ABSTRACT
Several microRNAs (miRs) play regulatory roles in cervical cancer. The present study investigated the molecular role of microRNA-29a in cervical cancer via modulation of cell division cycle 42 (CDC42) expression. The results showed significant (P < 0.05) downregulation of miR-29a human in cervical cancer cells. Overexpression of miR-29a inhibited the proliferation and colony formation of the cervical cancer cells via G0/G1 cell cycle arrest. In silico analysis and luciferase reporter assay showed CDC42 to be the molecular target of miR-29a. CDC42 was found to significantly (P < 0.05) overexpressed in cervical cancer cells. Nonetheless, miR-29 overexpression inhibited the expression of CDC42 in DoTc2 cells. Silencing of CDC42 inhibited the proliferation of DoTc2 cells. However, overexpression of CDC42 could prevent the tumour-suppressive effects of miR-29a. Finally, miR-29a was found to suppress the tumour-growth in vivo further confirming the tumour-suppressive effects of miR-29. Collectively, the findings of the present study revealed the tumour-suppressive effects of miR-29 of cervical cancer.

1. Introduction
Cervical cancer ranks among the most dominant malignancies of female population throughout the world. Ranked as 4th most commonly diagnosed disorder among women, cervical cancer is reported to affect more than half a million women per year and results in 270,000 deaths annually [1–3]. In comparison to the developed countries, cervical cancer is more prevalent in developing countries where from more than 80% of total deaths from cervical cancer are reported each year [4]. The optimal treatment strategy presently employed against cervical cancer consists of radical hysterectomy in combination with surgical resection of lymph nodes with or without radio/chemo-therapies. During recent years, a shift in understanding the progression of cervical cancer in terms of intensive molecular characterization of regulatory molecules governing the cervical cancer growth and propagation, has led to identification of a large number of specific cancer regulatory non-coding RNAs [5]. Considering this, the non-RNA classes like micro-RNAs (miRs) are appreciated for their prognostic value and therapeutic potential against cervical cancer [6]. They also have been proposed to aid in the monitoring of therapeutic response to cervical cancer treatment. The miRs act either as oncogenes or tumour-suppressors to affect the over-all process of tumorigenesis [7]. The miRs have been shown to act via post-transcriptional/translational targeting of eukaryotic genes crucial for cellular processes like cell division, differentiation and apoptosis to name a few [8,9]. The miR-29a has been shown to exhibit the tumour suppressive role in different human cancers and its down-regulation was found to be associated with cancer growth and progression [10,11]. The role of miR-29 in the development of pancreatic and gastric cancer has also been reported [12,13]. Koshizuka et al. reported that miR-29 targets the integrin β1-mediated oncogenic signalling to exert tumour-suppressive effects of miR-29 in head and neck squamous cell carcinoma [14]. Similarly, Xu et al. reported that miR-29 targets fibroblast growth factor 2 (FGF2) to inhibit the growth of nasopharyngeal carcinoma [15]. In yet another study, miR-29 has been shown to target ribosomal protein S15a (RPS15A) to suppresses the growth of hepatocellular carcinoma [16]. Inhibition of hepatocellular growth by miR-29 has also been shown to be mediated via post-transcriptional suppression of IGF2 mRNA-binding protein (IGF2BP) [17]. Despite these studies, the role of miR-29a via modulation of CDC42 expression has not been studied in cervical cancer. Against, this backdrop the present study was designed to explore the expression profile, role and therapeutic implications of miR-29/CDC42 axis in cervical cancer.
2. Materials and methods

2.1. Propagation of cell lines

A panel of human cervical cancer cell lines (C-33-A, CaSki, DoTc2, HeLa and SiHa) and normal human cervical epithelial cell line (NCEC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco’s Modified Eagle Medium (DMEM, Gibco) was used for propagation of all cell lines except HeLa cell line for which MEM (Gibco) was used. The culture media contained 10% supplementation of fetal bovine serum (FBS). The cell culturing was performed in humidified incubator at 37°C with 5% CO2 saturation.

2.2. Transfection

The miR-29a mimics, its negative control (miR-NC), si-CDC42 and its negative control (si-NC) were designed and purchased from Gene Pharma Company, China. Over-expression of CDC42 was performed by transfecting the DoTc2 cancer cells with pcDNA3.1 vector containing ORF of CDC42 (pcDNA-CDC42) while vector alone was used as control. The cancer cells were transfected with the help of Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol.

2.3. RNA extraction, cDNA synthesis and RT–PCR analysis

Trizol reagent (Thermo Fisher Scientific) was used for the isolation of total RNA from cell lines. RNA was treated with DNase I (Takara) for eliminating genomic DNA and was subsequently reverse-transcribed into cDNA using Reverse Transcriptase M-MLV (TaKaRa). SYBR Green PCR-mix (Thermo Fisher Scientific) was used for performing qRT-PCR on QuantStudio 5.0 real-time-PCR System (Thermo Fisher Scientific). The conditions for carrying out the PCR were 95°C for 25 sec, followed by 41 cycles of 95°C for 15 sec, and 57°C for 1 min. For estimating the relative expression of miR-29a, U6 snRNA served as the internal reference while the CDC42 gene expression was quantified in relation to human β-actin.

2.4. Cell viability assay

The proliferation of cancer cells was determined by CCK-8 assay kit (Dojindo Molecular Technologies) as per manufacturer’s instructions. Briefly, about 4000 transfected cells were added per well of 96-well plate. Each well was inoculated with 10 μL CCK-8 assay solution after 0, 24, 48, 72 or 96 h incubation at 37°C. This was followed by the addition of 10 μL CCK-8 and subsequent incubation at 37°C incubation for additional 2.5 h. Finally, absorbance was read at 450 nm for each well using spectrophotometer. Every sample was assessed using three replicates.

2.5. Colony formation assay

For analyzing the colony formation, 250 transfected DoTc2 cells with culture medium were seeded in each well of a 6-well plate. The culture medium was replaced after every 4 days. The cell culturing was performed for 14 days to allow colony formation. The colonies obtained were fixed with 70% ethanol and the stained using 0.1% crystal violet solution. Counting of colonies was performed manually from five randomly selected fields. Three replicates were kept for each treatment group.

2.6. Cell cycle analysis

To study the distribution of DoTc2 cells in different phases of cell cycle, the transfected cells were cultured for 48 h at 37°C in 12-well plate. The cells were homogenized by trypsinization and harvested by centrifugation. The ice-cold PBS was used for washing the cells thrice, and cells were fixed with methanol for 2 h at 4°C. The fixed cells were then stained with propidium iodide (PI)/RNase mix followed by analysis with fluorescence-activated cell sorter scan (FACS) through flow cytometry. The ELITE software was used for determining the percentage of cells in each mitotic phase of the cell cycle.

2.7. 5-ethynyl-2-deoxyuridine assay

The proliferative viability of transfected cells was estimated with 5-ethynyl-2-deoxyuridine (EdU) labelling/detection kit (Ribobio, Guangzhou, China). Precisely, the cancer cells were cultured at 2.5 × 10^4 cells/well density in 96-well plate for 48 h at 37°C. EdU labelling solution (50 μM) was added and the incubation at 37°C incubation was prolonged for another 2 h. The cells were treated with paraformaldehyde (4%) and Triton X-100 (0.5%) and then stained with anti-EdU working solution. The nuclei of transfected cells were stained with DAPI solution. The EdU-positive cells were detected using fluorescent microscopy.

2.8. In vivo mice xenograft study

BALB/c-nude mice (4–6 week old) were used for generating the mice tumorigenic xenografts of cervical cancer through subcutaneous injections of DoTc2 cancer cells into right mice scapulas. The experimental usage of animals was approved by the institutional
Ethical Committee on Animal care and Experimentation under approval number CMU/53/2019. For analyzing the effects of the miR-29a over-expression on mice tumour growth, the mice tumours were administered with either the injections of miR-29a mimics or its negative control after every 2 days. The administration was continued for a total of 3 weeks and the mice were then sacrificed. The relative tumorigenesis was then assessed by determining the size, volume and average weight of mice tumours. The expression of cyclin D1 was assessed by immuno-histochemical staining from mice tumours.

2.9. Target identification of miR-29a
To specifically predict the potential molecular targets of miR-29a, the in silico analyses were performed using TargetScan (www.Targetscan.org) and miRanda (www.microrna.org/microrna/home.do) online software tools. The TargetScan analysis was further used for predicting the miR-29a binding site in CDC42 3′-UTR.

2.10. Dual luciferase assay
To confirm the target prediction, luciferase assay was performed. Firstly, the reporter plasmids carrying wild-type CDC42 3′-UTR (WT) and mutant 3′ UTR (MUT) were constructed using pGL3 reporter plasmid. Each reporter construct was co-transfected with miR-29a mimics or miR-NC into DoTc2 cancer cells. Finally, the luciferase activity of the transfected cells was analyzed by Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection, as per the manufacturer’s protocol.

2.11. Western blotting
The transfected cells DoTc2 cells were lysed and total protein were extracted by a cell RIPA lysis buffer (Beyotime, China). The concentration of each protein sample was determined through Bradford assay. Equal proteins were separated using 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The blotting to polyvinylidene difluoride (PVDF) membranes was performed and the primary and secondary antibody treatments were performed to determine the relative protein concentration. B-actin protein served as the internal control for western blot study.

2.12. Statistical analysis
Each experiment was performed used at least three replicates. The statistical tests like Student’s t-test and one-way ANNOVA were performed using Graphpad Prism 7.0 software. The final values were presented as mean ± SD. P < 0.05 indicated the statistically significant difference between two data points.

3. Results
3.1. Overexpression of miR-29a reduced proliferation of cervical cancer cells
From the quantitative-real-time PCR study, it was found that miR-29a has significant (P < 0.05) transcriptional downregulation in cervical cancer cells when compared with its expression in normal cervical cell line (Figure 1(A)). Thus, to verify whether downregulated miR-29a expression levels account for cancerous features of cervical cancer cells, miR-29a was overexpressed in DoTc2 cervical cell line (Figure 1(B)). The DoTc2 cancer cells over-expressing miR-29a were seen to depict markedly lower proliferation (Figure 1(C)). The colony formation from such cells was also reduced significantly (P < 0.05) (Figure 1(D)). Taken together, miR-29a negatively regulates the proliferation of cervical cancer cells.

3.2. miR-29a overexpression triggered G0/G1 arrest to DoTc2 cell proliferation
To confirm if the growth inhibitory effects of miR-29a on cervical cancer cells were resulting from arrest of the cell division, the mitosis of DoTc2 cancer cells overexpressing miR-29a was analyzed and compared with the negative control cells. Higher percentage of cancer cells overexpressing miR-29a were seen to be accumulating at G0/G1 sub-phase of cell cycle comparison to the negative control cells suggesting G0/G1 cell cycle arrest (Figure 2(A)). The EdU assay indicated that the number of cancer cells positively stained with EdU, representing the proportion of cells in actively dividing state, was significantly (P < 0.05) declined under miR-29a overexpression (Figure 2(B)). The tumour-suppressive effects of miR-29a overexpression were thus inducted through cell cycle arrest at G0/G1 sub-phase.

3.3. miR-29a exhibited tumour-suppressive role in vivo mice xenografts
To infer whether deduced in vitro role of miR-29a in regulating cervical cancer cell growth correlates with its in vivo effects on cervical cancer tumorigenesis, mice xenograft models of cervical cancer overexpressing miR-29a expression were developed. In comparison to the control tumours, the xenograft tumours over-expressing miR-29a exhibited significantly (P < 0.05) lower size and tumour weigh (Figure 3(A)). The tumour volume was also shown to be inhibit significantly (P < 0.05) upon miR-29 overexpression (Figure 3(B)). Similarly, the average tumour weight was significantly (P < 0.05) lower under miR-29a over-expression (Figure 3(C)). The proliferation marker, cyclin D1 exhibited significantly lower expression in mice tumours administrated with miR-29a up-regulation (Figure 3(D)). The results are evocative of in vivo tumour suppressive
Figure 1. miR-29a inhibits the proliferation cervical cancer cells. (A) Relative expression of miR-29a in cervical cancer cell lines (C-33A, CaSk, DoTc2, HeLa and SiHa) in comparison to normal cervical epithelial cell line, NCEC. (B) RT-PCR analysis of miR-29a expression from miR-29a mimics transfected DoTc2 cancer cells in comparison to negative control cells. (C) analysis of viability of DoTc2 cancer cells transfected with miR-29a mimics or miR-NC by CCK-8 viability assay. (D) colony formation assay and relative percentage of colonies formed by DoTc2 cancer cells transfected with miR-29a mimics or miR-NC. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05 for normal vs. cancer cells and miR-NC vs miR-29a mimics).

role of miR-29a and indicate the therapeutic utility of miR-29a against cervical cancer.

3.4. CDC42 is targeted by miR-29a in cervical cancer

A number of human genes were predicted to be targeted by miR-29a by *in silico* analysis (Figure 4(A)). CDC42 was selected for further characterization for its reported role in cell cycle progression [18]. Online bioinformatics revealed the CDC42 3′-UTR binding site of miR-29a (Figure 4(B)). The binding site appeared to be more or less conserved in many animals as deduced from the online sequence alignment studies (Figure 4(C)). Dual luciferase assay proved that CDC42 is actually targeted by miR-29a in cervical cancer (Figure 5(A)). The expression of CDC42 gene was found to be negatively correlating with the expression of miR-29a in cancer cell lines further supporting the post-transcriptional targeting of CDC42 by miR-29a (Figure 5(B)). The western blotting of CDC42 also suggested the same (Figure 5(C)). The CDC42 expression was significantly (*P < 0.05) lower in DoTc2 cancer cells overexpressing miR-29a in comparison to that in negative control cells. The results thus clearly specify that miR-29a targets CDC42 in cervical cancer.

3.5. CDC42 acts as functional miR-29a target and assuages the role of miR-29a in cervical cancer

Whether the cervical cancer cell growth inhibitory effects of miR-29a overexpression were actually mediated through CDC42, CDC42 was silenced in DoTc2 cancer cells, and their proliferation rates were estimated. The CDC42 knockdown led to significant (*P < 0.05) decline in cancer cell growth (Figure 6(A)). Lastly, when the cervical cancer cells were co-transfected to overexpress both miR-29a and CDC42, the cells proliferated much like as control cancer cells suggesting CDC42 overexpression preventing tumour-suppressive effects of miR-29 (Figure 6(B)). Together, these finding suggest that the growth regulatory role of miR-29a is exerted though CDC42 in cervical cancer.

4. Discussion

The miRs represent a class of short single-nucleotide RNA species which are transcribed by RNA polymerase
Figure 2. miR-29a overexpression induced G0/G1 cell cycle arrest of cervical cancer cells. (A) Relative proportion of miR-29a overexpressing DoTc2 cells representing different stages of cell cycle in comparison to negative control cells (B) EdU assay for the assessment of proliferation of DoTc2 cancer cells transfected with miR-29a mimics or miR-NC. The experiments were performed in triplicate and expressed as mean ± SD (∗P < 0.05 for miR-NC Vs miR-29a mimics).

Figure 3. miR-29a inhibits tumour growth in vivo. (A) Analysis of relative size of mice tumours rescued from mice xenografts administered with intra-tumour injections carrying miR-29a mimics or miR-NC (B) analysis of relative volume of xenograft mice tumours under administration of miR-29a mimics or miR-NC carrying intra-tumour injections for varied periods (C) assessment of average tumour weight obtained under administration of miR-29a mimics or miR-NC carrying intra-tumour injections (D) immune-histochemical analysis of cyclin D1 from mice tumours injected with miR-29a mimics or miR-NC carrying intra-tumour injections. The experiments were performed in triplicate and expressed as mean ± SD (∗P < 0.05 for miR-NC Vs miR-29a mimics).
in eukaryotes and are involved in diverse biological and physiological processes [19]. The miRs were reported to not only regulate the tumorigenesis but are actively involved in regulating the molecular cross-talk between cancer cells and their micro-environment responsible for driving development and propagation of human cancers [20]. Cancer biologists have proposed the miRNA-replacement cancer therapy wherein the miRNA dysregulation might be corrected through miRNA overexpression or silencing to accurately exert the anti-cancer effects at molecular level [21]. The downregulation of miR-29a was elucidated to feature the malignancy of various human cancers yet many others were shown to be associated with significant miR-29a upregulation [22–24]. The conflicting reports might either suggest tissue-specific regulatory role of miR-29a or support its regulatory flexibility to behave as oncogene or tumour-suppressor in human cancers [12–17]. The dual regulatory role of miR-29a might be exercised through differential signalling pathways. In our present work, we found that miR-29a was noticeably downregulated in cervical cancer in consistent with its expression pattern in gastric and prostate cancers [25,26]. The repression of miR-29a was shown to be responsible for tumorigenic behaviour of cervical cancer cells. The overexpression of miR-29a in vitro cell line system and in vivo mice xenograft models confirmed the tumour-suppressive regulatory potential of miR-29a.
against cervical cancer advocating its cancer therapeu- tic utility against the same. The cell cycle progression was shown to be inhibited in cervical cancer cells over-expressing miR-29a enlightening the possible mechanism of growth inhibitory role of miR-29a. The tumour-suppressive effects of miR-29a have been proposed to result from the molecular targeting of a specific gene behaving as oncogene in human cancer. For instance, Insulin-like growth factor 1 receptor (IGF1R) was found to be targeted by miR-29a in hepatocellular cancer to inhibit the growth and migration of liver cancer cells [27]. In a similar fashion, miR-29a was here found to post-transcriptionally target the cell division cycle 42 (CDC42) protein in cervical cancer which modulated the tumour-inhibitory role of miR-29a. A Rho-GTPase, CDC42 protein was found to exhibit proto-oncogenic behaviour and contribute to growth and proliferation of human cancers like colon and breast cancer [28,29]. The CDC42 transcriptional knockdown has been shown to induce mitotic cell cycle arrest and apoptosis to inhibit the cancer cell growth [30]. Therefore, it is evident that miR-29a targets CDC42 to implement its tumour suppressive effect in cervical cancer. Similar conclusion was drawn by Li et al. in 2017 about miR-29a and CDC42 interactional inter-play in non-small cell lung cancer [31]. To conclude, the current study explored the regulatory link of miR-29a/CDC42 in cervical cancer for its cancer regulatory role and depicted its possible therapeutic utility as alternate and effective anti-cancer strategy. Future studies, exploring the expression of miR-29a and CDC42 in different cervical cancer tissues may prove further insights about the role of miR-29/CDC42 axis in cervical cancer. Moreover, identification of the drug candidates that can modulate miR-29 expression may prove essential in the management of cervical cancer.

5. Conclusion

Collectively, the results of the current study revealed the downregulation of miR-29 in cervical cancer. Overexpression of miR-29a exerts tumour-suppressive effects in cervical cancer cells by targeting CDC42. The study suggests that downregulation of miR-29a in cervical

Figure 5. miR-29a interacts with CDC42 (A) Dual luciferase reporter assay for the confirmation of miR-29a interaction with CDC42 UTR (B) relative expression of CDC42 in cervical cancer cell lines (C-33-A, CaSkI, DoTc2, HeLa and SiHa) in comparison to normal cervical epithelial cell line, NCEC (C) western blotting of CDC42 from DoTc2 cancer cells transfected with miR-29a mimics or miR-NC. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05 for normal vs. cancer cells and miR-NC Vs miR-29a mimics).
Figure 6. CDC42 overexpression prevents tumour-suppressive effects of miR-29. (A) CCK-8 assay for the analysis of viability of DoTc2 cancer cells transfected with si-CDC42 or si-NC (B) CCK-8 assay for the analysis of viability of DoTc2 cancer cells transfected with miR-29aa-mimics, miR-NC or miR-29a mimics + pcDNA-CDC42. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05 for si-NC Vs si-CDC42 and si-NC Vs miR-29 mimics + pcDNA-CDC42).

cancer might emerge as an effective prognostic measure for this malignancy. The results further proved miR-29a/CDC42 axis as potential therapeutic target for the management of cervical cancer.

Competing interests
The authors declare no competing interests.

Disclosure statement
No potential conflict of interest was reported by the author(s).

ORCID
Fang Song http://orcid.org/0000-0002-0845-6278

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