Mechanism of folate deficiency-induced apoptosis in mouse embryonic stem cells: Cell cycle arrest/apoptosis in G1/G0 mediated by microRNA-302a and tumor suppressor gene Lats2

Yan Liang\textsuperscript{a,b}, Yuanyuan Li\textsuperscript{b}, Zhengli Li\textsuperscript{b}, Zhuo Liu\textsuperscript{b}, Zhiping Zhang\textsuperscript{c}, Shaoyan Chang\textsuperscript{d}, Jianxin Wu\textsuperscript{a,b,*}

\textsuperscript{a} Department of Biochemistry, Peking University Capital Institute of Pediatrics Teaching Hospital, Beijing 100020, China
\textsuperscript{b} Department of Biochemistry, Capital Institute of Pediatrics, Beijing 100020, China
\textsuperscript{c} Reproductive Medicine of Center, Children's Hospital of Shanxi, Women Health Center of Shanxi, Taiyuan, Shanxi 030013, China
\textsuperscript{d} Department of Molecular Immunology, Capital Institute of Pediatrics, Beijing 100020, China

A R T I C L E   I N F O

Article history:
Received 23 May 2012
Received in revised form 9 July 2012
Accepted 16 July 2012
Available online 22 July 2012

Keywords:
Folate deficiency
miR-302a
Apoptosis
Cell cycle
Lats2

A B S T R A C T

Deficiencies in maternal diet, such as inadequate intake of folate, can inhibit normal development and lead to developmental defects. MicroRNAs (miRNAs) may play a role in mediating the effects of folate deficiency in the growing mammalian embryo, although conclusive evidences to support that possibility are not yet available. The goal of the present study was to investigate whether and how folate deprivation alters the properties of mouse embryonic stem cells (mESCs) in culture. For this purpose, mESCs were cultured in folate-deficient or complete culture medium. The results show that folate-deficient mESCs have a significantly higher rate of apoptosis, accumulate in G0/G1 and fail to proliferate. Expression profiling revealed several miRs and many mRNAs are differentially expressed in folate-deficient cells. RT-PCR data confirmed differential expressions of 12 miRNAs in folate-deficient cells. Furthermore, bioinformatics analyses and in vitro studies suggested that miR-302a plays a critical role in mediating the effects of folate on cell proliferation and cell cycle-specific apoptosis by targeting Lats2 gene. Together, these results suggest that the effects of folate deficiency on mammalian development may be mediated by miRNAs that regulate proliferation and/or cell cycle progression in mESCs.

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1. Introduction

Mammalian embryonic stem cells (ESCs) are pluripotent cells that localize to the inner cell mass at the blastocyst stage of embryogenesis. Under defined conditions, ESCs have the unusual ability to self-renew and maintain pluripotency indefinitely. Because clonal populations of mouse ESCs (mESCs) recapitulate these properties for many cell generations in cell culture, they are frequently used as an experimental model for embryonic development (Evans and Kaufman, 1981). Although mESCs have been used to study various aspects of mammalian development, they have not yet been used to examine whether and how nutrient deprivation interferes with normal development of a growing embryo.

Abbreviations: mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells; GO, gene ontology; FDR, false discovery rate; ECCs, embryonic carcinoma cells; iPSCs, induced pluripotent stem cells.

* Corresponding author at: Department of Biochemistry, Peking University Capital Institute of Pediatrics Teaching Hospital/Capital Institute of Pediatrics, Beijing 100020, China. Tel.: +86 10 85695593; fax: +86 10 85610322.
E-mail addresses: sarahs60113@126.com (Y. Liang), liuyuan2075@yahoo.com.cn (Y. Li), jocyelyn@126.com (Z. Li), liuzhuozhuo2005@163.com (Z. Liu), zhp754500@163.com (Z. Zhang), changyan2001@163.com (S. Chang), wjxianxin@gmail.com, cibioblab@163.com (J. Wu).

1357-2725/ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2012.07.014
model of mammalian embryonic development (Marsit et al., 2006; Shokkoff and Gallicano, 2010).

miRNAs are 17–24 nucleotide, single-stranded, non-coding RNA molecules that bind to and promote degradation and/or inhibit translation of target mRNAs (Lewis et al., 2005). miRNAs play critical roles as regulators of cell proliferation and differentiation, apoptosis, cell cycle progression, and dysexpression in embryos and adult tissues have been linked to developmental defects and other diseases, including cancer, in later life stages (Alvarez-Garcia and Miska, 2005; Bartel, 2004; Houbaviy et al., 2003). It has been estimated that a typical embryonic cell expresses >110,000 miRNAs, each of which regulates hundreds of target genes. At least three specific miRNA clusters, miR-290, miR-302 and miR-370, are conserved in human and mouse ES cells (Calabrese et al., 2007; Houbaviy et al., 2003). However, the specific roles of mESC-specific miRNAs during embryonic development are not well understood, and miRNA biology is currently a very active area of research.

In this report, the results show that folate-deficient mESCs accumulate in G0/G1, fail to proliferate, and have a significantly higher rate of apoptosis. Expression profiling revealed 60 up-regulated miRNAs, 34 down-regulated miRNAs, 2316 up-regulated miRNAs and 2544 down-regulated miRNAs in folate-deficient cells. RT-PCR confirmed that let-7a, mir-15a, 15b, 26, 29a, 34a, 130b, 125a-5p, 124, 290, and 302a are differentially expressed in folate-deficient cells. Bioinformatics analyses suggested a critical role for miR-302a in mediating the effects of folate on cell cycle progression. Consistent with this, in vitro, miR-302a was confirmed in regulating mESC proliferation, apoptosis and cell cycle phase by directly targeting Lats2 gene. Thus, our findings suggested a new layer of molecular regulatory mechanisms, focusing on the effects of folate in mESCs.

2. Materials and methods

2.1. Ethical aspects

The protocols and provisions for cell culture studies using mESCs were reviewed and approved by the Institutional Review Board of Capital Institute of Pediatrics at Beijing, P.R. China.

2.2. Cell culture media and other chemicals

Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (11960-069), L-glutamine (25300-081), non-essential amino acids (11140-50), B-mercaptoethanol (21985), penicillin-streptomycin (15140-122), ES qualified fetal bovine serum (100-125), and TrypLE™ Express (12605-010) were from Gibco/Invitrogen (Carlsbad, CA, USA). Folate-free Dulbecco’s Modified Eagle Medium (D-2554) was from Sigma (St. Louis, MO, USA). Leukemia inhibitory factor (LIF ESgro, ESG1107) was from Milipore (Billerica, MA, USA). Gelatin was from Amresco (Solon, OH, USA).

2.3. mESC culture and treatments

mESCs and mitomycin C-treated primary mouse embryonic fibroblast (MEF) feeder cells were obtained from Beijing Stem Cell Bank (Beijing, China) and cultured as previously described (Evans and Kaufman, 1981; Martin, 1981). Briefly, MEFs were plated on 0.1% gelatin-coated dishes in MEF medium (DMEM containing, 10% fetal qualified bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin). The following day, ES-D3 cells were suspended in ES cell medium (DMEM containing 15% fetal qualified bovine serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM mercaptoethanol, leukemia inhibitory factor 1000 U/mL, and 100 U/mL penicillin/streptomycin and plated on the feeder layer. After 24 h in culture (50–60% confluent) the medium was replaced, and after a further 24 h the cells (80–90% confluent) were split by 1/4–1/5, and one aliquot of mESCs were transferred to folate free DMEM supplemented with 15% dialyzed fetal bovine serum. Besides, other components of the culture medium were the same with the above. Cells cultures were maintained at 37 °C and 95% humidity with 5% CO2 for 4 days.

2.4. Intracellular folate concentration

Intracellular folate was determined with a competitive binding immunoassay, as described previously (Gu et al., 2002). Briefly, intracellular folate was extracted by a lysis buffer (Fermentas, Ontario, CA) form an aliquot containing 5 × 105 cells, and quantified using the Elecsys Folate Reagent Kit and Roche Elecsys 2010 Chemistry Analyzer (Roche, Basel, Switzerland).

2.5. Cell proliferation

Cell proliferation was quantified using a commercially available chromogenic assay (Cell Counting Kit-8, Doinido Laboratories, Tokyo, Japan), according to the manufacturer’s instructions. Briefly, 1500 cells were aliquoted into wells of a 96-well plate and incubated under identical conditions as experimental samples in culture. Assay solution (10 μL) was added to each well, incubated for 2 h, and then analyzed for total absorbance (450 nm) per well. The number of viable cells is proportional to the A450 of the reduced tetrazolium salt indicator dye: (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt). All assays were performed in triplicate.

2.6. Flow cytometry

mESCs were grown in culture under the indicated conditions and harvested at 72 or 96 h. Cell apoptosis was quantified using the Annexin V Apoptosis Detection Kit I (BD556547) and Cell Quest software. Cell cycle stages were analyzed using CycleTEST™ PLUS DNA Reagent Kit (BD340242) and Modifit software. All instruments and reagents were from Becton Dickinson Biosciences (San Diego, CA, USA).

2.7. Western blotting

SDS-PAGE and Western blots were performed according to standard procedures. Briefly, cells were lysed and the protein concentration was determined using the BCA assay (Thermo Scientific, Waltham, MA, USA). Aliquots of protein (40 μg) were separated by SDS PAGE and the separated proteins were transferred to PVDF membrane (Millipore, Billerica, MA, USA). Membranes were blocked and then incubated with primary antibodies against caspase-3 (1:2000), Lats2 (1:500) or β-actin (1:1000). Finally, membranes were probed with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:5000). All antibody reagents were from Cell Signal Technology (Boston, MA, USA), except Lats2 antibody from Abcam (Cambridge, MA, USA). Bands were detected by ECL Western Blot Kit (Cwbio, Beijing, China). Blots were visualized and quantified using Quantity One software (Bio-rad, Hercules, CA, USA).

2.8. Total RNA isolation and microarray

Total RNA was isolated as previously described (Zhang et al., 2010). Purified RNA was labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.16.0) (Exiqon, Denmark). After washing, slides were scanned using the Axon GenePix 4000B microarray
scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. miRNAs whose expression changed more than 2-fold were considered to be differentially regulated under the chosen experimental condition.

Approximately 5 μg total RNA was hybridized to Roche NimbleGen mouse musculus 12 × 135 v2 array (Roche, Basel, Switzerland). The following protocols/reagents were used: (1) reverse transcription using Invitrogen Superscript ds-cDNA synthesis kit; (2) ds-cDNA labeling with NimbleGen one-color DNA labeling kit; (3) array hybridization/washing using the NimbleGen Hybridization System and NimbleGen wash buffer kit; (4) the Axon GenePix 4000B microarray scanner (Molecular Devices Corporation generated TIFF files, which were imported into and analyzed using NimbleScan software (version 2.5). miRNAs with expression changed more than 1.5-fold were considered to be differentially regulated.

2.9. Real-time PCR

The step-loop RT-PCR method for miRNA and SYBR green real-time RT-PCR for mRNA were used to directly quantify expression of selected miRNAs and mRNA, respectively, with Reverse Transcription Kit (Promega, Madison, WI, USA) and power SYBR® Green PCR Master Mix (ABI, Foster City, CA, USA). Mouse U6 and β-actin served as internal controls. The fold-change for each miRNA and mRNA was calculated by the 2-ΔΔCt method (Chen et al., 2005; Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The primers used for step-loop RT-PCR were shown in supplementary material Table S1. All PCR reactions were carried out using an Applied Biosystems 7000 PCR system (Carlsbad, CA, USA).

Supplementary material related to this article, in the online version, at http://dx.doi.org/10.1016/j.biocel.2012.07.014.

2.10. Gene ontology analysis

Putative target genes for 12 differentially expressed miRNAs were predicted using TargetScan 6.0, and putative functions were assigned to the target genes using gene ontology (GO) data from NCBI. Fisher’s exact and chi square tests were used to calculate P-values for GO category assignments. GO assignments with a P-value <0.001 and an FDR <0.05 (false discovery rate) were selected. Enrichment (Re) is another measure of the significance and specificity of the GO assignment. Within each significant GO category, enrichment (Re) was given by: Re = (n/N)/((N/n), where n is the number of differentially-expressed genes in the particular category, n is the total number of genes in the category, N is the number of differentially-expressed genes in the entire dataset (i.e., all transcripts represented on the microchip), and N is the total number of genes in the microarray (Schlitt et al., 2003).

2.11. miRNA–gene network and miRNA–GO network

The relationship between miRNA expression and putative target gene expression were analyzed with reference to the Sanger microRNA database. An adjacency matrix was calculated for miRNA and genes, where A = [aij] is the attributed relationship between gene “i” and miRNA “j”, and aij represents the relative weight of gene “i” and miRNA “j” (Enright et al., 2003). Each network node was assigned a “degree”, based on connectivity of one miRNA to its target genes or vice versa. miRNA–GO network summarizes relationships between GO terms. Parameters and definitions were the same as for the miRNA–gene network.

2.12. Luciferase activity assay

The 1623 bp Lats2-3′-UTR fragment including two conserved binding sites for miR-302a was amplified by PCR from mouse 3T3 genomic DNA, with the following primers: F: 5′CGGCTCGAGGGGCCAACGTTATCTTCT3′; R: 5′GAATTCGCGCGCCAGGCTTTAAGTTTTATATAATAGTCGACTAG3′. The amplified fragments were inserted into the pmiR-REPORT™ vector (RiboBio, Guangzhou, China) using the Xhol and NotI sites. A mutant 3′-UTR fragment, with mutations in seed binding sites was generated using the following primers: Lats2-MUT (192–198): F: 5′ATGGAAAGTCTCTTTTTTGTGGATACCCAG-3′; R: 5′ATCCACAAAATGAGCCTTTCATCAGCTACTAAT3′; Lats2-MUT (291–297): F: 5′TCGTACCTTCATCTCCACGCTAG-AAAAGAAG3′; R: 5′TTGGGACATGGGTCAGATACAACACACCATGG3′. Similarly, the fragment of Lats2 3′-UTR mutant was inserted into the pmiR-REPORT™ control vector at the same sites. For reporter assays, HEK 293 cells were cotransfected with wild-type (mutant) reporter plasmid and miR-Ribo™ mimics (miR-Ribo™ negative control) using FuGene HD reagent (Roche, Basel, Switzerland). Firefly and Renilla luciferase activities were measured in cell lysates using the Dual-Luciferase Reporter Assay system. Luciferase activity was measured 48 h post-transfection using dual-glo luciferase reporter system according to the manufacturer’s instructions (Promega, Madison, WI, USA).

2.13. Overexpression of miR-302a in folate-deficient mES cells

Folate deficient mESCs were plated at 5000 cells per well in a 96-well plate and grown overnight. Cells were transfected with 75 nM miR-302a mimics or miR-Ribo™ negative control, using the following miRNAs sequences: miR-302a mimics (sense: 5′UAAGUGUCUCAUGUUUUGYG3′; antisense: 5′ACCAAAAAACAGGAGCGACUUU3′) or negative control (sense: 5′UUCUGCCACUGUUCAGGUT3′; antisense: 5′ACUGACUGCUCCUGAGATT3′). Transfected cells were incubated for 24 h, transferred to fresh culture medium, incubated for another 24 h and then quantified by CCK-8 assay. For apoptosis and cell cycle analysis, 5 × 10³ cells were incubated overnight in a single well of a 6-well plate, treated as above.

2.14. Alkaline phosphatase staining

To assess whether these cells progressing through the cell cycle were normal or aberrant cells, alkaline phosphatase staining (AP staining) was performed with an AP kit (SinDaSen 1101-050, Shanghai, China) according to the manufacturer’s instructions. Blue or purple colonies revealed positive AP staining, indicating the undifferentiated status of these cells, whereas differentiated cells appeared colorless.

2.15. Statistical analysis

Statistical analyses were performed using Microsoft Excel Program to calculate mean and standard deviation (SD). Statistical significance of differences was evaluated by Student’s t-test. Values with a P < 0.05 (two-tailed analysis) were considered statistically significant.

3. Results

3.1. mESCs grow poorly in folate-deficient media

Cell number and viability were evaluated at 24 h intervals for 4 days. Viable cells were quantified in both cultures using a CCK-8 Kit assay. No difference in growth of the two cultures was observed for the first 48 h (Figs. 1 and 2, P > 0.05). However, cell proliferation was significantly reduced after 72 or 96 h growth in folate-deficient medium (Figs. 1 and 2, P < 0.001). In addition, approximately half of the cells deprived of exogenous folate appeared to be dead after 96 h (data not shown).
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1</th>
<th>G2/M</th>
<th>S</th>
<th>Early apoptosis</th>
<th>Late apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.49 ± 0.44</td>
<td>15.81 ± 1.47</td>
<td>71.37 ± 0.70</td>
<td>9.43 ± 0.70</td>
<td>3.37 ± 0.47</td>
</tr>
<tr>
<td>FD (72 h)</td>
<td>21.09 ± 0.63</td>
<td>5.45 ± 0.53</td>
<td>73.46 ± 1.04</td>
<td>30.43 ± 0.70</td>
<td>8.5 ± 0.36</td>
</tr>
<tr>
<td>FD (96 h)</td>
<td>25.98 ± 1.42</td>
<td>5.09 ± 0.67</td>
<td>67.37 ± 0.67</td>
<td>22.93 ± 0.32</td>
<td>18.3 ± 1.00</td>
</tr>
</tbody>
</table>

* Indicates statistically significant difference from control (P < 0.001).
∆ Indicates statistically significant difference from folate deficiency (72 h) (P < 0.01).

Fig. 1. mESCs were cultured in complete or folate-deficient medium for 24, 48, 72, or 96 h and photographed at 100×. Scale bars, 200 μm. Representative fields at each time point are shown.

Fig. 2. mESCs were cultured in complete or folate-deficient medium for 24, 48, 72, or 96 h. Aliquots were removed and cell number estimated using a chromogenic method based on Aβ630. Assays were performed in triplicate and the mean ± SD was calculated. Asterisk (*) indicates statistically significant difference from control (P < 0.001).

To investigate whether poor cell growth correlates with folate deprivation, the intracellular folate concentration was measured at 24 h intervals after folate-starvation began. Although intracellular folate did not decrease significantly within the first 24 h in folate-deficient medium (Fig. 3, **23.60 ± 1.11 ng/mL, P > 0.05, 24 h vs 0 h**), the concentration of intracellular folate decreased steadily at later time points, with values of **9.4 ± 0.55, 2.16 ± 0.31** and **1.27 ± 0.16 ng/mL** at 48, 72 and 96 h time points, respectively (Fig. 3, **P < 0.05, 48, 72, 96 h vs 0 h**). These data demonstrate that the rate of mESC growth correlates with intracellular folate concentration. These results also suggest that mESCs may be more sensitive to low folate than TK6 and other differentiated cells (data not shown; see references: Gu et al., 2002; Huang et al., 1999; Lin et al., 2006; Marsit et al., 2006; Novakovic et al., 2006).

3.2. Folate-deficiency increases apoptosis and inhibits cell cycle progression

The effects of folate-deficiency on growth of mESCs were evident after approximately 3 days (72 h) in culture. Therefore, we measured apoptosis and analyzed cell cycle distribution of mESCs after 72 or 96 h in folate-deficient medium. The data show that more folate-deficient cells underwent apoptosis and had an abnormal cell cycle distribution. After 72 h, the percentage of folate-deficient cells in early stages of apoptosis was **30.43 ± 0.70% (P < 0.01)**, and **21.09 ± 0.63%, 5.45 ± 0.53%** of cells were in G0/G1 and G2/M, respectively (P < 0.01, Figs. 4 and 5, Table 1). After 96 h, the percentage of cells in early and late apoptosis was **22.93 ± 0.32% and 18.3 ± 1.00%**, respectively, and the percentage of cells in G0/G1 and G2/M was **25.98 ± 1.42% and 5.09 ± 0.67%**, respectively (Figs. 4 and 5, Table 1). In contrast, approximately 10% and 70% control mESCs were in G1 and S phases, respectively, indicating a rapid proliferation rate in control, but a slow proliferation rate in folate-deficient mESCs. These data suggest that folate-deficiency increases apoptosis rates, causes cell cycle arrest and accumulate at a G0/G1 cell cycle checkpoint in mESCs.

3.3. miRNA and mRNA profiles of folate-deficient mESCs

miRNA and mRNA expression profiles were evaluated in folate-deficient (72 h) and control mESCs. There were 60 up-regulated
miRNAs and 34 down-regulated miRNAs; meanwhile, a total of 2316 mRNAs were up-regulated and 2544 mRNAs down-regulated in folate deficient group. After screening these miRNAs and genes for possible inter-relationships, several miRNAs and mRNAs with known roles related to mESC proliferation, apoptosis, cell proliferation and cell cycle progression were identified. Finally, 12 of these were further analyzed. For example, expression of 10 up-regulated mRs, let-7a, miR-15a, 15b, miR-16, miR-29a, 29b, miR-34a, miR-130b, miR-125a-5p and miR-124, and 2 down-regulated miRs, miR-290 and miR-302a, were quantified by stem-loop RT-PCR (Fig. 6). The data were self-consistent, thus, validating the results of the microarray experiment. The data were also consistent with our earlier studies on miRNA expression in brain, which suggested that miRs 124, 125a-5p, 29a and 16, were also overexpressed in human anencephaly (Zhang et al., 2010).

3.4. A distinct miRNA–gene network and miRNA–GO network in folate-deficient mESCs

A set of 356 target genes were explored by predicting target genes of 12 miRNAs and negative correlation analysis with mRNA profile. Further, GO terms were analyzed and a summary of GOs data was demonstrated on supplementary material Fig. S1. This analysis identified 356 putative target genes for 12 differentially expressed miRs, indicating a variety of biological processes were involved. GO term analysis revealed 217 up-regulated GOs, of which small GTPase mediated signal transduction had the highest enrichment score. Of the 78 down-regulated GO terms, GO: G1/S transition of mitotic cell cycle had the highest score (supplementary material Fig. S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2012.07.014.

Target predictions and GO analysis were used to generate a miRNA–gene network and a miRNA–GO network. In the former, the QK gene (miR-124, 130b, 15a, 15b, 16, 29a, 29b targeted QK gene, \( n = 7 \)) and miR-124 (\( n = 102 \)) were the most highly connected nodes (Fig. 7), although a large proportion of these were unrelated to cell proliferation, cell cycle, and apoptosis. However, in the miRNA–GO network, miR-302a had the highest degree, suggesting that miR-302a may have the greatest impact on the shape/connectivity of this network (Table 2). Lats2 gene, one target gene of miR-302a, had the highest enrichment in the cell cycle and apoptosis [GO-terms], with enrichment value 98.90 (supplementary material Fig. S1). These data suggest that folate-deficiency has a significant impact on expression of miRNAs and genes related to apoptosis and cell cycle progression/arrest and cell proliferation. Therefore, additional experiments focused on the possible roles of miRNA-302a and Lats2 as regulators of apoptosis and/or cell proliferation/differentiation.

3.5. Lats2 is a putative direct target of miR-302a

The TargetScan algorithm predicted two binding sites for miR-302a in the Lats2 3′-UTR; one well conserved site at

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**Table 2**

<table>
<thead>
<tr>
<th>miRNA–GO-network summary.</th>
<th>Style</th>
<th>Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-302a</td>
<td>Down</td>
<td>140</td>
</tr>
<tr>
<td>mmu-miR-124</td>
<td>Up</td>
<td>98</td>
</tr>
<tr>
<td>mmu-miR-15a</td>
<td>Up</td>
<td>89</td>
</tr>
<tr>
<td>mmu-miR-15b</td>
<td>Up</td>
<td>89</td>
</tr>
<tr>
<td>mmu-miR-16</td>
<td>Up</td>
<td>89</td>
</tr>
<tr>
<td>mmu-miR-29a</td>
<td>Up</td>
<td>75</td>
</tr>
<tr>
<td>mmu-miR-29b</td>
<td>Up</td>
<td>75</td>
</tr>
<tr>
<td>mmu-let-7a</td>
<td>Up</td>
<td>68</td>
</tr>
<tr>
<td>mmu-miR-130b</td>
<td>Up</td>
<td>66</td>
</tr>
<tr>
<td>mmu-miR-290-3p</td>
<td>Down</td>
<td>47</td>
</tr>
<tr>
<td>mmu-miR-125a-5p</td>
<td>Up</td>
<td>43</td>
</tr>
<tr>
<td>mmu-miR-34a</td>
<td>Up</td>
<td>18</td>
</tr>
</tbody>
</table>

**Style** means the up-regulated or down-regulated miRNAs; **degree** means the contribution one miRNA to the GOs around. The key microRNAs in the network always have the biggest degrees.
194–200 and one poorly conserved site at 293–299. To test this prediction, the Lats2 3′-UTR was cloned into a luciferase reporter plasmid (Fig. 8A), and the ability of miR-302a to inhibit expression of the adjacent hILuc coding region was quantified. For this purpose, the luciferase reporter plasmid pmiR-RB-REPORT™-Lats2-3′-UTR or a mutant reporter plasmid carrying point mutations in the putative miR-302a binding sites was co-transfected with miR-302a mimics (Fig. 8B). The results show that miR-302a suppresses luciferase activity by approximately 50% when the reporter plasmid carried the wild type Lats 3′-UTR (Fig. 8C, P < 0.01), but no significant suppression was observed when the reporter plasmid carried a mutant Lats 3′-UTR (i.e., pmiR-RB-REPORT™-mut-lats2-3′-UTR). These results suggest that miR-302a binds directly to the predicted binding site(s) in the Lats2 3′-UTR and negatively regulates Lats2 expression.

3.6. miR-302a directly influences cell proliferation, apoptosis and cell cycle progression

To examine whether miR-302a plays a direct role in the response to low intracellular folate, folate-deficient mESCs were transfected with miR-302a mimics or a negative control miR. Cell growth and cell cycle distribution were compared in the two cultures. RT-PCR assays confirmed an approximately 5-fold increase after transfection with the miR-302a mimics (Fig. 9A). Approximately 48 h after transfection, the number and the size of colonies was greater in cultures transfected with the miR-302a mimic and cell proliferation increased significantly (Fig. 9B and C, P < 0.05). Consistent with this, the fraction of cells undergoing early apoptosis, late apoptosis or necrosis was much higher in negative control cells than in cells transfected with the miR-302a mimics (Fig. 9D, Table 3). Furthermore, the cell cycle distributions were altered in folate-deficient cells after transfection with the miR-302a
mimics, from 45.42 ± 1.09%, 1.06 ± 0.31%, 53.51 ± 0.95% in G0/G1, G2/M and S to 18.90 ± 0.90%, 15.86 ± 0.77% and 65.27 ± 0.22% in G0/G1, G2/M and S, respectively (Fig. 9E, Table 3). These data suggest that miR-302a promotes cell proliferation, inhibits apoptosis, and may partly rescue mESCs from low folate-induced cell cycle arrest and/or apoptosis. qRT-PCR and Western blotting confirmed that expression of Lats2 and caspase-3 increased in folate-deficient cells and decreased when these cells were transfected with the miR-302a mimic (Figs. 9F and G, P < 0.05). These data suggest that miRNA-302a regulates Lats2, possibly

Fig. 7. miRNA–gene network: circles represent predicted target genes and squares represent miRNAs. Red indicates upregulated miRNAs or mRNAs and blue indicates downregulated miRNAs or mRNAs.

Fig. 8. Luciferase reporter assay for interaction between miR-302a and the Lats2 3′-UTR. (A) pmir-RB-REPORT™ dual-luciferase reporter vector. (B) Wild type predicted binding sites for miR-302a and the corresponding mutated binding sites are shown; these sequences were cloned into the polylinker of the vector shown in (A). (C) Relative luciferase activity of reporter plasmids carrying wild-type or mutant Lats2 3′-UTR in cells cotransfected with negative control (NC) or miR-302a mimic. Assays were performed in triplicate. Normalized data are shown as mean ± SD.

Table 3
Apoptosis rates and cell cycle distributions after transfection with miR-302a mimics (mean ± SD%).

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1</th>
<th>G2/M</th>
<th>S</th>
<th>Early apoptosis</th>
<th>Late apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>45.42 ± 1.09</td>
<td>1.06 ± 0.31</td>
<td>53.51 ± 0.95</td>
<td>23.57 ± 0.90</td>
<td>25.23 ± 0.79</td>
</tr>
<tr>
<td>miR-302a mimics</td>
<td>18.90 ± 0.90†</td>
<td>15.86 ± 0.77</td>
<td>65.27 ± 0.22†</td>
<td>14.43 ± 0.43†</td>
<td>14.6 ± 0.63†</td>
</tr>
</tbody>
</table>

† Indicates statistically significant difference from negative control (P < 0.001).
at the level of transcription, while lats2 and caspase-3 might be co-regulated.

3.7. miR-302a transfected folate deficient cells showed the undifferentiated status

Approximately 95% miR-302a transfected folate deficient clones contained alkaline phosphatase activity (Fig. 9H) by counting the blue or purple stained cell colonies (undifferentiated ES cells) versus colorless colonies (differentiated ES cells) under the inverted microscope. This indicated the transfected folate deficient cells kept the morphological characteristic of ESC at least, however, more morphological characteristics (mouse ESC markers: Oct4, Sox2, Nanog, SSEA-1) and functional competency, including in vitro differentiation and teratoma formation in SCID mice, etc., will need further assessments (Reubinoff et al., 2000).

4. Discussion

Embryonic development is a complex process, influenced by the placenta and the maternal nutrient supply, both of which can strongly influence fetal growth and development. One nutrient that is critical during fetal development is folate, a vitamin best known for its ability to prevent neural tube defects. One prevailing theory is that an inadequate level of folate in the embryo leads to hypomethylation of genomic DNA, which adversely affects several DNA metabolic processes including DNA synthesis and DNA repair. This would explain why low folate is associated with DNA damage and cell death (Gu et al., 2002; Huang et al., 1999; Antony; James et al., 2003; Rondo and Tomkins, 2000). However, the exact mechanism by which low folate lowers cell viability and leads to developmental defects is not yet understood.

Recently, an increasing amount of data also indicates the importance of miRNAs in response to environmental stress, including nutrient deprivation. Nutrient–gene interactions enable various nutrients to transiently influence the expression of specific subsets of genes (Chiou, 2007; Cui et al., 2012; Marsit et al., 2006). This study uses mESCs to explore the downstream consequences of low folate during mammalian development. In particular, we provide evidence that specific miRNAs and their target genes play roles in the response of mESCs to low folate, and that miR-302a regulates cell cycle progression in mESCs by targeting Lats2, a tumor suppressor that negatively regulates the G1/S transition. Furthermore, miR-302a was down-regulated in a time and dose dependent manner in response to folate deficiency, as early as 12 h (approximately 1.5-fold, Fig. 3 and supplementary material Fig. S2). Thus, it is reasonable that miRNAs may be early sensors in response to nutrient stress, as well as crucial intermediate regulators.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2012.07.014.

mESCs were chosen for these experiments, because they are totipotent cells that can self-renew or differentiate in vitro and in vivo in response to developmental signals (Rossant, 2008). The results presented here demonstrate that folate-deficient mESCs fail to proliferate, undergo cell cycle arrest in GO/G1 and have a higher rate of apoptosis. Although these data do not agree with previous suggestions that folate deficiency causes cell cycle arrest in S phase (Benito et al., 1996; Courtemanche et al., 2004; Huang et al., 1999; Ingram et al., 1997; Lin et al., 2006), we propose that this discrepancy can be readily explained, as follows.

First, ESCs have an unusual cell cycle characterized by a generation time of 12–15 h, a short G1 and a relatively high proportion of S-phase cells (Rohwedel et al., 1996; Savatier et al., 1994). In addition, while somatic cells proliferate only in response to exogenous mitogens, ESCs proliferate in a mitogen-independent manner (Tiscornia and Izpisua, 2010). Second, mESCs have a rapid G1/S transition, due to low expression of cdkn1a, lats2 and rbl2, critical regulators of the cyclin E-Cdk2 complex (Burdon et al., 2002; Wang et al., 2008). Third, ESCs have a distinct miRNA profile (Young, 2011), and mESC-specific miRNAs may play critical roles during embryogenesis (Houbaviy et al., 2003). Furthermore, mESCs express multiple cell cycle–specific miRNAs (ESCC miRNAs), including miR-290, miR-302 and the miR-17–92 cluster; these miRs accelerate cell cycle progression (especially the G1–S transition), promote proliferation and may modulate an overlapping group of target genes, due to a shared, conserved seed sequence (Hatfield et al., 2005; Murchison et al., 2005; Tiscornia and Izpisua, 2010; Wang et al., 2008). Therefore, it is possible that ESCCC miRNAs cooperate to repress multiple inhibitors of the G1/S transition, promote S phase progression and reduce the length of the cell cycle. One last critical point is that genom hypomethylation resulting from low folate may significantly dysregulate expression of ESCC-specific miRNAs (Marsit et al., 2006).

It is estimated that ESCs express approximately 110,000 miRNAs, and approximately half of these are encoded by four loci: miR21, the miR17–92 cluster, the miR15b/16 cluster and the miR290–295 clusters. Furthermore, ESC-specific miRNAs, including the conserved miR-290, miR-302, and miR-370 clusters, are thought to play roles in regulating cell proliferation during embryonic development (Calabrese et al., 2007; Houbaviy et al., 2003). Here, for the first time, we show that folate–depletion alters the miRNA expression profile of mESCs. In particular, we identified 60 or 34 miRNAs differentially up- or down-regulated in folate-deficient mESCs. Interestingly, these are not the same miRNAs that were previously linked to neural tube defects during mammalian development (Cui et al., 2012).

GO analysis revealed the miRNA–gene network and miRNA–GO network in folate-deficient mESCs (supplementary material Fig. S1, Fig. 7 and Table 2). In the miRNA–gene network, miRNA-124 had the largest number of target genes, although a large proportion of these were unrelated to cell proliferation, cell cycle, and apoptosis. Therefore, more bioinformatics analyses of the predicted miRNA–gene and miRNA–GO networks are extremely necessary. Nevertheless, GO annotations are remarkably useful for the mining of functional and biological significance embedded in large microarray datasets. Here, GO-network analyses suggest miR-302a play a key role in regulating the G1/S transition of mitotic cell cycle by targeting Lats2 gene.

Three levels of evidence support the conclusion that miR-302a regulates Lats2: first, TargetScan predicts functional binding sites for miR-302a in the Lats 3′-UTR; second, miR-302a suppressed Renilla luciferase activity when the luciferase reporter plasmid carries the wild type Lats 3′-UTR (P < 0.01), while no significant suppression was observed when the predicted miR-302a binding sequences included point mutations (Fig. 8); and third, functional assays show that both folate-deficient mESCs express less Lats2 mRNA and protein after transfection with miR-302a mimics (Fig. 9), one after another (Wan et al., 2010). Furthermore, transfection with the miR-302a mimic rescued the phenotype of folate-deficient cells, reducing the frequency of apoptosis and stimulating cell proliferation. Together, these data suggest that miR-302a regulates Lats2, possibly at the transcriptional and translational levels.

The miR-302 family includes seven miRNAs that share a highly conserved S′ seed region: UUAUGUGCU, belonging to the miR302–367 cluster, which was identified to be expressed in mESCs, hESCs, their malignant counterparts ECCs and iPSCs, but not in differentiated cells and other adult cell lines confirming its specificity embryonic developmental stages (Houbaviy et al., 2003; Suh et al., 2004; Wilson et al., 2009). Furthermore, miR-302 is an ESCC miR, in that it targets critical G1/S cell cycle regulators, including cyclinD1 and Cdkn1a (Card et al., 2008; Wang
Fig. 9. Effect of miR-302a transfection on mESC cell cycle arrest. (A) Folate deficient mESCs were transfected with miR-302a mimic and miR-302a was quantified in transfected cells; (B and C) CCK-8 assay and representative images (250×, scale bars 200 μm) of mESCs in the indicated experimental group. Asterisk (*) indicates statistically significant difference from control (P < 0.001). (D) Flow cytometry was performed and analyzed as described in Figs. 4 and 5; (F and G) RT-PCR and Western blots quantifying Lats2 and caspase-3 mRNA and protein, respectively, in the indicated experimental group. B-actin and U6 are internal controls. (H) Alkaline phosphatase (AP) staining. Undifferentiated cells appear red or purple, whereas differentiated cells appear colorless (40×, scale bars 100 μm). Asterisk (*) indicates statistically significant difference from control group, while triangle (△) indicates statistically significant difference from negative control group. FD: folate-deficient group; NC: negative control group; miR-302a mimics: miR-302a mimics transfected group.
et al., 2008), thus biasing cells toward the CyclinE–Cdk2 pathway instead of the Cdk4/Cdk6–CyclinD pathway (Fluckiger et al., 2006). Previous studies also reported that expression of the miR-302–367 cluster reprogrammed somatic and cancer cells toward a less differentiated state (Anokye-Danso et al., 2011; Lin et al., 2008; Subramanyam et al., 2011), possibly via altered epigenetic programming, as described for iPS cells (Huang and Lin, 2012). Nevertheless, the mechanism by which miR-302 modulates mESC proliferative capacity is not yet fully understood, and additional studies are clearly needed.

**Lats**, also known as **warts** or **Kpn**, was first identified as a tumor suppressor component of the Hippo pathway that regulates cell growth and shape in *Drosophila melanogaster* (Justice et al., 1995). Lats2 is a serine/threonine kinase that regulates cell proliferation, apoptosis, cell migration and organ size (Visser-Grieve et al., 2012). Lats2 is essential during embryogenesis, and disruption of Lats2 is lethal to the developing embryo (McPherson et al., 2004). Recent studies show that activated Lats2 causes mdm2-p53-dependent cell cycle arrest and apoptosis involving aspp1, p27 or p73 (Aylon et al., 2010; Aylon and Oren, 2011; Okada et al., 2011; Tschop et al., 2011). Thus, Lats2 negatively regulates the G1/S transition, while Lats1 is thought to regulate the G2/M transition (Xia et al., 2002). Lats2 may directly or indirectly modulate the kinase activity of Cyclin E/Cdk2 (Li et al., 2003). However, the mammalian Hippo pathway is poorly understood (Tschop et al., 2011), and additional research on the Hippo pathway, and its possible connection to low folate-induced developmental defects, is needed.

In summary, based on the evidence presented here, we propose that the effect of folate deficiency on mammalian development may be mediated by altered expression of mESC-specific miRNAs, which may be early sensors in response to nutrient stress. However, additional research is urgently needed to better understand the complex inter-relationships between miRNAs and their target mRNAs, and how miRNAs regulate and balance mESC proliferation and mESC differentiation at different stages of embryogenesis.

**Conflict of interest statement**

None declared.

**Acknowledgements**

This work was supported by the National Basic Research Program of China (grant no. 2007CBI1903); National Natural Science Foundation of China (grant no. 30671156) and the Beijing Natural Science Foundation (grant no. 3072014).

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