The retinoblastoma 1 gene (RB1) modulates the proliferation of chicken preadipocytes

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ABSTRACT
1. The objective of this study was to reveal the role of chicken RB1 (Gallus gallus RB1, gRB1) in the proliferation of preadipocytes.
2. To measure gene expression of gRB1 in the proliferation of chicken preadipocyte, quantitative real-time PCR was used. The expression levels of gRB1 transiently increased during this process.
3. To detect the effect of gRB1 on the proliferation of chicken preadipocytes, MTT assay and cell-cycle analysis were performed. MTT assay showed that overexpression of gRB1 significantly suppressed (P < 0.05) the proliferation of chicken preadipocytes, and knockdown of gRB1 promoted the proliferation of chicken preadipocytes. Cell-cycle analysis showed that the proportion of preadipocytes in the G1 and G2 phases significantly increased (P < 0.05), and the proportion of preadipocytes in the S phase significantly decreased (P < 0.05) after up-regulation of the expression of gRB1. The proportion of preadipocytes in the S phase significantly increased (P < 0.05) after down-regulation of gRB1.
4. Quantitative real-time PCR was used to detect the effect of gRB1 on the expression of genes related to proliferation of chicken preadipocytes. Gene expression analysis showed that gRB1 knockdown promoted markers indicating proliferation of Ki-67 (MKi67) expression at 96 h (P < 0.05), and overexpression of gRB1 reduced MKi67 expression at 72 h (P < 0.05).
5. This study demonstrated that gRB1 inhibited preadipocyte proliferation at least in part by inhibiting the G1 to S phase transition.

Introduction
The selection for rapid growth in meat-type chickens has been accompanied by increased abdominal fat deposition (D’Andre et al. 2013). Excessive abdominal fat deposition can decrease feed conversion efficiency and carcass quality, leading to consumer rejection of meat (Demeure et al. 2013; de Verdal et al. 2013; Ramiah et al. 2014). Excessive deposition of abdominal fat is mainly due to the proliferation and differentiation of adipocytes in adipose tissues. Clarifying the genetic mechanisms related to proliferation and differentiation of adipocytes can help control excessive accumulation of abdominal fat.

In a previous study, a quantitative trait locus (QTL) with major effects on abdominal fat traits was mapped into a 3.7-Mbp (172.6–176.3 Mbp) region in chicken chromosome 1 using an F2 population of a broiler × layer cross (Liu et al. 2007). In this 3.7-Mbp region, 5 genes, including retinoblastoma 1 (RB1), were detected (Liu et al. 2008). In the examination of genome-wide selection signatures of abdominal fat content with the chicken 60 k single nucleotide polymorphism chip and the extended haplotype homozygosity assay, a number of genes in the significant core regions were detected, and the RB1 gene was detected again (Zhang et al. 2012). In addition, polymorphism analysis of the RB1 gene of Northeast Agricultural University broiler lines divergently selected for abdominal fat content indicated that the polymorphisms of RB1 were associated with abdominal fat content (unpublished data). RB1 is a tumour suppressor, encoded by the RB1 gene, which was first discovered in retinoblastoma tumours (Friend et al. 1986). The RB1 gene may participate in the biological process of cell cycle, neuronal differentiation and apoptosis (Weinberg 1995; Andrusiak et al. 2011; Yu et al. 2012). The information above suggests that RB1 plays an important role in the deposition of abdominal fat in broiler chickens. However, the mode of action of RB1 in chicken abdominal fat deposition remains unknown, hence, the aim of this study was to analyse the function of RB1 in the proliferation of chicken preadipocytes.

Materials and methods
Ethics statement
All animal work was conducted according to the guidelines for the Care and Use of Experimental Animals established by the Ministry of Science and Technology of the People’s Republic of China (approval number: 2006–398), and was approved by the Laboratory Animal Management Committee of Northeast Agricultural University.

Preparation and culture of cells
In the current study, the immortalised chicken preadipocyte line (ICP1) was used to analyse the function of RB1 in the proliferation of chicken preadipocytes. Primary chicken preadipocytes were isolated from the abdominal adipose tissue of 10-day-old Arbor Acres (AA) broilers, and were then infected with either chicken telomerase reverse transcriptase (chTERT) alone or in combination with chicken...
telomerase RNA (chTR) to establish an ICP1 (Wang et al. 2017). ICP1 survived >100 population doublings in vitro and displayed high telomerase activity with no sign of replicative senescence (Wang et al. 2017). This cell line shows great promise as an in vitro model for the investigation of chicken adipogenesis and lipid metabolism. ICP1 cells were maintained in Dulbecco’s Modified Eagle’s Medium Nutrient Mixture F12 (Ham) (DMEM F12) supplemented with 10% foetal serum and 1% penicillin and streptomycin. ICP1 cells were cultured until 90% confluence and then seeded in cell culture plates at a density of $1 \times 10^5$ cells/cm$^2$.

**Construction of RB1-overexpression plasmid and synthesis of siRNA-RB1**

To carry out the overexpression and RNA interference (RNAi) experiments, the RB1-overexpression plasmid was constructed and siRNA-RB1 was synthesised. The full-length coding sequence of chicken RB1 (Gallus gallus RB1, gRB1; GenBank accession number: NM_204419) was amplified from chicken abdominal adipose tissue cDNA using a pair of specific primers: sense, 5′-ACGTCCGACAACGGTCACTGAGGCGCC-3′; anti-sense, 5′-CCGCTGAGCAGCCCTGTTCTGTACGAAC-3′. The PCR product was cloned into pEasy-T1 Simple vector (TransGen, Beijing, China) and verified by direct sequencing. The full-length coding sequence of gRB1 was excised from the pEasy-T1-gRB1 plasmid by digesting with Sall and Xhol, and subcloned into the pCMV-HA vector (Clontech, Mountain View, CA, USA) to obtain the RB1-overexpression vector, pCMV-HA-gRB1.

The siRNA of the gRB1 selected for RNAi and the negative control were designed and synthesised by GenePharma Company (Shanghai, China; Table 1).

**RNA isolation and real-time RT-PCR**

To detect the expression levels of RB1, markers of Ki-67 (MKi67), Cyclin D1, proliferating cell nuclear antigen (PCNA) and transcription factor E2F1 (E2F1) genes used for real-time RT-PCR are shown in Table 2.

**Western blot analysis**

Western blot analysis was used to examine the expression of gRB1 overexpression. Chicken preadipocytes transfected with pCMC-HA-PPARα (M1, positive control), pCMV-HA-gRB1 (M2, gRB1 overexpression), or pCMV-HA (M3, control) vector for two days were homogenised in Radio Immunoprecipitation Assay buffer (PBS, pH 7.4, containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and a protease inhibitor cocktail) supplemented with protease inhibitors (1 mmol/l phenylmethylsulphonyl fluoride, 0.002 g/l aprotinin, and 0.002 g/l leupeptin). Cellular debris and lipids were eliminated by centrifuging the solubilised samples at 13,000 × g for 60 min at 4°C. Cell lysates were separated by 5–12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. To block non-specific binding, the membrane was incubated in blocking buffer (PBS with 5% nonfat dry milk) for 1 h at room temperature. After incubation with the primary antibody for HA-tag (1:200; Clontech) or β-ACTIN (1:1000; TransGen), a secondary horseradish peroxidase-conjugated antibody was added, and then a BeyoECL Plus kit (Beyotime, Jiang Su, China) was used for visualising the protein bands.

**The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

The MTT assay was used to examine the effects of overexpression or knockdown of gRB1 on ICP1 cells proliferation. ICP1 cells were transfected with the gRB1-overexpression plasmid or siRNA nucleotides for the knockdown of RB1 expression, as well as the control plasmid or negative control nucleotides (siNC), (TransGen). The primers for RB1, MKi67, Cyclin D1, PCNA, and E2F1 genes used for real-time RT-PCR are shown in Table 2.

**Table 2. Primer sequences used for real-time qRT-PCR.**

| Gene Name | GenBank Accession | Primers (5′-3′) |
|-----------|------------------|----------------|
| RB1       | NM_204419        | GCCTATATTTCTGAGGACAAC AAGACGCGACGAAACACCT |
| Cyclin D1 | NM_205381        | GCCTATATTCTGAGGACAAC AAGACGCGACGAAACACCT |
| PCNA      | NM_204170        | GTGCCTGGACCTGGTGT CCGTATCCGCATTGTCTTCT |
| MKi67     | XM_004020367     | AGCGTTGCGAAGATCCTGGCTT |
| NONO      | NM_001031532     | AGAAGACGAGCAAGAACGAAAC AGAAGACGAGCAAGAACGAAAC |
| E2F1      | NM_205219        | GGAATGGGGTGGTGGTGGGAGAT AGCGAGGGAGGGAAAAACAC |

1siNC = negative control; RB1 = Retinoblastoma 1.
respectively. After a 24-h incubation, these cells were passaged and seeded in 96-well plates at a concentration of 5000 cells per well. At the designated time points (12, 24, 48, 72, and 96 h), 20 ml of MTT solution (5 mg/ml; Sigma) were added to the medium, and the cells were incubated at 37°C for 4 h. After removal of the medium, 200 ml of dimethyl sulphoxide was added to each well, and the plates were shaken on a rocking platform at 60 × g for 15 min. The cell solution was collected and absorbance was recorded with an enzyme-labelled instrument (Bio-Rad, Hercules, CA, USA) at 492 nm. The experiments were repeated three times. For each biological repeat, there were two groups; a treatment and control, and cells were collected at 12, 24, 48, 72, and 96 h. At each time point, there were three wells for technical duplication. Only the result of one biological repeat was shown in the current study, because the results of the three biological repeats were similar to each other.

**Cell-cycle analysis**

After overexpression or knockdown of gRB1 for 48 h, ICP1 cells were trypsinised and subsequently fixed with ice-cold 70% ethanol for at least 1 h. After extensive washing, the cells were suspended in propidium iodide (PI) staining solution (Beyotime), slowly fully resuspended, and bated at 37°C for 30 min in the dark for subsequent FACSscan analysis (Becton-Dickinson, San Jose, CA, USA). Cell-cycle analysis was performed by the ModFit LT software (Verity Software House, Topsham, ME, USA). The experiments were repeated biologically three times. Each biological repeat included three wells as technical duplications. Only the result of one biological repeat was shown in the current study, because the results of the three biological repeats were similar to each other.

**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Comparisons between the two groups were performed using the unpaired two-tailed Student’s t-test. Statistical analysis among more than two groups was performed using ANOVA. All statistical analysis was carried out using SAS 9.1 (SAS Institute Inc., Cary, NC) software.

**Results**

**gRB1 gene expression during chicken preadipocyte proliferation**

The expression of gRB1 during the proliferation of chicken preadipocytes was analysed using real-time PCR. The cells were collected at 24, 48, 72 and 96 h, and the NONO gene was used as the internal reference. The results showed that the mRNA expression level of gRB1 in chicken preadipocytes initially increased and then decreased, with a peak at 72 h (Figure 1).

**Effect of gRB1 on chicken preadipocyte proliferation**

To explore the effect of gRB1 on the proliferation of chicken preadipocytes, overexpression and RNAi experiments were carried out. The pCMV-HA-PPARα plasmid with a length of 52.19 kDa, which were successively constructed previously, was used as the positive control, and the pCMV-HA was used as the negative control. The western blot analysis showed that pCMV-HA-gRB1 expressed the RB1 protein in chicken preadipocytes (Figure 2(a)). The MTT assay results showed that overexpression of gRB1 significantly suppressed the proliferation of ICP1 cells at 24 and 48 h (Figure 2(b)).

For the RNAi experiment, three interference fragments were used, which were named siRB1-409, siRB1-551 and siRB1-1680. The interference effect of these three different siRNAs was examined and the results indicated that siRB1-551 and siRB1-1680 significantly knocked down the gRB1 gene expression level compared with the control group (P > 0.05; Figure 2(c)). siRB1-1680 had the strongest interference effect, hence it was used to investigate the role of the gRB1 gene in the proliferation of chicken preadipocytes in the following experiment. The interference effect of siRB1-1680 on gRB1 expression was analysed at different time points and it was found that the knockdown effect was the most significant at 48 h (Figure 2(d)). The MTT results indicated that knockdown of gRB1 promoted the proliferation of ICP1 cells and this effect was significant at 96 h compared with the negative control (Figure 2(e)).

**Effect of gRB1 on the cell cycle of chicken preadipocytes**

The MTT results indicated that gRB1 suppressed the proliferation of preadipocytes. To explore whether the suppression was caused by changes in the cell cycle, the effects of gRB1 on the cell cycle of preadipocytes (ICP1) were analysed using overexpression and RNAi methods. The effects of gRB1 on the cell cycle of preadipocytes (ICP1) were examined at 48 h after transfection, because the overexpression and interference effects were the most significant at that time point. The overexpression results showed that the proportion of preadipocytes (ICP1) in the G1 and G2 phases significantly increased (P < 0.05), and the proportion of preadipocytes (ICP1) in the S phase significantly decreased (P < 0.05; Figure 3(a)). The RNAi results showed that the proportion of preadipocytes (ICP1) in the S phase significantly increased (P < 0.05) after the downregulation of the gRB1 gene (Figure 3(b)).
Effect of gRB1 on the expression levels of genes related to chicken preadipocyte proliferation

In the current study, the effects of gRB1 on the expression levels of genes related to the proliferation of chicken preadipocytes, including MKi67, PCNA, E2F1 and Cyclin D1, were analysed. ICP1 cells were seeded in 6-well plates. The time point at which cell confluence reached 30–50% was 0 h. At this time point, siRB1-1680, siNC, pCMV-HA-gRB1, or pCMV-HA was transfected into the preadipocytes. The preadipocytes were collected at 24, 48, 72, and 96 h, and RNA was extracted. The NONO gene was used as the internal reference. The results showed that the expression level of MKi67 significantly decreased at 72 h (P < 0.05) and the expression levels of E2F1, PCNA and Cyclin D1 did not significantly change after gRB1 overexpression (Figure 4(a)). In contrast, the expression level of MKi67 significantly increased at 96 h (P < 0.05), the expression levels of E2F1, PCNA and Cyclin D1 did not significantly change after gRB1 gene knockdown (Figure 4(b)).

Discussion

In the current study, overexpression and RNAi methods were used to reveal the role of gRB1 gene expression in the proliferation of chicken preadipocytes. The MTT results showed that overexpression of gRB1 suppressed preadipocyte proliferation and gRB1 knockdown promoted the proliferation of chicken preadipocytes, indicating that gRB1 suppressed the proliferation of preadipocytes in chickens. These results are consistent with studies that showed that mouse RB1 inhibited the proliferation of 3T3-L1 cells (Shang et al. 2007). Knockdown of RB1 in porcine could increase the mRNA levels of adipogenic markers, such as peroxisome proliferator-activated receptor gamma.
(PPARγ), fatty acid binding protein 4, adipocyte (aP2), lipoprotein lipase and adiponectin (Hu et al. 2015). Additionally, human RB1 inhibited the proliferation of cancer cells, including retinoblastoma (Yang et al. 2013), prostate cancer (Sharma et al. 2007), and lung cancer cells (Feng et al. 2011).

The current results indicated that the proportion of preadipocytes in the G1 phase significantly increased ($P < 0.05$) and the proportion of preadipocytes in the S phase significantly decreased ($P < 0.05$) after overexpression of the gRB1. When the expression level of gRB1 gene was knocked down, the proportion of preadipocytes in the S phase significantly increased ($P < 0.05$) and the proportion of preadipocytes in the G1 phase had a tendency to decrease. These results indicated that gRB1 inhibited the cell cycle of preadipocytes, mainly by inhibiting the G1 to S phase transition, resulting in G1 arrest. These results are consistent with studies on mammals that showed that RB1 is a negative regulator of the cell cycle, as it primarily binds to E2F, thereby preventing the progression of cells through the G1/S phase, resulting in cell-cycle arrest (Krek et al. 1994; Cho et al. 2012; Zhang et al. 2013; Lu et al. 2014).

In the current study, 4 cell-cycle-related genes, including MKi67, PCNA, E2F1, and CyclinD1, were selected to determine the mechanism of the effect of gRB1 on the cell cycle. MKi67 is a nuclear protein that is associated with and may be necessary for cellular proliferation (Scholzen and Gerdes 2000; Sánchez-Muñoz et al. 2013). Additionally, inactivation of MKi67 leads to inhibition of ribosomal RNA synthesis (Rahmanzadeh et al. 2007; Bando et al. 2013). The results of the current study showed that gRB1 overexpression inhibited the expression level of MKi67 at 72 h, and that gRB1 knockdown promoted the expression level of MKi67 at 96 h. These results indicated that gRB1 decreased the expression level of MKi67. This result is consistent with studies showing that RB1 suppressed the proliferation of human cells and that MKi67 protein promoted cell proliferation (Scholzen and Gerdes 2000; Zhang et al. 2013).

PCNA, a good indicator of cell proliferation, is a cofactor of DNA polymerase δ, and plays an important role in the initiation of cell proliferation (Broich et al. 1996; Chieffi et al. 2000; Bologna-Molina et al. 2013; Zhao et al. 2014). In the current study, the expression level of PCNA did not significantly change after knockdown or overexpression of the gRB1 gene. This result indicates that gRB1 may affect preadipocytes proliferation through a different way compared with PCNA.

E2F1 acts as a transcription factor of genes involved in cell-cycle progression, DNA replication, DNA repair and apoptosis (van den Heuvel and Dyson 2008; Hallstrom and Nevins 2009). In human cells, E2F1 binds preferentially to RB1 protein (pRb) in a cell-cycle-dependent manner and can mediate both cell proliferation and p53-dependent/independent apoptosis (Sherr and McCormick 2002). Here, the mRNA expression level of E2F1 did not significantly change after knockdown or overexpression of the gRB1 gene. This suggests that the interaction between gRB1 and E2F1 may not be at the transcriptional level, but at the translation level, similar to the case in mammals (Sherr and McCormick 2002). In mammals, pRb interacts with the E2F1 protein and plays important functions in DNA replication and cell proliferation (Nevins 2001; Sherr and McCormick 2002).

Cyclin D1 is a cell-cycle protein and is an important regulatory factor of the G1 to S phase transition (Coats et al. 1996; Shirali et al. 2013). Cyclin D1 and E2F participate in the regulation of cell-cycle-based networks of pRb (Ko and Prives 1996). Here, the expression level of Cyclin D1 did not significantly change after knockdown or overexpression of the gRB1 gene. This result indicated that the interaction between gRB1 and Cyclin D1 may not be at the transcriptional level, but at the translation level, as is the case in mammals (Dowdy et al. 1993; Siegert et al. 2000). In mammals, Cyclin D1 may be able to independently regulate the activity of pRb (Siegert et al. 2000).
In summary, these results showed that gRBI is a negative regulator of chicken preadipocyte proliferation, and that it inhibits the cell cycle of preadipocytes through G1 arrest.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**References**

Andrusiak, M. G., K. A. McClellan, D. Duga-Tiesh, L. M. Julian, S. P. Rodriguez, D. S. Park, T. E. Kennedy, and R. S. Slack. 2011. "Rb/ E2F Regulates Expression of Neogenin during Neuronal Migration." *Molecular and Cellular Biology* 31 (2): 238–247. doi:10.1128/MCB.00378-10.

Bando, M., H. Iwakura, H. Ariyasu, H. Koyama, K. Hosoda, S. Adachi, K. Nakao, K. Kangawa, and T. Akrami. 2013. "Overexpression of Intraislet Ghrelin Enhances β-cell Proliferation after Streptozotocin-Induced β-cell Injury in Mice." *American Journal of Physiology, Endocrinology and Metabolism* 305 (1): 140–148. doi:10.1152/ajpendo.00412.2013.

Bologna-Molina, R., A. Moncada-Taylor, N. Molina-Frechero, A. D. More-Estevé, and S. Gáñache-Acuña. 2015. "Comparison of the Value of PCNA and Ki-67 as Markers of Cell Proliferation in Ameloblastic Tumors." *Medicina oral, patología oral y cirugía bucal* 18 (2): 174–179. doi:10.431medoral.18573.

Brochi, G., A. M. Lavezzi, B. Biondo, and L. D. Pignatari. 1996. "PCNA–A Cell Proliferation Marker in Vocal Cord Cancer. Part II: Recurrence in Malignant Laryngeal Lesions." *In Vivo (Athens, Greece)* 10 (2): 175–178.

Cheffi, P., R. Franco, D. Fuglione, and S. Stabano. 2000. "PCNA in the Testis of the Frog, Rana Esculenta: A Molecular Marker of the Mitotic Testicular Epithelium Proliferation." *General and Comparative Endocrinology* 119 (1): 11–16. doi:10.1006/gcen.2000.7500.

Cho, H., S. S. Hayami, G. Toyokawa, K. Mafiemi, Y. Yamane, T. Suzue, N. Ohshima, et al. 2012. "RBI Methylation by SMYD2 Enhances Cell Cycle Progression through an Increase of RBI Phosphorylation." *Neoplasia* 14 (6): 476–486.

Coats, S., W. M. Flanagan, J. Nourse, and J. M. Roberts. 1996. "Requirement of p27Kip1 for Restriction Point Control of the RB and P53 Pathways in Retinoblastoma Tumor Suppressor Status Is a Critical Determinant of Therapeutic Response in Prostate Cancer Cells." *Cancer Research* 67 (13): 6192–6203. doi:10.1158/0008-5472.CAN-06-4424.

Sherr, C. J., and F. McCormick. 2002. "The RB and p53 Pathways in Cancer." *Cancer Cell* 2 (1): 103–112.

Shirol, S., M. Aghaie, M. Sharafi, M. Fathi, M. Sohrabi, and M. Moussafir. 2013. "Adenovirus Induces Apoptosis via cyclinDependent Kinase 6 and Bcl-2/Bax Pathways in Human Ovarian Cancer Cell Line OVCAR-3." *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine* 34 (2): 1085–1095. doi:10.1007/s13277-013-0650-1.

Siegert, L. J., J. J. Rushhton, W. R. Sellers, W. G. Kaelin Jr, and P. D. Robbins. 2000. "Cyclin D1 Suppresses Retinoblastoma Protein-Mediated Inhibition of TAFII250 Kinase Activity." *Oncogene* 19 (50): 5703–5711. doi:10.1038/sj.onc.1203966.
Van den Heuvel, S., and N. J. Dyson. 2008. “Conserved Functions of the pRB and E2F Families.” *Nature Reviews. Molecular Cell Biology* 9 (9): 713–724. doi:10.1038/nrm2469.

Wang, W., T. Zhang, C. Wu, S. Wang, Y. Wang, H. Li, and N. Wang. 2017. “Immortalization of Chicken Preadipocytes by Retroviral Transduction of Chicken TERT and TR.” *PloS one* 12 (5): e0177348. doi:10.1371/journal.pone.0177348.

Weinberg, R. A. 1995. “The Retinoblastoma Protein and Cell Cycle Control.” *Cell* 81 (3): 323–330.

Yang, Y., S. Tian, B. Brown, P. Chen, H. Hu, L. Xia, J. Zhang, et al. 2013. “The Rb1 Gene Inhibits the Viability of Retinoblastoma Cells by Regulating Homologous Recombination.” *International Journal of Molecular Medicine* 32 (1): 137–143. doi:10.3892/ijmm.2013.1374.

Yu, Y., Q. G. Ren, Z. H. Zhang, K. Zhou, Z. Y. Yu, X. Luo, and W. Wang. 2012. “Phospho-Rb Mediating Cell Cycle Reentry Induces Early Apoptosis following Oxygen-Glucose Deprivation in Rat Cortical Neurons.” *Neurochemical Research* 37 (3): 503–511. doi:10.1007/s11064-011-0636-6.

Zhang, H., S. Z. Wang, Z. P. Wang, Y. Da, N. Wang, X. X. Hu, Y. D. Zhang, et al. 2012. “A Genome-Wide Scan of Selective Sweeps in Two Broiler Chicken Lines Divergently Selected for Abdominal Fat Content.” *BMC Genomics* 13: 704. doi:10.1186/1471-2164-13-704.

Zhang, Y. F., A. R. Zhang, B. C. Zhang, Z. G. Rao, J. F. Gao, M. H. Lv, Y. Y. Wu, S. M. Wang, R. Q. Wang, and D. C. Fang, 2013. “MiR-26a Regulates Cell Cycle and Anoikis of Human Esophageal Adenocarcinoma Cells through Rb1-E2F1 Signaling Pathway.” *Molecular Biology Reports* 40 (2): 1711–1720. doi:10.1007/s11033-012-2222-7.

Zhao, H., M. S. Chen, Y. H. Lo, S. E. Waltz, J. Wang, P. C. Ho, J. Vasiliauskas, R. Plattner, Y. L. Wang, and S. C. Wang. 2014. “The Ron Receptor Tyrosine Kinase Activates c-Abl to Promote Cell Proliferation through Tyrosine Phosphorylation of PCNA in Breast Cancer.” *Oncogene* 33 (11): 1429–1437. doi:10.1038/onc.2013.84.