Published in final edited form as:

Toxicol Mech Methods. 2017 September ; 27(7): 511–517. doi:10.1080/15376516.2017.1324932.

Altered miRNA expression in aniline-mediated cell cycle progression in rat spleen

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Abstract

Aniline exposure is associated with toxicity to the spleen, however, early molecular events in aniline-induced cell cycle progression in the spleen remain unknown. miRNAs have been implicated in tumor development by modulating key cell cycle regulators and controlling cell proliferation. This study was, therefore, undertaken on the expression of miRNAs, regulation of cyclins and cyclin-dependent kinases (CDKs) in an experimental condition that precedes a tumorigenic response. Male SD rats were treated with aniline (1 mmol/kg/day by gavage) for 7 days, and expression of miRNAs, cyclins and CDKs in rat spleens were analyzed. Microarray and/or qPCR analyses showed that aniline exposure led to significantly decreased miRNA expression of let-7a, miR-24, miR-34c, miR-100, miR-125b and greatly increased miR-181a. The aberrant expression of miRNAs was associated with significantly increased protein expression of cyclins A, B1, D3 and E. Furthermore, remarkably enhanced expression of CDKs like CDK1, CDK2, CDK4, CDK6, especially p-CDK1 and p-CDK2 as well as alternations in the expression of pRB, p27 and CDC25A in the spleens of aniline-treated rats was also observed. The data suggest that aniline exposure leads to aberrant expression of miRNAs in the spleen which could be important in the regulation of cell cycle proteins. Our findings thus, provide new insight into the role of miRNAs in cell cycle progression, which may contribute to aniline-induced tumorigenic response in the spleen.

Keywords

Aniline; miRNA; cyclin; cyclin-dependent kinase; cell cycle; spleen

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Participated in research design: MF Khan, G Wang, J Wang

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Conflict of Interest

The authors have no conflicts of interest to declare.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs containing about 22 nucleotides that regulate gene expression at post-transcriptional level through base pairing with complementary sequences in the 3' untranslated region (UTR) of target mRNAs (Di Leva et al. 2014, Jansson and Lund 2012). In recent years, miRNAs have gained widespread attention due to their novel and crucial potency of biological regulation and pathological processes (Bueno and Malumbres, 2011, Di Leva et al. 2014, Esquela-Kerscher and Slack 2006, Jansson and Lund 2012). In fact, miRNAs are implicated in almost every biological processes, including cell proliferation, differentiation, survival and apoptosis, and alterations in their expression have been associated with the occurrence of different diseases, such as cancer, autoimmune and cardiovascular diseases (Bueno and Malumbres 2011, Di Leva et al. 2014, Garo and Murugaiyan 2016, Esquela-Kerscher and Slack 2006, Jansson and Lund 2012). Recently, miRNAs have attracted increasing attention in the field of cancer research, and have been proposed as key modulators in cancer pathogenesis, diagnostic biomarkers, prognosis, potential cancer therapeutic targets and response to therapy (Di Leva et al. 2014, Jansson and Lund 2012, Ravegnini et al. 2015, Svoronos et al. 2016). In view of their potential involvement in cell proliferation and carcinogenesis, miRNAs are excellent targets to assess their role in cell cycle regulation.

Aniline, a widely used industrial chemical, has been known to cause splenic toxicity characterized by splenomegaly, hyperplasia, fibrosis, and a variety of sarcomas on chronic exposure in rats (Bus and Popp 1987, Di Girolamo et al. 2009, Goodman et al. 1984, Khan et al. 1997, 1999, 2006, Ma et al. 2008, 2011, Wang et al. 2005, 2011, 2015, Weinberger et al. 1985). Our recent studies show that subchronic/chronic aniline exposure leads to iron overload and oxidative stress with consequent increases in oxidative DNA damage and cellular proliferation, particularly dysregulation of cyclins and cyclin-dependent kinases (CDKs) in the spleens. Such events could potentially lead to a mutagenic and/or carcinogenic response in the spleen (Ma et al. 2008, 2011, Wang et al. 2010, 2011, 2015). Early cellular and molecular events involved in the dysregulation of cell cycle and aberrant cellular proliferation following aniline exposure are not known. Cell cycle is a complex and precisely regulated process, but its dysregulation may result in an uncontrolled cell cycle progression leading to a carcinogenesis phenotype (Malumbres and Barbacid 2001, 2009, Wang et al. 2011, Williams and Stoeber 2012). This study was, therefore, undertaken to assess involvement and association of specific miRNAs in cell cycle regulation, and dysregulation of cyclins and CDKs in the spleen in an experimental condition that precedes aniline-induced tumorigenic response.

Materials and methods

Animals and treatments

Seven-week old male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and acclimated in a controlled environment animal room (temperature, 22°C; relative humidity, 50%; photoperiod, 12-h light/dark cycle) for 7 days prior to dosing. Twelve animals were randomly divided into 2 groups. One group of 6 rats received 1 mmol/kg/day aniline hydrochloride (~97%; Aldrich, Milwaukee, WI) in 0.5 ml of drinking

water by gavage for 7 days, while the other 6 rats (control group) received 0.5 ml of water only (Khan et al. 1997, 2003, Wang et al. 2008, 2010, Wu et al. 2005).

Choice of dose and duration of exposure was based on earlier studies (Khan et al. 1997, 2003, Wang et al. 2008, 2010, Wu et al. 2005). The rats were euthanized 24 h following last dose and the spleens were collected, weighed and divided into several portions for use in various analyses. Portions of the spleen were snap-frozen in liquid nitrogen and stored at -80°C for RNA isolation and protein extraction. All animal experiments for this study were conducted in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

RNA isolation and microarray analysis

Total RNA, including miRNAs from fresh-frozen spleens was extracted by using the *mirVana* miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions (Wang et al. 2015). Microarray analysis was performed using miRCURY LNATM microRNA Arrays (Exiqon, Woburn, MA) in accordance with the manufacturer's instructions (Bhushan and Kandpal 2011).

Analysis of miRNA expression analysis using quantitative real-time PCR

The cDNA was synthesized from total RNA by reverse transcription (RT) using a miRCURY LNA Universal RT kit (Exiqon) in accordance with the manufacturer's instructions (Wang et al. 2015). Quantitative real-time PCR was performed with the converted cDNA using miRNA LNA PCR primers and SYBR Green Master Mix (Exiqon) on an Eppendorf Mastercycler Realplex (Eppendorf). Twenty μl of reaction mix consisting of 8 μl of diluted cDNA, 2 μl of primers and 10 μl of SYBR Master Mix (Exiqon) was subjected to qPCR. The PCR was performed under the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, and 60°C for 1 min. The target sequence of each miRNA is shown in Table 1 and U6 was used as an internal control. All samples were run in triplicate and results were converted to relative values ($\log_2$ ratios) between control and aniline-treated samples (Wang et al. 2011, 2015).

Western blot analysis

The protein expression of cyclinA/B1/D3/E, CDK1/2/4/6, phosphor-CDK1 (p-CDK1), phosphor-CDK2 (p-CDK2), retinoblastoma proteins (pRB), phosphor-pRB (pRB-p), p27 and Cell Division Cycle-25A (CDC25A) was determined by using Western blot analysis as described earlier (Khan et al. 2006, Wang et al. 2008, 2011, 2015). Briefly, the spleen lysates (50 μg of proteins) were fractionated on 10% SDS-PAGE gels and transferred to PVDF membranes (Amersham, Arlington Heights, IL). After blocking with non-fat dry milk (5%, w/v), the membranes were probed with antibodies (Santa Cruz Biotechnology, 6 A β -actin antibody (Sigma) was used to confirm even loading. The quantification of blot signals was performed by densitometry and normalized using the β -actin signal (Wang et al. 2008, 2011, 2015).

Quantification of cyclin A, B1, D3 and E mRNAs using real-time RT-PCR

Quantification of cyclins A, B1, D and E gene expression was performed using real-time RT-PCR essentially as described in our previous studies (Ma et al. 2008, Wang et al. 2010, 2011, 2015). The tissue total RNA was extracted from rat spleens using a RiboPure kit (Ambion, Austin, TX) and cDNA was synthesized from the RNA using the SuperScript first-strand synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR was performed using a Mastercycler Realplex (Eppendorf, Westbury, NY) and nucleotide sequences of primers used in the real-time PCR process are listed in Table 2. For each sample, parallel reactions were processed in triplicate for the detection of 18S and rat cyclin A, B1, D3 and E. The reaction samples in a final volume of 25 μ l contained 2 μ l cDNA templates, 2 μ l primer pair, 12.5 μ l iQ SYBR Green Supermix, and 8.5 μ l water. PCR conditions were as follows: 95°C for 2 min for template denaturation, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s and an additional 20 s hold for fluorescent signal acquisition. The fold changes in cyclins A, B1, D3, E cDNA (target gene) relative to the 18S endogenous control were determined as fold change = 2^{-C_T} , where $C_T = (C_T \text{ aniline} - C_T \text{ 18S}) - (C_T \text{ control} - C_T \text{ 18S})$.

Statistical analysis

All data are expressed as means $\pm$ SD. Comparison between the groups was made by *p* value determination using Student's two-tailed *t*-test (GraphPad InStat 3 software, La Jolla, CA). A *p* value of < 0.05 was considered to be statistically significant.

Results

miRNA profile in rat spleen following aniline exposure

miRNAs are known as a key regulators of multiple biological and pathological processes, including cell proliferation and cancer development (Bueno and Malumbres 2011, Di Leva et al. 2014, Garo and Murugaiyan 2016, Esquela-Kerscher and Slack 2006, Jansson and Lund 2012). Increasing number of miRNAs has been implicated in regulating cell cycle progress via modulating critical components of its regulatory machinery and alteration of miRNA levels can contribute to pathological conditions, including tumorigenesis (Bueno and Malumbres 2011, Jansson and Lund 2012, Li et al. 2015). As reported in our earlier studies (Khan et al. 1997, 2003, Wang et al. 2010, Wu et al. 2005), we observed increased spleen weight, cell number and cellular proliferation following aniline exposure in this study also (data not shown). To evaluate the potential role of miRNA in aniline-mediated cell proliferation, we analyzed and profiled the expression of miRNAs in the spleens from control and aniline-treated rats. The generated map for the entire range of miRNAs showed differences between miRNA profiles of aniline-treated and the control spleens (Fig. 1A). Based on *p* values and fold changes, we identified 3 miRNAs with specific differential expression signatures between aniline-treated and controls (Fig. 1B). Interestingly, expression of all three miRNAs i.e., Let-7a (decreased 59%), miR-34c (decreased 78%), and miR-125b (decreased 61%), showed significant decreases ($p < 0.05$). These miRNAs are known to suppress tumorigenesis by targeting key components of the cell cycle regulatory engine (Bueno and Malumbres 2011, Jansson and Lund 2012, Lal et al. 2009, Li et al. 2014, Li et al. 2015, Zhao et al. 2015).

Comparison in the expression of cell cycle regulatory miRNAs between aniline-treated and control rats

To further validate the results of miRNA expression profiling and explore the potential of aberrant miRNA expression in cell cycle progression following aniline exposure, qRT-PCR was performed on matched samples. We analyzed the expression of several miRNAs which have been confirmed to be associated with regulating cell cycle progression (Table 3; Bueno and Malumbres 2011, Jansson and Lund 2012, Lal et al. 2009, Li et al. 2014, Li et al. 2015, Zhao et al. 2015) and found the expression patterns (Fig. 2) similar to those observed in the profiled miRNA expression in Fig. 1. Aniline exposure led to significantly decreased expression of Let-7a (decreased 64%), miR-24 (decreased 59%), miR34c (decreased 84%), miR-100 (decreased 63%), miR-125b (decreased 64%), whereas miR-181a expression was increased by 118% in comparison to controls (Fig. 2), suggesting significantly aberrant expression of miRNAs and providing mechanistic evidence for better understanding of aniline-mediated splenotoxicity.

Effect of aniline exposure on protein and mRNA expression of cyclins A, B, D and E

Cyclins are key components of the cell cycle machinery controlling cell cycle progression (Chulu and Liu 2009, Malumbres and Barbacid 2001, 2009, Wang et al. 2011, 2015, Williams and Stoeber 2012, Wolgemuth et al. 2013) and our previous studies demonstrated remarkable increases in the expression of cyclins following subchronic aniline exposure (Wang et al. 2011, 2015). To evaluate the early response to aniline exposure and elucidate the potential molecular mechanisms, we determined the protein expression of cyclins A, B1, D3 and E in the rats treated with aniline for 7 days. As evident from Fig. 3A, aniline treatment resulted in significantly increased expression of cyclins A, B1, D3 and E by 186%, 248%, 195% and 113%, respectively, in comparison to controls.

Since Western analysis data showed significant increases in protein expression of cyclins A, B1, D3 and E in spleens following aniline exposure, it was of interest to evaluate if aniline exposure also influences the expression of cyclins at gene levels. Therefore, the mRNA expression of cyclins A, B1, D3 and E was determined by using real-time PCR and the results are presented in Fig. 3B. Aniline treatment led to 7.9, 7.3, 3.7 and 5.4-fold increases, respectively, in the mRNA expression of cyclins A, B1, D3 and E in comparison to the controls, providing further support to the observed increases in protein expression.

Aniline exposure increases the expression of CDKs

CDKs are another central components of the cell cycle regulatory machinery and play a key role in driving cell cycle progression (Chulu and Liu 2009, Malumbres and Barbacid 2001, 2009, Wang et al. 2011, 2015, Williams and Stoeber 2012). To evaluate the roles of CDKs in regulating splenocyte proliferation, CDK1/2/4/6 and phosphor-CDK1/2 expression was, therefore, analyzed by Western blot and data are presented in Fig. 4. The protein expression of CDK1/2/4/6 in aniline-treated rats showed remarkable increases of 100%, 176%, 91% and 109%, respectively, as compared to the control rats. More importantly, aniline exposure led to even greater increases in p-CDK1 (262%) and p-CDK2 (220%) expression compared to controls. The results further suggest that even short-term aniline exposure stimulates the expression of cell cycle regulatory proteins which promote cell cycle progression.

Aniline exposure led to alterations in pRB, p27 and CDC25A expression

Cell proliferation is controlled by a complex regulatory machinery. Apart from the central components like cyclins and CDKs, pRB, p27 and CDC25A also play important roles in cell cycle progression by governing cell cycle transition from one phase to the next, and alterations in their expression have been implicated in the genesis or progression of many human malignancies (Abukhdeir and Park 2008, Bueno and Malumbres 2011, Malumbres and Barbacid 2009). Therefore, to evaluate the potential of pRB, p27 and CDC25A in aniline-mediated cell proliferation, the expression of pRB, pRB-p, p27 and CDC25A was determined by Western blot analysis. As evident from Fig. 5, aniline treatment caused significant alterations in the protein expression of pRB-p (increased 2.9-fold), p27 (decreased 72%) and CDC25A (increased 5.2-fold) in the spleens, suggesting aniline-induced aberrant expression of pRB-p, p27 and CDC25A, and thus, their potential role in cell cycle progression.

Discussion

Even though increasing evidence has demonstrated that aniline exposure is associated with splenotoxicity including splenomegaly, hyperplasia, fibrosis, and eventually tumor formation on chronic exposure in rats (Bus and Popp 1987, Di Girolamo et al. 2009, Goodman et al. 1984, Khan et al. 1997, 1999, 2006, Wang et al. 2005, 2011, 2015, Weinberger et al. 1985), the early cellular and molecular events involved in splenic damage remain largely unknown. Splenic sarcomas are the most serious consequence of chronic aniline exposure, and cell cycle dysregulation could be the early molecular event potentially leading to tumor development (Bus and Popp 1987, Di Girolamo et al. 2009, Goodman et al. 1984, Khan et al. 1997, 1999, 2006, Wang et al. 2011, 2015, Weinberger et al. 1985). This led us to investigate the cell cycle regulatory machinery and regulatory miRNAs in the rat spleens following short-term aniline exposure for better understanding of the early potential mechanisms in the pathogenesis of aniline-mediated tumorigenic response.

Cell cycle progression is a complex process which is tightly controlled by cell cycle regulatory machinery, particularly by two most important groups of proteins cyclins and CDKs complexes (Chulu and Liu 2009, Malumbres and Barbacid 2001, 2009, Stark and Taylor 2006, Wang et al., 2011, 2015, Williams and Stoeber 2012). Dysregulation of certain cyclin-CDK complexes results in either continued proliferation or unscheduled re-entry into the cell cycle, two properties characteristic of most tumor cells (Chulu and Liu 2009, Malumbres and Barbacid 2001, 2009, Williams and Stoeber 2012). In this study, we not only observed significant increases in both protein and mRNA expression of cyclins (A, B1, D3, and E), but also found greatly increased expression of CDKs (including CDKs 1/2/4/6), especially p-CDK1 and p-CDK2 in the spleens of aniline-treated rats. Established evidences support that cyclin D-CDK4, cyclin D-CDK6 and cyclin E-CDK2 drive G1 progression through the restriction point, while S phase is initiated by cyclin A-CDK2, whereas cyclin B-CDK1 regulates progression through G2 and entry into mitosis (Williams and Stoeber 2012, Wang et al. 2011, 2015). Therefore, their overexpression, as observed in the present experimental condition, is highly significant and could contribute to initiation of cellular proliferation and/or uncontrolled cellular proliferation in the spleen.

Apart from the cyclin-CDK complexes, cell proliferation and progression through each cell cycle and transition from one phase to the next are also monitored by regulatory pathways, called checkpoints, which maintain the correct order of events (Malumbres and Barbacid 2009, VanArsdale et al. 2015, Wang et al. 2011, Williams and Stoeber 2012). The pRB plays a key role in preventing G1-phase cells from entering S-phase and also contributes to G2-M transition through the regulation of E2F responsive genes (Giacinti and Giordano 2006, Malumbres and Barbacid 2009, VanArsdale et al. 2015, Wang et al. 2011). Phosphorylation of pRB leads to the inactivation of pRB via releasing E2F from an inhibitory complex, enabling it to promote the transcription necessary for progression into late G1 and S phase (Giacinti and Giordano 2006, VanArsdale et al. 2015, Wang et al. 2011). CDK inhibitors (CDKIs) such as P27, which can reversibly halt cell cycle progression, is involved in the regulation G1-S transition and regulatory kinase of G2 checkpoint by interacting with specific cyclin-CDK complexes (Malumbres and Barbacid 2009, Wang et al. 2015, Williams and Stoeber 2012). CDC25 plays an important role in transition from one phase to the next by dephosphorylating different residues to activate CDKs. While CDC25B and CDC25C promote G2/M progression, CDC25A plays a more extensive role in assisting both G1/S and G2/M transition (Malumbres and Barbacid 2009, Shen and Huang 2012, Sur and Agrawal 2016). Significantly increased expression of pRB-p and CDC25A along with decrease in P27 expression, as observed in this study, are critical and highly valuable in the understanding of a potentially uncontrolled cell progression pattern.

There is increasing evidence that a single miRNA may target up to several hundred mRNAs, thus miRNAs could function as master coordinators, efficiently regulating fundamental cellular processes, including cell proliferation, development and apoptosis (Bueno and Malumbres 2011, Chafin et al. 2014, Jansson and Lund 2012, Zhao et al. 2015). Even though increasing number of miRNAs are still being studied, several miRNAs, including Let-7a, miR-24, miR-34c, miR-100, miR-125b and miR-181a, are confirmed to target transcripts that encode proteins directly or indirectly involved in cell cycle progression, cellular proliferation and cancer progression (Bueno and Malumbres 2011, Chafin et al. 2014, Tan et al. 2012, Zhao et al. 2015). While miR-181a enhances cell proliferation as an oncogene, miRNAs Let-7a, miR-24, miR-34c, miR-100 and miR-125b in fact inhibit cell progression as tumor-suppressors via a variety of signaling pathways (Bueno and Malumbres 2011, Chafin et al. 2014, Lal et al. 2009, Tan et al. 2012, Zhao et al. 2015). The tumor-suppressive Let-7a is known to inhibit cell proliferation via targeting multiple cell regulators including cyclins A/D, CDKs 2/4/6, CDC25A and E2F and activating oxidative stress signaling pathways (Bueno and Malumbres 2011, Cannell et al. 2010, Chafin et al. 2014, Lal et al. 2009). miR-24 inhibits cell cycle progression through targeting cyclins A/B, CDKs 1/2/4/6 and E2F (Bueno and Malumbres 2011, Lal et al. 2009). miR-34c is a member of the miR-34 family and has a tumor suppressor function. Similarly, miR-34c influences cell growth mainly by downregulating cell cycle-related proteins to induce a cell cycle arrest (Bueno and Malumbres 2011, Cannell et al. 2010, Zhao et al. 2015). While miR-100 modulates cell cycle mainly by inducing an arrest in the G2/M phase via targeting polo-like kinase 1 (PLK1), miR-125b inhibits cell proliferation by arresting both G1/S and G2/M phases through influencing CDC25A expression and oxidative stress such as NF- κ B, PI3K/Akt signaling pathways (Bueno and Malumbres 2011, Li et al. 2014, Petrelli et al.

2012, Tan et al. 2012, Zhao et al. 2015). However, miR-181a promotes cell progression by regulating cell cycle inhibitor p27 (Bueno and Malumbres 2011, Cuesta et al. 2009). Interestingly, our data show that aniline exposure leads to significantly decreased splenic miRNA expression of Let-7a, miR-24, miR-34c, miR-100, miR-125b and greater increases in the expression of miR-181a. Moreover, the alterations in these miRNA expression were associated with greater increases in cyclinA/B1/D3/E, CDK1/2/4/6 as well as aberrant expression of pRB-p, CDC25A and P27. Therefore, aberrant expression of miRNAs including Let-7a, miR-24, miR-34c, miR-100, miR-125b and miR-181a in the spleens following aniline exposure, as observed in this study, may provide mechanistic evidence towards better understanding of relationship among aniline exposure, oxidative stress (signaling pathways), aberrant miRNA expression, dysregulation of cell cycle machinery and tumorigenesis in the spleens.

In conclusion, our data show that aniline exposure causes aberrant expression of miRNAs including Let-7a, miR-24, miR-34c, miR-100, miR-125b and miR-181a. The aberrant expression of miRNAs was associated with increases in cyclins such as cyclinA/B1/D3/E, CDKs like CDK1/2/4/6, particularly p-CDK1/2. These novel findings following a short-term exposure along with our earlier findings help us in postulating that aniline-induced oxidative stress, as a result of iron overload, leads to aberrant regulation of miRNAs which may trigger specific signaling pathways such as AP-1 and NF- κ B, resulting in increased cyclins' expression and CDKs' activities, and consequently result in greater cell proliferation in the spleen (Fig. 6). However, further studies are needed to fully validate our hypothesis and better understand the precise molecular mechanisms of aniline-mediated tumorigenic response, such as the initiation of cell cycle entry and progression, and the regulatory role of specific miRNAs in each phase of cell cycle by using miRNA mimics/inhibitors and use of specific gene inhibitors to block target pathways or knock down/inhibit target genes.

Acknowledgements

This work was supported by Grant ES06476 from the National Institute of Environmental Health Sciences (NIEHS), NIH, and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

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Figure 1.
(A) Hierarchical clustering of differentially expressed miRNA genes in the spleens of control and aniline-treated rats. The resulting values were assessed for p value < 0.05 and a fold change between -2 and 2. Red donates increased levels of expression comparing aniline-treated to controls and blue denotes decreased expression. (B) Microarray analysis of three major cell cycle related miRNA genes in the spleens of control and aniline-treated rats. Values are mean ± SD. *p<0.05 vs. respective controls.

Figure 2. Real-time PCR analysis of miRNAs Let-7a, miR24, miR-34c, miR-100, miR-125b and miR-181a expression in rat spleens following aniline exposure. Values are means $\pm$ SD. * $p < 0.05$ vs. respective controls.

Figure 3.

Cyclins A, B1, D3 and E protein and gene expression in rat spleens following aniline exposure. (A) Western blot determination of cyclins A, B1, D3 and E protein expression in control or aniline-treated rats and densitometric analysis of the relative expression of these cyclins. Values are mean $\pm$ SD; * $p < 0.05$. (B) Cyclins A, B1, D3 and E gene expression in rat spleens following aniline exposure. The fold change in mRNA expression (2^{-C_T}) was determined by real-time PCR analysis. Values are means $\pm$ SD ($n = 3$). * $p < 0.05$ vs. respective controls.

Figure 4.

The relative protein expression of CDKs in rat spleens following aniline exposure. (A) Western blot determination of CDKs1/2/4/6 protein expression in control and aniline-treated rats. (B) Western blot determination of p-CDK1 and p-CDK2 protein expression in control and aniline-treated rats. Values are mean $\pm$ SD. * $p < 0.05$ vs. respective controls.

Figure 5. Protein expression of pRB-p, pRB, p27 and CDC25A in the rat spleens following aniline exposure. Data include Western blot detection of pRB-p, pRB, p27 and CDC25A in the spleens of control and aniline-treated rats and the densitometric analysis of the protein bands. Values are mean $\pm$ SD. * $p < 0.05$ vs. respective controls.

Figure 6.
The plausible mechanisms of aniline-mediated cell proliferation in the spleen.

Table 1.

Target sequences of miRNAs for real-time PCR analysis

Genes	Target sequences	Accession number
Let-7a	UGAGGUAGUAGGUUGUAUAGUU	MIMAT0000062
miR-24	UGGCUCAGUUCAGCAGGAACAG	MIMAT0000080
miR-34c	AGGCAGUGUAGUUAGCUGAUUGC	MIMAT0000686
miR-100	AACCCGUAGAUCCGAACUUGUG	MIMAT0000098
miR-125	UCCCUGAGACCCUUUAACCUGUGA	MIMAT0000443
miR-181a	AACAUUCAACGCUGUCGGUGAGU	MIMAT0000256
U6	Reference Gene	

Table 2.

Primer sequences of cyclins A, B1, D3 and E for real-time PCR analysis

Genes	Forward	Reverse
Cyclin A	aacgatgagcacgtccctac	cagctggcctcttctgagtc
Cyclin B1	catgctggactacgacatgg	ctccgtgtgggacaggtagt
Cyclin D3	ggccttggtagaagagcaa	gcacctcaagacctcaac
Cyclin E	gaaatcagaccgccagag	cgtgcagaaagtgtctatc
18S	Universal 18S Internal Standard	

Table 3.

Altered miRNAs and their known functions

miRNA	Function/targeted cell cycle regulation	References
Let-7a	Inhibits cell proliferation by targeting cyclins A/D, CDKs 2/4/6, CDC25A, E2F	Bueno et al. 2011, Cannell et al. 2010, Chafin et al. 2014, Lal et al. 2009
miR-24	Inhibits cell cycle progression via targeting cyclins A/B, CDKs 1/2/4/6, E2F	Bueno and Malumbres 2011, Lal et al. 2009
miR-34c	Influences cell proliferation by down-regulating cyclins D/E, CDKs 4/6	Bueno and Malumbres 2011, Cannell et al. 2010, Zhao et al. 2015
miR-100	Induces G2/M arrest via targeting PLK1	Li et al. 2015, Petreli et al. 2012
miR-125	Inhibits cell proliferation via influencing Inhibits cell proliferation via influencing	Li et al. 2014, Petreli et al. 2012, Tan et al. 2012, Zhao et al. 2015
miR-181a	Promotes cell proliferation by regulating cell cycle inhibitor p27	Bueno and Malumbres 2011, Guesta et al. 2009