

Dicer knockdown induces fibronectin-1 expression in HEK293T cells via induction of Egr1

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ABSTRACT

Background: Dicer is a multidomain ribonuclease III enzyme involved in the biogenesis of microRNAs (miRNAs) and small interfering RNAs (siRNAs); depletion of Dicer was found to impair the migration of endothelial cells.

Methods: siRNA transfection, cell migration assay, real-time RT-PCR, chromatin immunoprecipitation, Western blotting, ELISA, caspase-3 activity assay, and annexin-V-FITC assay were utilized.

Results: Knockdown of Dicer impairs the migratory capacity of HEK293T cells and induces fibronectin-1. The upregulation of fibronectin-1 is dependent on Egr1. Fibronectin-1/Dicer double-knockdown cells showed a marked increase in apoptosis compared with fibronectin-1 single knockdown cells.

Conclusions: Decreased Dicer expression induces fibronectin-1 expression via an Egr1-dependent manner.

General significance: Our data suggest that upregulation of fibronectin-1 protects Dicer knockdown HEK293T cells against apoptosis.

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1. Introduction

Dicer is a ribonuclease III-like enzyme that plays a key role in the biogenesis of miRNAs and siRNAs [1]. In addition, Dicer is essential for the regulation of chromatin structure and function, DNA replication timing, genome stability, and cellular senescence [2–6]. Targeted knockout of Dicer leads to the inability to maintain the stem cell population during early mouse development [7], aberrant T cell differentiation [8], meiosis arrest of mouse oocytes [9], dilated cardiomyopathy [10], poor proliferation of primordial germ cells and spermatogonia [11], aberrant B cell development [12], glomerular disease [13], degeneration of the mouse retina [14], and neurological disorders [15].

Depletion of Dicer was found to impair the migration of endothelial cells [16,17], and fibronectin-1 (FN1) was upregulated in Dicer knockdown endothelial cells [18]. Here, we demonstrate that knockdown of Dicer impairs the migratory capacity of HEK293T cells and induces fibronectin-1 expression. We also explore the molecular mechanisms of Dicer knockdown-induced upregulation of fibronectin-1 and the biological significance of elevated fibronectin-1 expression.

2. Materials and methods

2.1. Cell culture and RNAi

HEK293T cells were cultured in RPMI 1640 medium supplemented with 10% FBS. siRNAs were prepared by in vitro transcription with the

Silencer siRNA Construction kit (Ambion). Target sequences of siRNAs were as follows: Dicer siRNA (AAGCTTACCTTCTCCAGGCT), fibronectin-1 siRNA (GTGGTCCTGTCGAAGTATT), Egr1 siRNA (CCATGGACAAC-TACCCTAA), and the control siRNA was designed according to the sequence of the non-silencing control siRNA (AATTCTCCGAACGTGT-CACGT) from Qiagen. Transfection was performed using siPORT NeoFX (Ambion), according to the manufacturer's instructions.

2.2. Migration assay

Cells were trypsinized 48 h after transfection with siRNAs and placed in the upper chamber of a modified Boyden chamber (2×10^4 cells per chamber; pore size, 8 μm ; BD Biosciences) coated with 10 $\mu\text{g}/\text{mL}$ fibronectin-1. The chamber was placed in serum-free DMEM. After 6 h, cells were fixed with 4% paraformaldehyde. Cells on the upper part of the chamber that did not migrate were removed with a cotton swab, and cells on the lower part of the chamber were stained with propidium iodide. To test whether the secreted fibronectin-1 inhibited cell migration, different concentrations of fibronectin-1 were added to the medium in upper chamber.

2.3. Western blotting and ELISA

Cells were lysed in RIPA buffer. Total protein was measured using the Bradford protein assay. Samples were denatured at 100 °C for 5 min. Equal amounts of total protein were loaded to each well for electrophoresis in SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride microporous membranes (Millipore). Membranes were then incubated with primary antibody, followed by

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incubation with horseradish peroxidase–linked secondary antibodies. The primary antibodies included anti-Dicer (ab14601; Abcam), anti-fibronectin-1 (Abcam), anti-Egr1 and anti- β -actin (both from Santa Cruz Biotechnology, Inc.). Fibronectin-1 in the culture supernatant was quantified using the ELISA kit (EK0349; Boster, Wuhan, China).

2.4. Quantitative real-time RT–PCR

Total RNA was prepared using TRIZOL (Invitrogen) and incubated with RNase-free Dnase I (Fermentas) for 30 min. The DNA-free RNA was reverse transcribed using MMLV reverse transcriptase (TOYOBO Bio-Technology) according to the manufacturer's instructions. Samples prepared without reverse transcription served as negative control templates. SYBR green PCR was performed in triplicate, using the ABI PRISM 7300 Sequence Detection system (Applied Biosystems). All samples were normalized to the signal generated from GAPDH. Primer sequences (forward and reverse) were as follows: Dicer, TCCACGAGTCACAATCAACACGG and GGGTCTGCATTAGGAGCTAGATGAG; ERG1, GCCTGCGACATCTGTGGAA and CGCAAGTGGATCTTGGTATGC; GAPDH, ATGACATCAAGAAGGTGGTG and CATACCAGGAAATGAGCTTG; fibronectin-1, GGAGTTGATTATACATCACTG and TTTCTGTTTGATCTGGACCT; ITGA5, TGCCTCCCTCACATCTTC and TGCTTCTGCCAGTCCAGC.

2.5. RNAPol–ChIP

Chromatin immunoprecipitation was performed as reported previously [20]. DNA immunoprecipitated with RNA pol II antibody (sc-899; Santa Cruz Biotechnology) was amplified with primer pairs in triplicate, and amplification was monitored using SYBR green fluorescence on the ABI PRISM 7300 Sequence Detection system. Samples prepared without antibody served as negative controls. All samples were normalized to the signal generated from β -actin. Primer sequences (forward and reverse) were as follows: Fibronectin-1, TCCTTGCTGGTATCATGGCAG and AGACCCAGGCTTCTCATACTGA; ACTB, TTCCTGGGCATGGAGTC and CAGGTCTTTCGGGATGTC.

2.5. Apoptotic assays

The activity of caspase-3 was determined using the Caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China). Cell lysates were incubated in a 96-well microtitre plate with 20 ng Ac-DEVD-pNA for 1 h at 37 °C. Caspase activity was measured by cleavage of the Ac-DEVD-pNA substrate to pNA, one unit is the amount of enzyme that will cleave 1.0 nmol of the colorimetric substrate Ac-DEVD-pNA per hour at 37 °C under saturated substrate concentrations. Annexin-V–FITC assay was conducted using annexin-V/PI apoptosis kit (Biovision) according to the manufacturer's instructions. The cells were labeled with annexin-V–FITC and propidium iodide for 5 min and washed once with PBS; the fluorescence was then detected with FACSaria (BD Biosciences).

2.6. Statistical methods

All statistical analyses were performed with Microsoft Excel software. All graphs represented the mean \pm SD from three independent experiments. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Knockdown of Dicer impairs the migratory capacity of HEK293T cells and induces the expression of fibronectin-1

Although migration of Dicer-deficient endothelial cells on a collagen matrix was not affected [16], it was significantly impaired

when fibronectin was used as the matrix [17,18]. To test whether Dicer could regulate the migration of other cells, we knocked down Dicer in HEK293T cells. Our results indicate that knockdown of Dicer inhibited the migration of HEK293T cells towards a fibronectin-1 matrix (Fig. 1A). Increased synthesis of fibronectin-1 inhibits smooth muscle cell migration [19], and fibronectin-1 is upregulated in Dicer knockdown endothelial cells [18]. Using quantitative RT–PCR, we found that fibronectin-1 was upregulated in Dicer knockdown HEK293T cells compared to control siRNA-transfected cells (Fig. 2A). The upregulation of fibronectin-1 was confirmed by Western blotting (Fig. 3B). In accordance with the previous finding that inhibition of cell migration by the AT₂ receptor occurs simultaneously with increased fibronectin-1 secretion [19], we found that the secreted fibronectin-1 in the culture medium was increased in Dicer knockdown cells (Fig. 2C). To confirm that the impact of fibronectin-1 on migration is caused by its secretion, we added fibronectin-1 to the culture medium and found that migration was inhibited in a concentration-dependant manner (Fig. 1B).

3.2. Egr-1 is required for the induction of fibronectin-1 in Dicer knockdown HEK293T cells

To dissect the molecular mechanisms responsible for the induction of fibronectin-1 in Dicer knockdown HEK293T cells, we performed chromatin immunoprecipitation, using anti-RNA polymerase II antibody (RNAPol–ChIP) [20] to check the transcription of this gene. Transcription of fibronectin-1 increased in Dicer knockdown cells (Fig. 2B). Egr1 binds to the fibronectin-1 promoter and is required for the induction of fibronectin-1 matrix production by hepatocyte

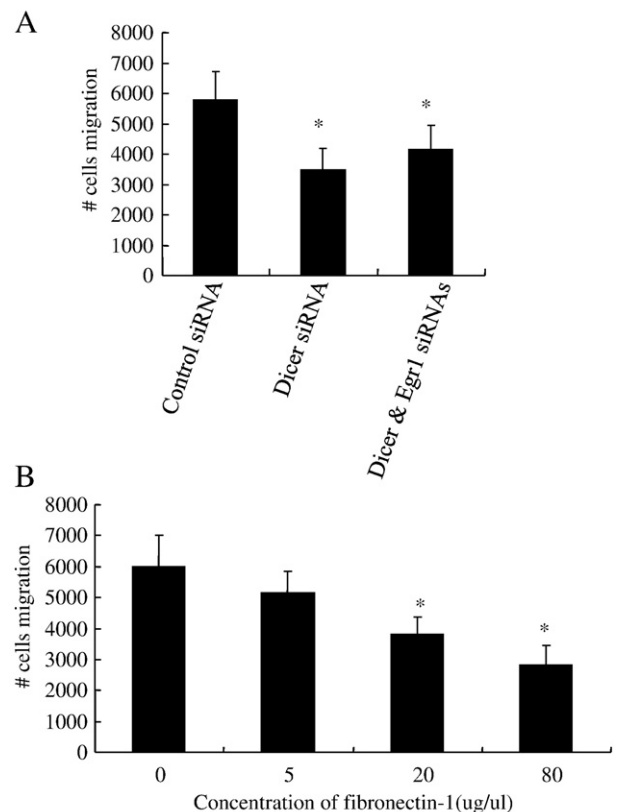


Fig. 1. Inhibition of HEK293T cell migration by knocking down Dicer expression or by fibronectin-1. (A) Cells were plated in the upper chamber 48 h after transfection. Six hours after plating, cells that had migrated to the bottom chamber were fixed and counted. * $P < 0.05$ compared to cells transfected with control siRNA. (B) Fibronectin-1 inhibited cell migration, fibronectin-1 was added to the medium in the upper chamber at different concentrations, and cell migration was carried out as described in A. * $P < 0.05$ compared to cells without addition of fibronectin-1.

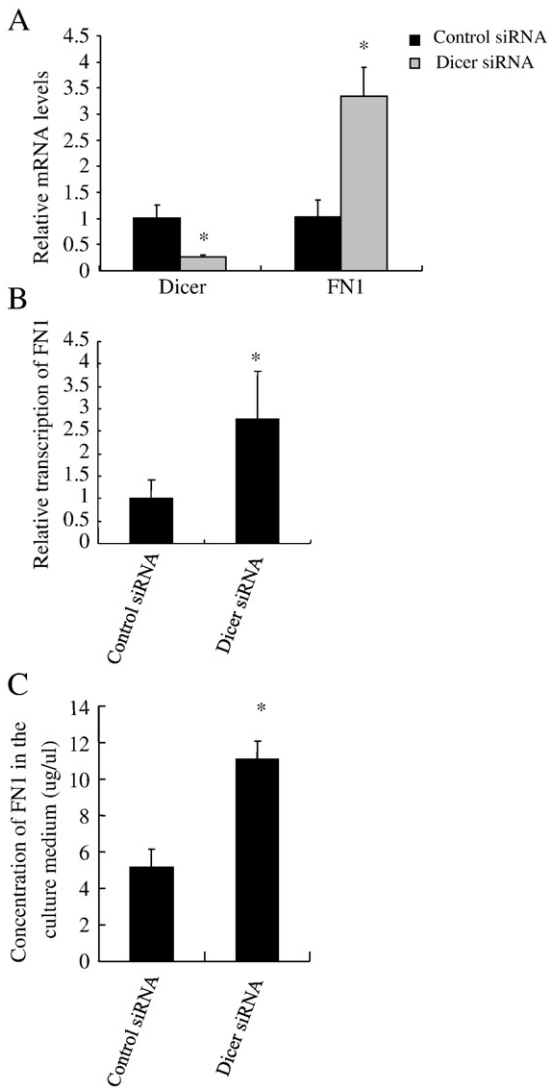


Fig. 2. Upregulation of fibronectin-1 in Dicer knockdown cells. (A) Real-time RT-PCR analysis of fibronectin-1 mRNA levels 48 h after Dicer siRNA transfection; (B) RNAPol-ChIP indicated that the transcription of fibronectin-1 was increased in Dicer knockdown cells compared to control cells. The value from cells transfected with control siRNA was arbitrarily set at 1. (C) ELISA indicated that the secreted fibronectin-1 in the culture medium was increased in Dicer knockdown cells. * $P < 0.05$ compared to cells transfected with control siRNA.

growth factor [21]. Our previous work indicated that Egr1 is upregulated in Dicer knockdown HEK293T cells [5]. Therefore, we deduced that the upregulation of fibronectin-1 is dependent on Egr1. To test this hypothesis, we simultaneously knocked down Dicer and Egr1 in HEK293T cells and found that knockdown of Egr1 prevented the upregulation of fibronectin-1 induced by Dicer knockdown (Fig. 3). Consequently, knockdown of Egr1 partially rescued the migratory capability of Dicer knockdown cells (Fig. 1A).

3.3. Fibronectin-1/Dicer double knockdown cells show a marked increase in apoptosis compared with fibronectin-1 single knockdown cells

Silencing Dicer expression decreases endothelial cell viability and sensitizes them to apoptosis in response to serum starvation [17,18]. To test whether decreased Dicer expression led to apoptosis in HEK293T cells, we checked the activity of caspase 3, whose activation plays a key role in initiation of cellular events during the early apoptotic process, in Dicer knockdown and control cells. Although

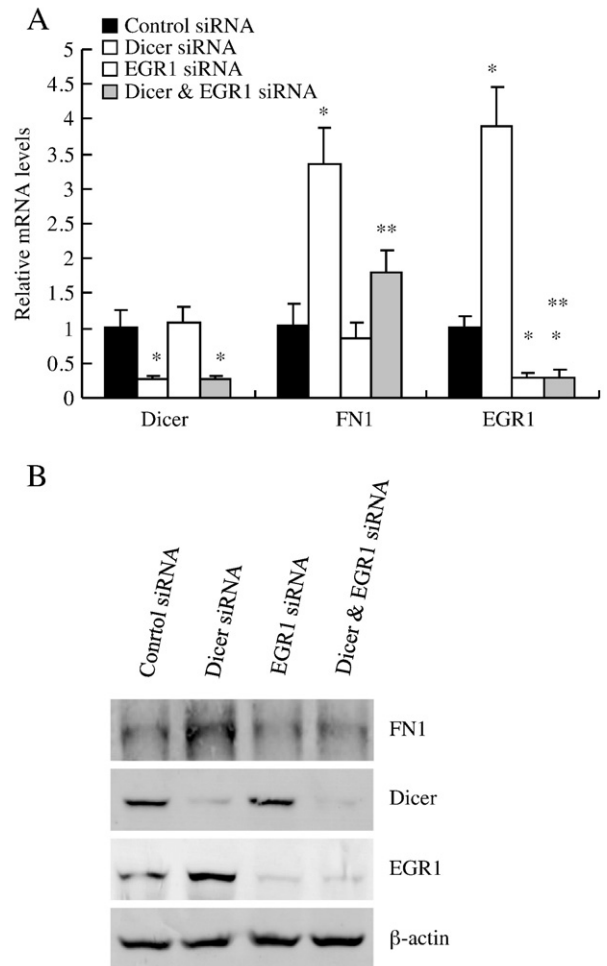


Fig. 3. Egr1 is required for the elevated transcription of fibronectin-1 in Dicer knockdown HEK293T cells. (A) Real-time RT-PCR indicated that the upregulation of fibronectin-1 in response to Dicer knockdown was blocked by Egr1 silencing. The mRNA level in cells transfected with control siRNA was arbitrarily set at 1. * $P < 0.05$ compared to cells transfected with control siRNA; ** $P < 0.05$ compared to cells transfected with Dicer siRNA alone. (B) Western blotting confirmed that depletion of Egr1 prevented the induction of fibronectin-1 in Dicer knockdown cells.

Dicer knockdown did not lead to a significant increase in caspase 3 activity, fibronectin-1 knockdown resulted in a 2-fold increase in caspase 3 activity. Simultaneous knockdown of Dicer and fibronectin-1 resulted in a marked increase in caspase 3 activity, and the increase in caspase 3 activity can be blocked partially by pre-coating the culture plates with 10 μ g/mL fibronectin-1 (Fig. 4A and B). Consistent with the caspase 3 assay, annexin-V staining indicated that knockdown of Dicer did not lead to apoptosis; however, simultaneous knockdown of Dicer and fibronectin-1 enhanced the apoptosis induced by fibronectin-1 knockdown, and the apoptosis can be rescued by pre-coating the culture plates with fibronectin-1 (Fig. 4C). Therefore, our observation suggests that upregulation of fibronectin-1 in Dicer knockdown cells may protect cells from apoptosis.

4. Discussion

In this study, we demonstrated that fibronectin-1 and EGR1 were upregulated in Dicer knockdown HEK293T cells, and that upregulation of fibronectin-1 by Dicer knockdown was prevented by genetic inhibition of EGR1, which indicated that upregulation of fibronectin in response to Dicer knockdown was caused by EGR1. Knockdown of Dicer compromises miRNA biogenesis in HEK293T cells [3], and

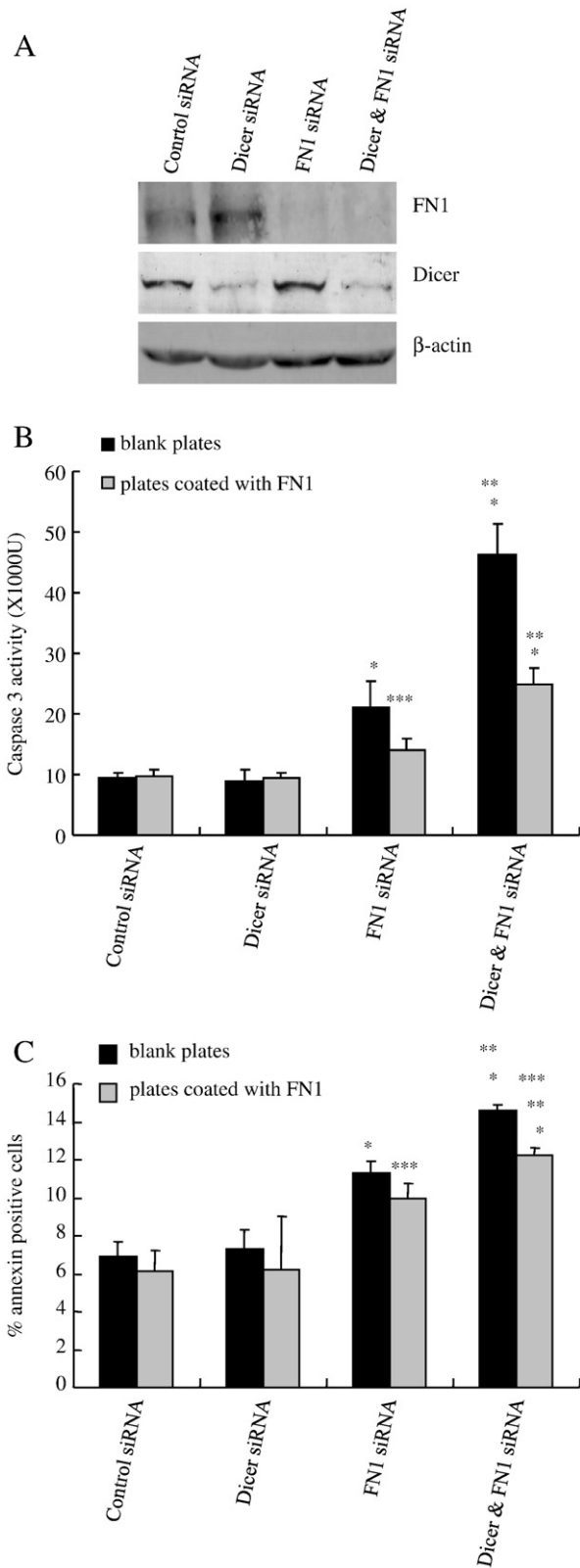


Fig. 4. Fibronectin-1 protects Dicer knockdown HEK293T cells against apoptosis. Cells were transfected with control siRNA, Dicer siRNA, fibronectin-1 siRNA, or co-transfected with Dicer siRNA and fibronectin-1 siRNA. The culture plates were either coated with BSA or fibronectin-1. Cell apoptosis was determined 48 h after transfection. (A) Western blotting confirmed the knockdown efficiency. (B) Caspase 3 activity. (C) Percentage of annexin-V-positive cells. * $P < 0.05$ compared to cells transfected with control siRNA; ** $P < 0.05$ compared to cells transfected with fibronectin-1 siRNA alone; *** $P < 0.05$ compared to blank plates.

miRNAs directly or indirectly regulate the expression of fibronectin-1 [22–24]. Given that knocking down EGR1 alone did not result in a significant decrease of fibronectin-1 and that upregulation of fibronectin-1 in response to Dicer knockdown was not prevented completely by double knockdown of EGR1 and Dicer, further study is needed to elucidate whether upregulation of fibronectin-1 is also the consequence of miRNA misregulation.

Depletion of Dicer was found to impair the migratory capacity of endothelial cells [16,17]. In this study, we demonstrated that knockdown of Dicer also inhibited the migration of HEK293T cells. Increased synthesis of fibronectin-1 inhibits smooth muscle cell migration [19]; therefore, the reduced migration of HEK293T cells may be the consequence of elevated synthesis and secretion of fibronectin-1. Although the expression of integrin α_5 , the major fibronectin receptor, was not significantly increased in Dicer knockdown cells (data not shown), we cannot rule out the possibility that depletion of Dicer differentially modifies other fibronectin receptors, thereby selectively impairing cell functions dependent on the fibronectin matrix [25].

Fibronectin-1 protects prostate cancer cells from TNF- α -induced apoptosis [26], and knockdown of fibronectin-1 induces mitochondria-dependent apoptosis in rat mesangial cells [27]. Consistent with these observations, we demonstrated that knockdown of fibronectin-1 in HEK293T cells led to apoptosis. Although Dicer knockdown did not cause apoptosis in HEK293T cells, fibronectin-1/Dicer double knockdown cells showed a marked increase in apoptosis compared with fibronectin-1 single knockdown cells. Therefore, we speculate that Dicer knockdown causes apoptosis if without upregulation of fibronectin-1.

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References

- [1] Y. Tomari, P.D. Zamore, Perspective: machines for RNAi, *Genes Dev.* 19 (2005) 517–529.
- [2] J.C. Peng, G.H. Karpen, H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability, *Nat. Cell Biol.* 9 (2007) 25–35.
- [3] K.F. Tang, Y. Wang, P. Wang, M. Chen, Y. Chen, H.D. Hu, P. Hu, B. Wang, W. Yang, H. Ren, Upregulation of PHLDA2 in Dicer knockdown HEK293 cells, *Biochim. Biophys. Acta* 1770 (2007) 820–825.
- [4] H.F. Jørgensen, V. Azaara, S. Amols, M. Spivakov, A. Terry, T. Nesterova, B.S. Cobb, B. Ramsahoye, M. Merkenschlager, A.G. Fisher, The impact of chromatin modifiers on the timing of locus replication in mouse embryonic stem cells, *Genome Biol.* 8 (2007) R169.
- [5] K.F. Tang, H. Ren, J. Cao, G.L. Zeng, J. Xie, M. Chen, L. Wang, C.X. He, Decreased Dicer expression elicits DNA damage and up-regulation of MICA and MICB, *J. Cell Biol.* 182 (2008) 233–239.
- [6] R. Mudhasani, Z. Zhu, G. Hutvagner, C.M. Eischen, S. Lyle, L.L. Hall, J.B. Lawrence, A. N. Imbalzano, S.N. Jones, Loss of miRNA biogenesis induces p19^{Arf}-p53 signaling and senescence in primary cells, *J. Cell Biol.* 181 (2008) 1055–1063.
- [7] E. Bernstein, S.Y. Kim, M.A. Carmell, E.P. Murchison, H. Alcorn, M.Z. Li, A.A. Mills, S. J. Elledge, K.V. Anderson, G.J. Hannon, Dicer is essential for mouse development, *Nat. Genet.* 35 (2003) 215–217.
- [8] S.A. Muljo, K.M. Ansel, C. Kanellopoulou, D.M. Livingston, A. Rao, K. Rajewsky, Aberrant T cell differentiation in the absence of Dicer, *J. Exp. Med.* 202 (2005) 261–269.
- [9] E.P. Murchison, P. Stein, Z. Xuan, H. Pan, M.Q. Zhang, R.M. Schultz, G.J. Hannon, Critical roles for Dicer in the female germline, *Genes Dev.* 21 (2007) 682–693.
- [10] J.F. Chen, E.P. Murchison, R. Tang, T.E. Callis, M. Tatsuguchi, Z. Deng, M. Rojas, S.M. Hammond, M.D. Schneider, C.H. Selzman, G. Meissner, C. Patterson, G.J. Hannon, D.Z. Wang, Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 2111–2116.
- [11] K. Hayashi, S.M. Chuva de Sousa Lopes, M. Kaneda, F. Tang, P. Hajkova, K. Lao, D. O'Carroll, P.P. Das, A. Tarakhovskiy, E.A. Miska, M.A. Surani, MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis, *PLoS ONE* 3 (2008) e1738.
- [12] S.B. Koralov, S.A. Muljo, G.R. Galler, A. Krek, T. Chakraborty, C. Kanellopoulou, K. Jensen, B.S. Cobb, M. Merkenschlager, N. Rajewsky, K. Rajewsky, Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage, *Cell* 132 (2008) 860–874.

- [13] J.J. Ho, P.A. Marsden, Dicer cuts the kidney, *J. Am. Soc. Nephrol.* 19 (2008) 2043–2046.
- [14] D. Damiani, J.J. Alexander, J.R. O'Rourke, M. McManus, A.P. Jadhav, C.L. Cepko, W.W. Hauswirth, B.D. Harfe, E. Strettoi, Dicer inactivation leads to progressive functional and structural degeneration of the mouse retina, *J. Neurosci.* 28 (2008) 4878–4887.
- [15] T.H. Davis, T.L. Cuellar, S.M. Koch, A.J. Barker, B.D. Harfe, M.T. McManus, E.M. Ullian, Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus, *J. Neurosci.* 28 (2008) 4322–4330.
- [16] Y. Suárez, C. Fernández-Hernando, J.S. Pober, W.C. Sessa, Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells, *Circ. Res.* 100 (2007) 1164–1173.
- [17] A. Kuehbacher, C. Urbich, A.M. Zeiher, S. Dimmeler, Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis, *Circ. Res.* 101 (2007) 59–68.
- [18] S. Asada, T. Takahashi, K. Isodono, A. Adachi, H. Imoto, T. Ogata, T. Ueyama, H. Matsuura, H. Oh, Downregulation of Dicer expression by serum withdrawal sensitizes human endothelial cells to apoptosis, *Am. J. Physiol. Heart Circ. Physiol.* 295 (2008) H2512–H2521.
- [19] C. Chassagne, C. Adamy, P. Ratajczak, B. Gingras, E. Teiger, E. Planus, P. Oliviero, L. Rappaport, J.L. Samuel, S. Meloche, Angiotensin II AT(2) receptor inhibits smooth muscle cell migration via fibronectin cell production and binding, *Am. J. Physiol. Cell Physiol.* 282 (2002) C654–C664.
- [20] J. Sandoval, J.L. Rodríguez, G. Tur, G. Serviddio, J. Pereda, A. Boukaba, J. Sastre, L. Torres, L. Franco, G. López-Rodas, RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription, *Nucleic Acids Res.* 32 (2004) e88.
- [21] C. Gaggioli, M. Deckert, G. Robert, P. Abbe, M. Batoz, M.U. Ehrenguber, J.P. Ortonne, R. Ballotti, S. Tartare-Deckert, HGF induces fibronectin matrix synthesis in melanoma cells through MAP kinase-dependent signaling pathway and induction of Egr-1, *Oncogene* 24 (2005) 1423–1433.
- [22] X. Zhang, S. Liu, T. Hu, S. Liu, Y. He, S. Sun, Up-regulated microRNA-143 transcribed by nuclear factor kappa B enhances hepatocarcinoma metastasis by repressing fibronectin expression, *Hepatology* 50 (2009) 490–499.
- [23] S.W. Shan, D.Y. Lee, Z. Deng, T. Shatseva, Z. Jeyapalan, W.W. Du, Y. Zhang, J.W. Xuan, S.P. Yee, V. Siragam, B.B. Yang, MicroRNA MiR-17 retards tissue growth and represses fibronectin expression, *Nat. Cell Biol.* 11 (2009) 1031–1038.
- [24] Q. Wang, Y. Wang, A.W. Minto, J. Wang, Q. Shi, X. Li, R.J. Quigg, MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy, *FASEB J.* 22 (2008) 4126–4135.
- [25] A. Kuehbacher, C. Urbich, S. Dimmeler, Targeting microRNA expression to regulate angiogenesis, *Trends Pharmacol. Sci.* 29 (2008) 12–15.
- [26] M. Fornaro, J. Plescia, S. Chheang, G. Tallini, Y.M. Zhu, M. King, D.C. Altieri, L.R. Languino, Fibronectin protects prostate cancer cells from tumor necrosis factor- α -induced apoptosis via the AKT/survivin pathway, *J. Biol. Chem.* 278 (2003) 50402–50411.
- [27] D. Wu, X. Chen, D. Guo, Q. Hong, B. Fu, R. Ding, L. Yu, K. Hou, Z. Feng, X. Zhang, J. Wang, Knockdown of fibronectin induces mitochondria-dependent apoptosis in rat mesangial cells, *J. Am. Soc. Nephrol.* 16 (2005) 646–657.