Kessler, P.A. Labosky, Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo, Genes Dev. 16 (2002) 2650–2661.
[4] D.M. Tompers, R.K. Foreman, Q. Wang, M. Kumanova, P.A. Labosky, Foxd3 is required in the trophoblast progenitor cell lineage of the mouse embryo, Dev. Biol. 285 (2005) 126–137.
[5] Y. Liu, P.A. Labosky, Regulation of embryonic stem cell self-renewal and pluripotency by Foxd3, Stem Cells 26 (2008) 2475–2484.
[6] G. Pan, J. Li, Y. Zhou, H. Zheng, D. Pei, A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal, FASEB J. 20 (2006) 1730–1732.
[7] R. Kos, M.V. Reedy, R.L. Johnson, C.A. Erickson, The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos, Development 128 (2001) 1467–1479.
[8] M. Barembaum, M. Bronner-Fraser, Early steps in neural crest specification, Semin. Cell Dev. Biol. 16 (2005) 642–646.
[9] P. Katiyar, A.E. Aplin, FOXD3 regulates migration properties and Rnd3 expression in melanoma cells, Mol. Cancer Res. 9 (2011) 545–552.
[10] E.V. Abel, A.E. Aplin, FOXD3 is a mutant B-RAF-regulated inhibitor of G(1)-S progression in melanoma cells, Cancer Res. 70 (2010) 2891–2900.
[11] A.S. Cheng, M.S. Li, W. Kang, V.Y. Cheng, J.L. Chou, S.S. Lau, M.Y. Go, C.C. Lee, T.K. Ling, E.K. Ng, J. Yu, T.H. Huang, K.F. To, M.W. Chan, J.J. Sung, F.K. Chan, Helicobacter pylori causes epigenetic dysregulation of FOXD3 to promote gastric carcinogenesis, Gastroenterology 144 (2013) 122–133 e129.
[12] D. Li, H. Mei, M. Qi, D. Yang, X. Zhao, X. Xiang, J. Pu, K. Huang, L. Zheng, Q. Tong, FOXD3 is a novel tumor suppressor that affects growth, invasion, metastasis and angiogenesis of neuroblastoma, Oncotarget 4 (2013) 2021–2044.
[13] P.J. Wijchers, J.P. Burbach, M.P. Smidt, In control of biology: of mice, men and Foxes, Biochem. J. 397 (2006) 233–246.
[14] S.S. Myatt, E.W. Lam, The emerging roles of forkhead box (FOX) proteins in cancer, Nat. Rev. Cancer 7 (2007) 847–859.
[15] A. Astolfi, M. Nannini, M.A. Pantaleo, M. Di Battista, M.C. Heinrich, D. Santini, F. Catena, C.L. Corless, A. Maleddu, M. Saponara, C. Lolli, V. Di Scioscio, S. Formica, G. Biasco, A molecular portrait of gastrointestinal stromal tumors: an integrative analysis of gene expression profiling and high-resolution genomic copy number, Lab. Invest. 90 (2010) 1285–1294.
[16] H. Zhao, D. Chen, J. Wang, Y. Yin, Q. Gao, Y. Zhang, Downregulation of the transcription factor, FoxD3, is associated with lymph node metastases in invasive ductal carcinomas of the breast, Int. J. Clin. Exp. Pathol. 7 (2014) 670–676.
[17] M.A. Nieto, Epithelial–mesenchymal transitions in development and disease: old views and new perspectives, Int. J. Dev. Biol. 53 (2009) 1541–1547.
[18] R. Kalluri, R.A. Weinberg, The basics of epithelial–mesenchymal transition, J. Clin. Invest. 119 (2009) 1420–1428.