MicroRNA-874–mediated inhibition of the major G₁/S phase cyclin, CCNE1, is lost in osteosarcomas

Tanushree Ghosh, Akhil Varshney, Praveen Kumar, Manpreet Kaur, Vipin Kumar, Ritu Shekhar, Raksha Devi, Priyanka Priyanka, Md. Muntaz Khan, and Sandeep Saxena

From the National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110067, India

Received for publication, July 24, 2017, and in revised form, October 24, 2017. Published, Papers in Press, November 6, 2017, DOI 10.1074/jbc.M117.808287

Edited by Xiao-Fan Wang

The tumor microenvironment is characterized by nutrient-deprived conditions in which the cancer cells have to adapt for survival. Serum starvation resembles the growth factor deprivation characteristic of the poorly vascularized tumor microenvironment and has aided in the discovery of key growth regulatory genes and microRNAs (miRNAs) that have a role in the oncogenic transformation. We report here that miR-874 down-regulates the major G₁/S phase cyclin, cyclin E1 (CCNE1), during serum starvation. Because the adaptation of cancer cells to the tumor microenvironment is vital for subsequent oncogenesis, we tested for miR-874 and CCNE1 interdependence in osteosarcoma cells. We observed that miR-874 inhibits CCNE1 expression in primary osteoblasts, but in aggressive osteosarcomas, miR-874 is down-regulated, leading to elevated CCNE1 expression and appearance of cancer-associated phenotypes. We established that loss of miR-874–mediated control of cyclin E1 is a general feature of osteosarcomas. The down-regulation of CCNE1 by miR-874 is independent of E2F transcription factors. Restoration of miR-874 expression impeded S phase progression, suppressing aggressive growth phenotypes, such as cell invasion, migration, and xenograft tumors, in nude mice. In summary, we report that miR-874 inhibits CCNE1 expression during growth factor deprivation and that miR-874 down-regulation in osteosarcomas leads to CCNE1 up-regulation and more aggressive growth phenotypes.

The poorly vascularized tumor microenvironment is associated with conditions of hypoxia and growth factor deprivation to which the cancer cells adapt for subsequent tumor progression and metastasis (1, 2). Serum withdrawal has been used to mimic growth factor deficiency, which results in stalling of the cell cycle progression (3–5). Movement of the cells from one phase to the next is regulated by cyclins and their catalytic subunits, the cyclin-dependent kinases (CDKs), which drive the transitions between the different phases (6). It has been known that one of the reasons for cell cycle stalling during growth factor deprivation is the decrease of cyclin E, and independent mechanisms have been proposed to curtail cyclin E–CDK activity during serum starvation; protein kinase C associates with cyclin E and recruits it to the perinuclear zone during serum starvation, which is reversed when cells enter the cell cycle (7–9).

The role of non-coding RNAs in cell cycle regulation during serum starvation has been investigated previously; microarray analysis shows that a large number of microRNAs are significantly and sustainably altered during serum starvation (10). The levels of miR-16 increase during arrest in G₀ phase and decrease upon release into the cell cycle, suggesting that the rapid up-regulation of miR-16 induces cell cycle arrest (11). Similarly, it has been reported that miR-34a targets Sirt1 in response to oxidative and genotoxic stress, exacerbating hypoxia and serum starvation–induced apoptosis during serum starvation (12). Similar miRNA expression patterns were observed during stalling of cell growth either by contact inhibition or serum starvation, suggesting that common growth pathways are targeted (13). Thus, there is accumulating evidence that miRNAs modulate major physiological pathways during stress, playing a key role in adaptation of cancer cells. miRNAs from multiple families (miR-16, miR-7, miR-132, and miR-144) have been identified that target cyclin E, with their down-regulation being linked to different cancers (14–17). Although it is widely accepted that miRNA-mediated cyclin E regulation has a vital role in oncogenesis, the role of cyclin E in regulating the cell cycle in the tumor microenvironment remains elusive. Moreover, no miRNA has been identified that targets CCNE1 during serum starvation. In recent years, it has been realized that long non-coding RNAs (lncRNAs) play a crucial role in tumorigenesis through their interactions with DNA, protein, and RNA. Functional annotation of mammalian cDNA has identified more than 25,000 RNAs that have a 5′ cap and splicing and polyadenylation sites but no open reading frame (18). Deregulated expression of several lncRNAs has been associated with cancers. One such oncogenic lncRNA is HOXATIR, up-regulation of which indicates poor prognosis and enhanced metastasis in breast cancer (19). One of the mechanisms of action of lncRNA is to titrate away specific miRNAs, thereby relieving the repression of miRNA target genes. It has been shown that long non-coding RNA, PTENP1, acts as competing endogenous RNA (ceRNA) to sponge miR-106b and miR-93 (20). It has been reported that lncRNA XLOC_008466 is up-regulated in human...
non-small cell lung cancer, where it functions as a ceRNA, directly binding to miR-874, although the target gene that affects cell proliferation remained unidentified (21).

Serum withdrawal resembles the growth factor deprivation characteristic of the poorly vascularized tumor microenvironment and has aided in the discovery of key growth modulatory genes and miRNAs that have a role in the oncogenic transformation (22). In the present study, we investigated the miRNA–mRNA regulatory networks in contrasting physiological growth states, cell cycle entry and exit, to draw a correlation between them and tested these regulatory networks during osteosarcoma oncogenesis. We observed that miR-874 targets CCNE1 during serum starvation, whereas in growth factor–sufficient conditions, miR-874 is down-regulated, resulting in CCNE1 prevalence and cell growth. We observed that the miR-874 regulation of CCNE1 is lost in osteosarcoma, leading to aggressive cancer-related phenotypes.

Results

A screen identifies miRNA–mRNA regulatory networks during serum starvation

To identify the miRNAs that are differentially regulated during growth factor deficiency, we followed a systematic approach as described in the legend to Fig. 1A. Asynchronous HCT116 cells were grown in serum-free medium for 48 h, after which cells were resupplemented with 10% fetal bovine serum and further grown for 12 h. The asynchronous, serum-starved, and serum-resupplemented cells were harvested for evaluating RNA and protein levels as well as cell cycle analysis; flow cytometry analysis established that serum-starved cells were arrested in G0/G1 phase, indicating cell cycle exit, whereas after serum restimulation, cells progressed into the cell cycle (Fig. 1B). Serum withdrawal led to an increase in the levels of CDK inhibitors, p21 and p27, which were down-regulated as cells progressed into the cell cycle after serum restimulation (Fig. 1C). We performed global miRNA expression profiling of asynchronous, serum-starved, and serum-resupplemented cells from two biological replicates using miRCURY LNA™ microRNA Array (7th generation), which is based on miRBase version 19.0. High concordance among the biological replicates demonstrated that the individual sample sets had low variability, as seen in the heat map (Fig. 1D). Of a total of 567 miRNAs that were analyzed, we identified 51 differentially expressed miRNAs in asynchronous, serum-starved, and serum-resupplemented HCT116 cells with a >2-fold change using a significance analysis of microarray (SAM) plot (Fig. 1E). A majority of these differentially regulated miRNAs were up-regulated following serum withdrawal (48) and down-regulated upon serum restimulation (44), whereas only three miRNAs exhibited an inverse expression pattern. The quantitative real-time PCR analysis confirmed differential expression of 13 miRNAs: miR-663a, miR-874-3p (miR-874), miR-1469, miR-3178, miR-3195, miR-3196, miR-3687, miR-4279, miR-4449, miR-4488, miR-4497, miR-4508, and miR-4516. Each of these miRNAs was found to be up-regulated in serum-starved cells in comparison with asynchronously growing cells, whereas the expression levels of these miRNAs were down-regulated in serum-resupplemented cells in comparison with serum-starved cells (Fig. 1F).

A repertoire of E2F-regulated genes primarily involved in DNA replication and cell cycle is known to be down-regulated in the G0 phase, and by using qRT-PCR we determined the levels of key cell cycle genes in asynchronous, serum-starved, and serum-resupplemented cells. The expression levels of major cell cycle and replication genes showed an inverse correlation to that of the differentially regulated miRNAs; serum deprivation resulted in down-regulation of the genes, and upon serum restimulation, the normal expression levels were restored (Fig. 1G). Immunoblotting confirmed the down-regulation of key cell cycle and replication proteins (Fig. 1H). Next, we sought to identify the putative targets of the serum starvation–induced miRNAs based on the mRNA target prediction algorithms, TargetScan, miRanda, miRWalk, and RNA22 (Fig. 1I). While deciding on the candidate miRNA to be pursued for further investigation, our decision was based on miRBase 21, which identifies “high-confidence” miRNAs based on several criteria, such as sufficient reads for each miRNA arm in the miRBase-collated deep sequencing data set, processing pattern of the mature sequences from the hairpin precursor form, and conservation of miRNA sequences across species, the methodology of which has been described in detail (23). Among the 13 validated miRNAs identified in our screen, only miR-874 was included in the high-confidence miRNA subset; therefore, we focused on this miRNA for further investigation. miR-874 is well conserved across mammals ranging from humans to rhesus monkeys to mice and has been detected in deep sequencing from multiple tissues: squamous cells, embryonic stem cells, cerebellum, heart, kidney, melanomas, etc. Another major factor in deciding on the candidate miRNA was the size; it has been reported that several miRNAs in the range of 17–19 nucleotides are possibly by-products of other non-coding RNAs like t-RNAs and sno-RNAs, originating from pseudo-precurtor miRNA genes that lack the typical pre-miRNA hairpin structure. In our study, whereas miR-874 was 22 nt long, a majority of other miRNAs (miR-3178, miR-3195, miR-3196, miR-4279, miR-4488, miR-4497, miR-4508, and miR-4516) were found to be shorter than 19 nt. Therefore, for further evaluations, we followed up with miR-874 that fulfilled the basic acceptance criteria for a high-confidence miRNA. To evaluate whether the predicted genes are targets of miR-874, we transfected double-stranded mimic of miR-874, which increased its intracellular levels (Fig. 1J). miR-874 overexpression led to a significant decrease in the transcript levels of cyclin E1, whereas the mRNA levels of the other predicted targets, Cdc25a, Mcm8, and Psp2, remained unaffected, thereby suggesting that CCNE1 is a target of miR-874 (Fig. 1K). The levels of CCNE2 did not decrease after miR-874 expression, indicating that it specifically targets CCNE1 for cell cycle regulation (Fig. 1K). Thus, our screen identified miR-874 as a serum starvation–induced miRNA.

miR-874 down-regulates cyclin E1

We have observed that the levels of miR-874 increase during serum starvation, whereas those of CCNE1 decrease, and this pattern is reversed during serum restimulation. We wanted to establish whether miR-874 was responsible for the oscillation in CCNE1 levels; therefore, we restored miR-874 levels during
miR-874 regulates CCNE1

serum restimulation and assayed the effect on CCNE1 levels. We transfected miR-874 twice in serum-starved cells followed by serum stimulation to allow them to enter the cell cycle (Fig. 2A, i). We observed that the presence of miR-874 prevented the

cyclin E1 increase during cell cycle reentry (Fig. 2A, ii and iii). However, unlike CCNE1, the increase in the levels of CCNE2 during cell cycle reentry was not affected by miR-874 overexpression (Fig. 2A, ii). We observed that although the levels of

RNA Extraction
miRNA microarray to identify differentially regulated miRNAs:
qRT-PCR to identify differentially regulated cell cycle genes
In silico miRNA target prediction & filtering
Comparative analysis of miRNA and target miRNA expression in osteosarcoma
Target validation and analysis of gain- and loss-of-miRNA function in vitro as well as in vivo
Define the role of miRNA in cancer cell survival

Serum Starved vs. Asynchronous Serum Starved

Relative Expression
Cyclin B
Cdc25a
Cyclin E1
p21
LC

Exp 2

Serum Starvation suppressed genes

Cyclin B
Cdc25a
Cyclin E1
p21
LC

Exp 1

Relative Expression
CCNE1 mRNA increase significantly in the 12-h period of serum resupplementation, the increase in protein levels was not proportionate to the increase in mRNA. We believe that although fresh mRNA is synthesized during serum restimulation, it is not completely translated in the 12-h period, as it has been previously reported that levels of many proteins, including cyclin E, are only partially restored during 12–16 h of serum restimulation (24–26). Thus, we conclude that miR-874 specifically targets CCNE1, and its down-regulation permits CCNE1 increase during cell cycle reentry.

**Inverse correlation between CCNE1 and miR-874 expression is conserved in human osteosarcoma**

Next, we wanted to test whether miR-874–mediated CCNE1 regulation is preserved in osteosarcoma, a condition where CCNE1 deregulation is well established (27). We compared the endogenous levels of miR-874 and CCNE1 in human normal osteoblastic cell line hFOB1.19 and two other human osteosarcoma cell lines that differ in their aggressiveness: U2OS (rapidly proliferating, highly invasive, and migratory) and KPD (slowly proliferating, less invasive, and migratory) (Fig. 2B, i) (28). The levels of CCNE1 mRNA and protein were significantly higher in U2OS in comparison with both KPD and hFOB1.19 (Fig. 2B, ii and iii). The expression of miR-874 in all three of the cell lines was inversely correlated to CCNE1 levels (i.e. miR-874 was considerably low in U2OS as compared with KPD and hFOB1.19) (Fig. 2B, iv). This indicates an inverse correlation between CCNE1 and miR-874 expression in human osteosarcoma. We also established that the oscillation of miR-874 and CCNE1 during cell cycle reentry and exit is conserved in osteosarcoma cell lines (Fig. 2C, i–iv).

**CCNE1 is a direct target of miR-874**

For evaluating whether CCNE1 is a direct target of miR-874, we mutated the binding site of miR-874 in CCNE1 (Fig. 3A). The entire 3′-UTR of CCNE1 carrying either the WT or mutated binding site for miR-874 was cloned into pmirGLO Dual-Luciferase vector. When the wild-type CCNE1 3′-UTR was introduced into U2OS cells along with miR-874 mimic or positive control miR-16 mimic, it exhibited a significant reduction in the luciferase activity, but the mutation in the binding site at the 3′-UTR abrogated the inhibition on luciferase activity caused by miR-874 overexpression (Fig. 3B). Thus, miR-874 inhibits CCNE1 expression through direct interaction with its 3′-UTR. We have determined the effect of miR-874 overexpression on CCNE1 levels, and next we assessed the effect of loss of function of miR-874 on CCNE1 levels. We transfected U2OS cells with mimic of miR-874 or single-stranded antisense inhibitor of miR-874, alone or in combination, and evaluated the effect on CCNE1 expression. As expected, we observed a marked decrease in CCNE1 levels as a result of miR-874 overexpression, whereas CCNE1 protein increased marginally upon miR-874 inhibition (Fig. 3, C and D). However, transfection of miR-874 inhibitor reversed the CCNE1 down-regulation caused by miR-874 mimic expression, confirming that miR-874 regulates endogenous CCNE1 levels. Thus, miR-874 represses CCNE1 by binding to a site in its 3′-UTR.

**miR-874–mediated down-regulation of CCNE1 is independent of E2F transcription factors**

It has been well established that the promoter for the CCNE1 gene harbors multiple binding sites for E2F transcription factors, and they regulate the expression of endogenous cyclin E (29, 30). Thus, it is possible that the decrease of CCNE1 levels after miR-874 overexpression is due to the down-regulation of E2F transcription factors. We evaluated the levels of E2F1, E2F2, and E2F3 after miR-874 overexpression in asynchronously growing U2OS cells. We observed that although the CCNE1 mRNA levels were down-regulated, the levels of E2F transcription factors did not alter significantly (Fig. 4A). We also evaluated the levels of E2F1, E2F2, and E2F3 during serum starvation, and as expected, the levels of all three of the E2F transcription factors were down-regulated in serum-deficient cells (Fig. 4B). In addition, miR-874 overexpression during serum resupplementation did not significantly alter the endogenous levels of E2F1, E2F2, and E2F3 mRNA (Fig. 4B). There

---

**Figure 1. A screen to identify differentially regulated miRNAs during serum starvation.** A, schematic outline of the strategy employed for identification of differentially regulated miRNAs upon serum starvation and serum resupplementation. B, HCT116 cells were serum-starved for 48 h, followed by resupplementation with fetal bovine serum for 12 h. Flow cytometry of propidium iodide–stained DNA demonstrates that serum starvation for 48 h led to an increase in the G0/G1 population, indicating cell cycle exit, whereas serum stimulation for 12 h resulted in cell cycle reentry, seen as a clear accumulation in the G2/M phases. C, immunoblot shows induction of CDK inhibitors p21 and p27 after serum withdrawal and down-regulation of these marker proteins after serum stimulation. D, heat map representation of expression of most significantly differentially regulated miRNAs in asynchronous, serum-starved, and serum-resupplemented HCT116. Each column corresponds to an individual sample, whereas each row represents an individual miRNA. Relative expression is represented as a colorgram (green, high expression; red, low expression). E, SAM plots of two comparison pairs: serum-starved versus asynchronous and serum-resupplemented versus serum-starved. The two parallel dashed lines represent the cut-off threshold specified by the false discovery rate, thus displaying the total number of up-regulated (red dots) and down-regulated (green dots) miRNAs for each plot. The SAM plot identified 48 up-regulated and three down-regulated miRNAs during serum starvation and three up-regulated and 44 down-regulated miRNAs during serum resupplementation. F, the relative expression of 51 top differentially regulated miRNAs in asynchronous, serum-starved, and serum-resupplemented HCT116 was confirmed using a quantitative RT-PCR assay, where the expression of miRNAs was normalized with an endogenous control, RNU48. G, the mRNA expressions of selected cell cycle and DNA replication genes were individually determined using qRT-PCR in asynchronous, serum-starved, and serum-resupplemented HCT116 cells, where the expression of genes was normalized with an endogenous control, β-c globulin. H, immunoblot of the same samples as described in C shows differential expression of some of the activator genes involved in the cell cycle pathway. I, computational integration of differentially regulated miRNA–mRNA pairs using four miRNA target prediction algorithms: miRanda, miRTWalk, RNA22, and TargetScan. Putative targets of each miRNA are represented by different color codes as indicated based on the number of algorithms predicting a binding site. J, HCT116 cells transfected on 3 consecutive days with miR-874 mimic or negative control mimic (NC) were harvested 24 h after the last transfection, and the -fold change in miRNA expression (×1000) is represented. K, effect of miR-874 overexpression on the mRNA levels of putative targets by qRT-PCR. The -fold change with respect to negative mimic transfected control is shown. Data are represented as the mean of two biological replicates ± S.D. (error bars).
miR-874 regulates CCNE1

was a 30% reduction in the levels of E2F3 after miR-874 mimic transfection; however, because the expression of CCNE1 is known to be controlled by all three transcription factors, it is unlikely to result in major CCNE1 down-regulation. Thus, the decrease in CCNE1 levels caused by miR-874 is not due to down-regulation of E2F transcription factors. Transcription factor–binding site prediction algorithms identify 16 putative E2F consensus–binding sites in the 10-kb region surrounding
miR-874 regulates CCNE1

A

miR-874 binding site

CCNE1 coding region

CCNE1 Mut-3'UTR

miR-874 binding site

CCNE1 3’UTR

5’ .. GUGGAUGGCAUCAAACAGGGCAA..3’

3’ .. AGCCAGGGAGCCCGGUCCCGUC..5’

miR-874 binding site

3’ .. AGCCAGGGAGCCCGGUCCCGUC..5’

5’ .. GUGGAUGGCAUCAAACAGGGCAA..3’

miR-874 binding site

CCNE1 Mut-3’UTR

5’ .. GUGGAUGGCAUCAAACAGGGCAA..3’

3’ .. AGCCAGGGAGCCCGGUCCCGUC..5’

miR-874 binding site

5’ .. GUGGAUGGCAUCAAACAGGGCAA..3’

3’ .. AGCCAGGGAGCCCGGUCCCGUC..5’

Figure 3. miR-874 down-regulates CCNE1 by targeting its 3’-UTR. A, pictorial representation of the binding of miR-874 to its target site in wild-type and mutant CCNE1 3’-UTR. B, CCNE1 3’-UTR possessing the intact or mutant (Mut) binding site for miR-874 cloned in pmirGLO Dual-Luciferase vector was cotransfected with control mimic, miR-874 mimic, or miR-16 mimic, as indicated, and the relative luciferase activity was determined. C and D, U2OS cells were transfected on 3 consecutive days with negative control mimic, miR-874 mimic, or miR-874 inhibitor, as indicated, and cyclin E1 transcript and protein levels were analyzed. Ectopic miR-874 mimic expression resulted in decreased CCNE1 mRNA levels, whereas the cotransfection of miR-874 inhibitor prevented this suppression of CCNE1. LC, loading control, a nonspecific band that displays equal protein load in different lanes. The numbers indicate levels of cyclin E1 relative to negative control mimic–transfected cells. Data are represented as the mean ± S.D. (error bars) of two biological replicates.

Figure 2. miR-874 and CCNE1 expression is inversely correlated in osteosarcoma. A, U2OS cells were serum-starved for 48 h, during which time the cells were transfected twice with miR-874 mimic or negative control mimic. After 48 h, cells were replenished with FBS and further grown for 12 h, following which cells were harvested. i, miR-874 mimic prevented miR-874 down-regulation during serum resupplementation. ii and iii, CCNE1 mRNA as well as protein levels were down-regulated after serum deprivation, whereas the levels were restored upon serum resupplementation in negative mimic–transfected sample. However, the natural restoration of CCNE1 levels upon serum resupplementation was completely abolished in miR-874–overexpressing cells. However, unlike CCNE1, the increase in the levels of CCNE2 during cell cycle reentry was not affected by miR-874 overexpression. LC, loading control, a nonspecific band that displays equal protein load in different lanes. The numbers indicate levels of cyclin E1 relative to asynchronously growing cells. B (i), proliferation rate of U2OS and KPD cell lines as measured by MTT assay over a period of 96 h. B (ii), relative expression of CCNE1 mRNA in U2OS (aggressive osteosarcoma), KPD (non-aggressive osteosarcoma), and control hFOB1.19 cell line (fetal osteoblast). CCNE1 transcript levels were higher in U2OS than KPD and hFOB1.19. B (iii), immunoblot showing significantly higher cyclin E1 protein level in U2OS cells. B (iv), the expression levels of mature miR-874 were significantly lower in U2OS compared with KPD and hFOB1.19, as evaluated by qRT-PCR. C, U2OS cells were serum-starved for 48 h, followed by resupplementation with FBS for 12 h. i, flow cytometry of propidium iodide–stained DNA from U2OS cells demonstrates an increase in the G0/G1 population upon serum starvation, whereas serum resupplementation for 12 h resulted in cell cycle reentry seen as a clear accumulation in the S phase. ii, qRT-PCR demonstrates that miR-874 expression is induced upon serum starvation, which is decreased after serum resupplementation. iii, CCNE1 mRNA levels are down-regulated after serum starvation and up-regulated upon serum stimulation. iv, immunoblot shows that cyclin E1 protein level is down-regulated after serum starvation and up-regulated upon serum stimulation. Data are represented as the mean ± S.D. (error bars) of two biological replicates.

J. Biol. Chem. (2017) 292(52) 21264–21281 21269
miR-874 regulates CCNE1

Figure 4. miR-874–mediated down-regulation of CCNE1 is independent of E2F transcription factors. A, U2OS cells were transfected on 3 consecutive days with negative control mimic or miR-874 mimic followed by evaluation of CCNE1, E2F1, E2F2, and E2F3 transcripts. Ectopic miR-874 mimic expression resulted in decreased CCNE1 mRNA levels, but the levels of E2F1, E2F2, and E2F3 mRNA were not suppressed, demonstrating that miR-874 down-regulation of CCNE1 is independent of E2F transcription factors. The numbers indicate levels of individual transcripts relative to negative control mimic–transfected cells. B, asynchronously growing U2OS cells were serum-starved for 48 h, followed by evaluation of E2F1, E2F2, and E2F3 transcripts. As reported previously, E2F transcription factors were down-regulated after serum starvation in comparison with asynchronously growing cells (compare asynchronous to serum-starved). During serum starvation, the cells were transfected twice with miR-874 mimic or negative control mimic, replenished with FBS, and further grown for 12 h, following which cells were harvested. The levels of E2F1, E2F2, and E2F3 mRNA after miR-874 mimic transfection have been expressed relative to negative control mimic–transfected serum-resupplemented cells. E2F1, E2F2, and E2F3 mRNA levels were not significantly different in negative control mimic– and miR-874 mimic–transfected serum-resupplemented cells. C (i and ii), E2F transcription factors do not regulate miR-874 at the transcriptional level. U2OS cells were transfected on 3 consecutive days with control GL2, E2F1, or E2F2 siRNA as indicated, followed by the evaluation of E2F1, E2F2, and pri-miR-874 transcripts. The levels of E2F1 or E2F2 transcripts have been expressed relative to control GL2-transfected cells. D, depletion of long non-coding RNA XLOC_008466 derepresses miR-874, leading to cyclin E1 inhibition. The putative sites of miR-874 binding to XLOC_008466 and CCNE1 mRNA are shown in the top panel. U2OS cells were transfected on 3 consecutive days with control GL2 or XLOC_008466 siRNA, and the levels of XLOC_008466, miR-874, and cyclin E1 transcript were analyzed. The y axis is discontinuous from 2 to 7 to accommodate all data points. Data are represented as the mean ± S.D. (error bars) of two biological replicates.

the genomic loci from where miR-874 is expressed (chromosome 5q31.2). We wanted to assay whether E2F transcription factors regulate the expression of primary transcript of miR-874. Therefore, we evaluated the levels of primary miRNA (pri-miR-874) upon E2F1 or E2F2 depletion by RT-PCR with primers that amplify the 201-nt region around the mature miR-874. We observed that E2F1 or E2F2 siRNA effectively reduced the levels of targeted gene, but the levels of pri-miR-874 were not significantly reduced (Fig. 4C, i and ii). The results indicate that although miR-874 harbors several E2F consensus–binding sites, its expression may not be regulated in an E2F-dependent manner.
miR-874 regulates CCNE1

XLOC_008466–miR-874–CCNE1 ceRNA network exists in osteosarcomas

It has been reported that IncRNA XLOC_008466 functions as a competing endogenous RNA binding to miR-874. We wanted to assay whether the XLOC_008466–miR-874 network affects CCNE1 expression in osteosarcomas (Fig. 4D). We depleted the endogenous levels of XLOC_008466 by transfecting a siRNA duplex that targets its 3’-end. Transfection of siRNA targeting XLOC_008466 decreased its endogenous levels to about half that of control cells (Fig. 4D). The decrease in XLOC_008466 levels resulted in a significant increase in miR-874 levels, establishing that XLOC_008466 functions as a natural sponge for miR-874. Concomitantly, up-regulation of miR-874 due to XLOC_008466 inhibition resulted in CCNE1 down-regulation. Thus, the XLOC_008466–miR-874–CCNE1 network exists in osteosarcoma, and it is likely that a balance of these antagonistic factors influences the progression of osteosarcoma.

miR-874 overexpression impedes S phase progression

Having identified CCNE1 as a direct target of miR-874, we next investigated the effect of miR-874 on cell cycle progression, particularly on S phase progression. U2OS cells transfected on 3 consecutive days with miR-874 mimic were either grown asynchronously or synchronized at the G1/S boundary by hydroxyurea treatment for 20 h and were subsequently released into S phase by further growing the cells in drug-free medium for 3 h. Cells at the three indicated time points were subjected to a short BrdU pulse of 20 min and were subsequently harvested for BrdU immunofluorescence. We observed that the BrdU incorporation in asynchronously growing cells transfected with miR-874 was reduced to 23% as compared with 49% in control cells (Fig. 5A, i and iii). When BrdU incorporation was measured distinctly upon S phase enrichment (HLI + 3 h), we observed that in comparison with 61% control cells that were BrdU-positive, only 15% of the miR-874–transfected cells incorporated BrdU (Fig. 5A, ii and iii). Thus, miR-874 expression impedes S phase progression.

Loss of miR-874–mediated control of cyclin E1 is a general feature of osteosarcomas

Next, we tested the miR-874–CCNE1 regulation in HOS and MG-63 osteosarcoma cell lines. MG-63 is an osteosarcoma cell line that displays a high proliferation rate and clonogenic ability under anchorage-independent conditions and shows >90% tumor formation in in vivo tumorigencity assays (28, 31, 32). HOS is a highly tumorigenic osteosarcoma cell line that displays high invasion and migration potential as well as high proliferation and clonogenic ability (28, 33). Considered as a highly aggressive cancer cell line, HOS is utilized as a control for assaying tumorigenic properties. First, we tested whether HOS and MG-63 display an inverse pattern of CCNE1 and miR-874 expression in comparison with human normal osteoblastic cell line hFOB1.19. We noted that the mRNA levels of CCNE1 were significantly higher in HOS and MG-63 in comparison with hFOB1.19 (Fig. 5B). On the other hand, the levels of miR-874 were considerably low in HOS and MG-63 as compared with hFOB1.19 and, thus, inversely correlated to CCNE1 levels. We observed that miR-874 overexpression led to a significant decrease in the transcript levels of cyclin E1 in HOS and MG-63 cells (Fig. 5C). Thus, the high levels of CCNE1 observed in aggressive osteosarcomas were reversed by the restoration of miR-874. These results establish that the loss of miR-874–mediated regulation of cyclin E1 is a characteristic feature of osteosarcomas. Moreover, ectopic expression of miR-874 in both HOS and MG-63 led to a marked decrease in the viable cell count (Fig. 5D, i and ii). Our results demonstrate that the restoration of miR-874 control of CCNE1 leads to suppression of osteosarcoma cell proliferation. Thus, by utilizing multiple osteosarcoma cell lines, we demonstrate that miR-874 is down-regulated in osteosarcomas, which results in elevated CCNE1 levels, and miR-874 restoration reverses high CCNE1 expression and the high rate of proliferation of aggressive osteosarcoma.

miR-874 inhibits tumorigenic phenotypes in osteosarcomas

To evaluate the effect of miR-874 on in vitro cell survival, we transfected miR-874 mimic, followed by γ-irradiation and colony count determination at 11 days. miR-874 restoration negatively affected the clonogenic cell survival, with at least 50% inhibition in the colony formation capacity in non-irradiated as well as γ-irradiated samples (Fig. 6A, i and ii). To assess the role of miR-874 in wound healing, a key determinant of malignancy, we performed a wound-healing assay in U2OS cells after transfection with miR-874 mimic. Confluent monolayers were scratched, followed by visualization of migrating cells into the wounded area. We observed that ectopic expression of miR-874 dramatically reduced the wound healing capacity of U2OS cells (Fig. 6B, i and ii). Collectively, our results indicate that miR-874 overexpression substantially inhibits cell movement and cell survival in osteosarcoma in vitro. We utilized in vitro transwell migration and invasion assays to investigate the effects of miR-874 on cell migration and invasion ability. We observed that the cell migration ability was suppressed by miR-874 overexpression in U2OS cells (Fig. 6C, i and ii). In addition, ectopic expression of miR-874 inhibited the invasion of U2OS cells through Matrigel (Fig. 6D, i and ii). Thus, by utilizing independent assays, we demonstrate that miR-874 overexpression inhibits tumorigenicity of osteosarcomas.

Restoration of CCNE1 suppresses the miR-874–mediated inhibition of S phase progression

Our data demonstrate that miR-874 inhibits cell proliferation and impedes S phase progression by targeting CCNE1. To further illustrate that the inhibitory effect of miR-874 on cell proliferation was primarily due to down-regulation of CCNE1, we utilized a retroviral vector to express the coding region of CCNE1, which lacks the site for miR-874 binding. U2OS cells stably expressing CCNE1 were transfected on 3 consecutive days with miR-874, followed by incubation with nucleotide analog, BrdU, to evaluate the rate of DNA synthesis. Endogenous CCNE1 levels were decreased in all samples transfected with miR-874 mimic, whereas the exogenously expressed CCNE1 expression remained unaffected by miR-874 overexpression (Fig. 7A). As shown previously, transfection of miR-
miR-874 regulates CCNE1

874 mimic reduced the cell viability to almost half that of control cells, but co-expression of miR-874–resistant CCNE1 partially reverses the suppression of loss of cell viability, demonstrating that the effect of miR-874 on cell viability was primarily due to down-regulation of CCNE1 (Fig. 7B). Similarly, the BrdU-positive population that was significantly decreased after miR-874 overexpression was restored after co-expression of miR-874–resistant CCNE1 (Fig. 7C). Collectively, these results prove that the anti-proliferative activity of miR-874 is primarily due to down-regulation of CCNE1.
miR-874 suppresses tumor formation and progression in nude mice

To explore the anti-tumorigenic activity of miR-874 in vivo, nude mice were injected with U2OS cells stably expressing miR-874. However, tumor formation was not seen in nude mice up to 45 days, possibly due to poor tumorigenicity of the cell line; thus, we carried out the in vivo functional study using HCT116-derived tumors in nude mice (28). We constructed a recombinant lentiviral vector stably expressing miR-874 (pLKO.1 miR-874) in HCT116. qRT-PCR confirmed a decrease in the expression level of CCNE1 in pLKO.1 miR-874 as compared with pLKO.1 control (Fig. 8A). HCT116 cells stably expressing either pLKO.1 control vector or pLKO.1 miR-874 were injected subcutaneously into the right flank of nude mice between 4 and 6 weeks of age. Tumor volume was measured every second day, and mice were sacrificed after 17 days, followed by excision of tumors. Notably, miR-874–treated mice showed significant inhibition of tumor growth as compared with the control group (Fig. 8, B–E). The levels of cyclin E1 were significantly reduced in tumors excised from miR-874–treated mice, indicating that the suppression of tumor induction and progression in vivo by miR-874 is primarily due to down-regulation of CCNE1 (Fig. 8F).

Discussion

Alteration in the expression levels of miRNAs and potential target genes are well characterized for several human cancers, but the regulatory circuits cannot be simply established, as multiple miRNAs could possibly target a gene and multiple genes could be potentially targeted by a miRNA. By analyzing the expression of miRNA and potential target mRNAs in contrasting physiological states, as we have done during cell cycle exit and cell cycle reentry, an interrelationship between them could be established. We investigated the miRNA–mRNA regulatory networks functional during serum starvation, which has been used to mimic growth factor deficiency in the tumor microenvironment, and tested them in osteosarcoma oncogenesis (4, 34). miR-874 has been reported to be down-regulated in multiple cancers (breast cancer, gastric cancer, and head and neck squamous cell carcinoma), with its targets including CDK9, STAT3, and HDAC1 (34–37). On the other hand, cyclin E has been known to be overexpressed in multiple malignancies, including carcinomas, lymphomas, leukemias, sarcomas, and adrenocortical tumors (38–41). Although the dysregulation of miR-874 and cyclin E expression during oncogenesis was individually reported before our study, their relation was not established. We discovered the miR-874–cyclin E1 regulation during growth factor deprivation and assayed it in human osteosarcoma cell lines that differ in their aggressiveness: KPD (slowly proliferating and less invasive cell line) and U2OS (rapidly proliferating and highly invasive cell line) (28). Comparative analysis of osteosarcoma cell lines with contrasting cancer-related phenotypes offers an alternative to specifically address the molecular changes during progression of oncogenesis. Although the osteosarcoma cell lines of varying aggressiveness are not isogenically matched, nonetheless, we have attempted to correlate miRNA expression with aggressive growth phenotypes. Comparisons were also made with a primary osteoblast cell line, but because the osteoblasts have progressed into a specific differentiation lineage, the differences in gene expression would also be likely to result from its differentiation commitment (42). Our work clearly indicates a strong relationship of CCNE1 and miR-874 expression with the aggressiveness of the cancer. Overexpression of miR-874 led to a decrease in the transcript levels of cyclin E1 in U2OS, HOS, and MG-63 cells, establishing that the loss of miR-874–mediated control of cyclin E1 is a general feature of osteosarcomas. The effect of CCNE1 repression due to miR-874 overexpression was apparent on the S phase progression, whereas co-expression of miR-874–resistant CCNE1 largely reversed the effect. Unlike the miR-16 family, which targets multiple cell cycle genes, miR-874 specifically targets cyclin E1, exemplifying the diverse nature of cell cycle control and its subversion during oncogenesis (43).

It has been reported that expression of miR-874 was negatively correlated with TNM (tumor, node, and metastasis) stage in cancer patients (44). Significantly, patients with high levels of miR-874 demonstrate better survival; data analysis from the Cancer Genome Atlas reveals that bladder urothelial carcinoma patients with high levels of miR-874 displayed an overall 50% survival rate over a period of 3000 days in comparison with a 25% survival rate of patients exhibiting low miR-874 expression (45). Although the survival data are not available for osteosarcomas, for other cancers, such as hepatocellular carcinoma and pancreas adenocarcinoma, patients with high levels of miR-874 have a significantly better survival rate in comparison with patients with low levels of miR-874. The accumulating information on miR-874 (poor expression in cancer tissues, inverse correlation with cancer metastasis, and, most importantly, the reversal of aggressive cancer phenotypes upon its restoration) clearly establishes that miR-874 is an important molecule during tumorigenesis. The significance of miR-874 in human cancers is also apparent from the multiple levels of regulation that is subjected to during carcinogenesis. XLOC_008466, a long non-coding RNA, functions as a competing endog...
miR-874 regulates CCNE1

A (i)

B (i)

C (i)

D (i)

U2OS migration

U2OS invasion

miR-874 mimic

Negative Control mimic

Cell invasive counts/field

Cell migration counts/field

Negative Control mimic

miR-874 mimic

Survival (%)
miR-874 regulates CCNE1

Experimental procedures

Cell culture, irradiation, cell synchronization, and cloning

U2OS, HOS, MG-63, and KPD (human osteosarcoma cell lines); HEK293T (human embryonic kidney cells with SV40 large T antigen cell line); and HCT116 (human colorectal cell line) were maintained in DMEM supplemented with 10% FBS along with 1% of 100 units/ml antibiotic and antimitotic solution at 37 °C in a humidified atmosphere containing 5% CO2. For ionizing radiation treatment, cells were exposed to either 2 or 4 gray of γ-radiation and were harvested 11 days post-treatment for a clonogenic survival assay. For serum starvation, cells were seeded at ~50% confluence in complete medium, and once cells adhered and attained morphology, the medium was removed, followed by two 1× PBS washes, and the cells were cultured in medium completely devoid of FBS for 48 h. For arresting the cells at the G1/S transition, cells were grown in DMEM supplemented with 2 mM hydroxyurea for 20 h, after which the cells were harvested for immunofluorescence. For cloning of human CCNE1 coding sequence, CCNE1 cDNA was amplified by PCR and cloned into murine leukemia virus long terminal repeat-driven plasmid, pMX-puro-3NLS-GST-HA. HEK293T cells were transfected with pMX-puro-CCNE1 along with helper plasmids expressing the viral VSV-G envelope protein as well as the Gag and Pol proteins to generate viral particles. To obtain a stable U2OS cell line expressing CCNE1, U2OS cells were infected with the viral particles and selected with 1 μg/ml puromycin after 48 h.

Transfection

For RNAi-mediated gene silencing, siRNAs against GL2, CCNE1, E2F1, E2F2, and XLOC_008466 were custom-synthesized by Dharmacon. For gain- and loss-of-function studies of miRNAs, miRIDIAN miRNA mimics and inhibitors were used. Cells were transfected with either 100 nm miRNA inhibitor or 50 nm siRNA or miRNA mimic using Lipofectamine 2000 reagent (Invitrogen) for 3 consecutive days. The cells were harvested 24 h after the last transfection for immunoblotting, flow-cytometric analysis, or RT-PCR. The siRNA sequences used were as follows: GL2, CGUACCGGGAAUACUCCGA; CCNE1, CCUCCCAA-AGUUGCACCAGU; E2F1, UAUCUGUACUCCGACGCU; E2F2, GACUGCGUAUGACACUUG; XLOC_008466, GUG-AGUUGCUUCAGGCCAG.

Figure 6. miR-874 overexpression inhibits tumorigenic phenotypes in osteosarcomas. A (i and ii), clonogenic cell survival assay to evaluate the effect of miR-874 expression. U2OS cells were transfected on 3 consecutive days with either negative control mimic or miR-874 mimic, after which they were γ-irradiated, allowed to grow for 11 days, and stained with crystal violet, after which the colonies were counted. i, quantification of i, which demonstrates that the colony-forming ability is significantly decreased upon miR-874 overexpression. B (i and ii), wound-healing assay to evaluate the effect of miR-874 expression on cell migration. U2OS cells were transfected on 2 consecutive days with either negative control mimic or miR-874 mimic and grown to confluence, after which a wound was created using a micropipette tip. The extent of wound healing was monitored every 24 h. ii, quantification of i, which demonstrates that the wound-healing capability is profoundly reduced upon miR-874 overexpression. Data are represented as the mean ± S.D. (error bars) of two biological replicates. C (i and ii), U2OS cells were transfected on 3 consecutive days with either negative control mimic or miR-874 mimic, and the numbers of cells migrating through a microporous membrane were counted at 24 h after third transfection. i, a representative field showing the DAPI-stained nucleus of migrated cells. ii, quantification of the number of migratory cells observed in i. Each point refers to number of cells in an individual field, whereas long and short horizontal bars represent the mean and S.D., respectively. D (i and ii), U2OS cells were transfected on 3 consecutive days with either negative control mimic or miR-874 mimic, and the numbers of cells invading through a Matrigel-coated membrane were counted at 24 h after the third transfection. i and ii, a representative field showing the DAPI-stained nucleus of invading cells and quantification as described above. Data are represented as the mean ± S.D. of two independent experiments with more than five fields analyzed for each sample. **, p < 0.001. Scale bar, 50 μm.

J. Biol. Chem. (2017) 292(52) 21264–21281 21275
miR-874 regulates CCNE1

**Figure 7. Restoration of CCNE1 partially rescues the miR-874–mediated inhibition of cell proliferation.** A, U2OS cells stably expressing HA-tagged coding sequence of CCNE1 or control protein, SLD5, were transfected on 3 consecutive days with either miR-874 mimic or negative control mimic. Immunoblotting with α-HA antibody (top) confirmed the expression of exogenous CCNE1 and SLD5. In addition, immunoblotting with α-CCNE1 (middle) demonstrates a decrease in the endogenous CCNE1 levels by miR-874 overexpression, whereas the exogenously expressed CCNE1 expression was not affected. B, trypan blue viability assay showing the number of viable cells in each sample described in A. Data are represented as the mean ± S.D. (error bars) of two biological replicates. *p values calculated using two-tailed t test show that the cell viability in miR-874–transfected samples expressing HA-tagged CCNE1 is significantly different from samples that do not express HA-tagged CCNE1 samples (*, p < 0.05). C, flow cytometry of BrdU-labeled transfected cells, as described in A. Cells were pulsed with BrdU, followed by staining with anti-BrdU antibody conjugated to FITC along with propidium iodide (PI). The dot plot displays BrdU incorporation (y axis) and DNA content (x axis), and the inset shows the cells incorporating BrdU. The data demonstrate that the effect of miR-874 on S phase progression was primarily due to inhibition of CCNE1.
miR-874 regulates CCNE1

A. Relative Expression

B. Tumor Volume

C. Tumor Weight

D. pLKO.1 control

E. pLKO.1 miR874

F. pLKO.1 control

G. Serum Starved: Cell Cycle exit

H. Serum re-supplemented: Cell Cycle re-entry

I. Primary Osteoblasts

J. Aggressive Osteosarcomas

K. Low miR-874

L. High CCNE1

M. S-phase delay

N. Poor Tumorigenicity

O. High Migration, invasion, proliferation

P. High Clonogenic Survival

J. Biol. Chem. (2017) 292(52) 21264–21281 21277
miR-874 regulates CCNE1

cells were treated with 2 N HCl for 15–20 min to denature the DNA, followed by a neutralization step of 5 min at room temperature with 0.1 M sodium tetraborate (pH 8.5). Cells were then washed with a blocking solution comprising 3% BSA in PBS containing 0.1% Triton X-100 followed by incubation with mouse anti-BrdU antibody (dilution 1:10 in blocking solution) conjugated to FITC for 1 h. After antibody staining, cells were washed with 1× PBS, and DNA was stained with propidium iodide and run on a FACS machine as described above.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). For miRNA analysis, total RNA was poly(A)-tailed using Escherichia coli poly(A) polymerase (New England Biolabs) before reverse transcription as described previously (48). The qRT-PCR were carried out in duplicates in a 10-μl volume for the expression analysis. The reaction mixture contained SYBR Select master mix (1X; Applied Biosystems), cDNA template, and forward and reverse gene- or miRNA-specific primers (0.1 μM each). The target sequence amplification temperature profile followed as was follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and amplification for 30 s at annealing temperature of 60 °C. Finally, a melt curve analysis was carried out at a temperature range of 60–95 °C for 20 min. The small nucleolar RNA, RNU48, and BMG (β-microglobulin) were used as internal controls for miRNA and mRNA quantification, respectively. Results were calculated using the ΔΔCt method to determine the -fold change in expression between the experimental and control groups.

Indirect immunofluorescence and microscopy

To study the BrdU incorporation, U2OS cells grown on coverslips were cultured in medium containing 100 μM BrdU for 20 min before harvesting. Cells were fixed with 4% formaldehyde for 10 min, followed by denaturation with 2 N HCl for 15–20 min and neutralization with 0.1 M sodium tetraborate (pH 8.5) for 2 min. Cells were then blocked with 3% BSA for 30 min and stained with mouse anti-BrdU antibody (BD Biosciences) for 1 h, followed by incubation with secondary antibody for 1 h. Finally, the cells were visualized under the microscope after mounting with Vectashield mounting reagent containing DAPI, which stains the nucleus. The secondary antibody was conjugated to Alexa Fluor 488 and purchased from Invitrogen.

Immunoblotting and antibodies

For Western blotting, the whole-cell lysates from cells of almost equal confluence were prepared in a proportionate volume of Laemml buffer and denatured at 95 °C, followed by SDS-PAGE. The gel was transferred onto a nitrocellulose membrane, blocked with 3% BSA prepared in 1× TBST. The membrane was then incubated with the appropriate antibody, washed, and probed with horseradish peroxidase–conjugated secondary antibody. Enhanced chemiluminescence was used to visualize the protein bands. Quantity One software was utilized to evaluate the levels of specific proteins, which were expressed after normalization with the protein-loading control. The following antibodies were used for Western blotting. Antibodies against CCNE1, CDK2, β-actin, CCNB1, and CDC25A were obtained from Santa Cruz Biotechnology, Inc. Antibodies against p21 and p27 were procured from Cell Signaling Technology. Antibody used to detect the HA epitope was purchased from Sigma.

miRNA microarray

Total RNA was isolated by the TRIzol method, and samples were outsourced to Exiqon for miRNA microarray analysis. Microarray analysis was conducted as dual-channel Hy3/Hy5 experiments on Exiqon’s miRCURY LNA microRNA Array 7th generation, comprising 3100 capture probes, complementary to human, mouse, and rat, and their related viral sequences from the version 19.0 release of miRBase. A 500-ng RNA sample was labeled with an Hy3 fluorophore along with Hy5-labeled reference RNA sample (Exiqon, Vedbaek, Denmark) using Exiqon’s miRCURY LNA microRNA HiPower labeling kit with the use of synthetic spike controls, Spike-in microRNA Kit version 2 (Exiqon), according to the manufacturer’s protocol. Hybridization of labeled RNA to the array was performed in SureHyb chambers (Agilent Technologies) for 16 h at 56 °C. Slides were washed according to the manufacturer’s instructions and scanned at 10 μm resolution using an Agilent G2505C DNA microarray scanner. Raw data were generated using Imagene version 9.0 software (BioDiscovery, Inc.), using an feature extraction protocol available on demand from Exiqon. These raw data have been submitted to the GEO database under accession number GSE103436.

miRNA target prediction

The putative miRNA targets were predicted using the following algorithms: TargetScan, miRanda, RNA22, and miRWalk.

Figure 8. miR-874 inhibits tumor growth in a mouse xenograft model. A. CCNE1 mRNA levels in HCT116 cells stably expressing miR-874 from lentiviral vector pLKO.1. B, tumor volume in nude mice subcutaneously injected with HCT116 cells expressing miR-874. Tumor volume was measured on alternate days, and on the 17th day, mice were sacrificed, tumors were excised, and the tumor weight was determined as shown in C, D, representative photographs of nude mice injected with HCT116 cells expressing pLKO.1 control or pLKO.1 miR-874. Arrowheads point to the tumor. E, macroscopic images of the tumors on the 17th day revealed that the tumors formed in miR-874–treated mice were significantly smaller than control mice. T1–T6 and T7–T12, tumors excised from control and miR-874–expressing mice, respectively. F, immunoblot of the excised tumors revealed that cyclin E1 protein levels were low in the miR-874–treated group as compared with the control group. The numbers indicate levels of cyclin E1 relative to control tumor T1. Data are represented as the mean ± S.D. (error bars) of two biological replicates. G, model depicting miR-874–mediated regulation of CCNE1 during cell cycle phases and osteosarcomas. During growth factor deprivation, miR-874 levels are induced, resulting in down-regulation of CCNE1 and cell cycle exit. However, during serum restimulation, miR-874 expression is decreased, leading to derepression of CCNE1 levels and resulting cell cycle reentry. An analogous miR-874–CCNE1 regulation exists in osteosarcomas; primary osteoblasts or less aggressive osteosarcomas display a regulation that is similar to serum-starved cells, where high levels of miR-874 inhibit the expression of CCNE1, resulting in poor tumorigenicity. Aggressive osteosarcomas exhibit a miR-874–CCNE1 regulation observed during cell cycle reentry (i.e. miR-874 is down-regulated, resulting in high CCNE1 levels and development of cancer-related phenotypes, such as increased migration and invasion).
Luciferase reporter assay

To construct a luciferase reporter plasmid, 3’-UTR full-length fragment of CCNE1 that hosts the predicted miR-874–binding site or mutated site was cloned downstream to the firefly luciferase into pmirGLO Dual-Luciferase miRNA target expression vector (Promega). For the luciferase reporter assay, U2OS cells were co-transfected with 750 ng of luciferase reporter plasmid harboring the 3’-UTR of the gene along with 50 nM miRNA mimic using Lipofectamine 2000 reagent. After 24 h of transfection, cells were harvested, and luciferase activity was measured in a Lumicount Luminometer (Packard) using the Dual-Luciferase reporter assay kit (Promega) according to the manufacturer’s instructions. Firefly luciferase activity was normalized to Renilla luciferase activity.

Cell proliferation assays

For MTT cell proliferation assay, 30,000 cells in 500 μl of medium/well were seeded in triplicates in 24-well cell culture dishes. The MTT substrate, thiazolyl blue tetrazolium bromide, was added to cells in culture at a final concentration of 0.5 mg/ml and incubated at 37 °C. After 3–4 h, the cells were resuspended in 500 μl of DMSO and shaken for 15 min. The quantity of formazan was measured by recording changes in absorbance at 570 and 630 nm (reference wavelength) using a microplate reader (BioTek PowerWave XS). For cell viability count, the trypan blue exclusion method was utilized where U2OS cells were transfected with miRNA mimic for 3 consecutive days, and 24 h after the last transfection, cells were collected and dissolved in 1 ml of 1× PBS, and 20 μl of cell suspension was stained with an equal volume of 0.4% trypan blue. Viable cells, which excluded trypan blue dye, were counted in quadruplicate using a glass hemocytometer.

Wound-healing assay

U2OS cells seeded in 6-well plates with an approximate confluence of 30–40% were transfected with 50 nM miRNA mimic using Lipofectamine 2000 reagent for 2 consecutive days and were cultured until confluence. A wound was then created by manually scraping the cell monolayer with a 200-μl pipette tip. The cultures were washed twice with 1× PBS and supplemented with fresh medium. Cell movement into the wound was observed at four preselected time points (0, 24, 36, and 48 h) in eight randomly selected microscopic fields for each condition and time point. Images were acquired with a Nikon TE2000-S inverted fluorescence microscope. The distance traveled by the cells was determined by measuring the wound width at different time points, and the experiment was performed in triplicates.

Clonogenic assay

U2OS cells transfected with miRNA mimic were counted, and 1000 cells were seeded in a 6-well culture dish in triplicates. Following 2 h of seeding, cells were irradiated with 0, 2, or 4 grays of γ-radiation. After 11–12 days of incubation, plates were gently washed with 1× PBS and stained with 0.1% of crystal violet. Colonies with over 50 cells were manually counted.

Invasion and migration assays

U2OS cells (3 × 10⁴) resuspended in 250 μl of serum-free medium were seeded into the upper well of the Matrigel-coated membrane of a transwell chamber (8-μm pore size, Corning), for assaying cell invasion. For migration assays, the cells were seeded into the upper well of a micillicell hanging cell culture insert of a transwell chamber (8-μm pore size; Millipore). Serum-containing medium was added to the lower chamber, and cells were incubated at 37 °C for 24 h. Subsequently, cells in the upper chamber were removed, and the cells migrating to or invading the bottom of the membrane were fixed with cold methanol and visualized under the microscope after mounting with Vectashield mounting reagent containing DAPI that stains the nucleus. Five random fields of each membrane were photographed and counted for statistical analysis.

Generation of lentivirus and infection into mammalian cell lines

For lentivirus preparation, lentiviral vector pLKO.1 containing mature miRNA sequences was co-transfected with packaging vector pMD2.G and envelope vector pSAX2 at a 4:3:1 ratio using Lipofectamine 2000 in HEK293T cells. Supernatant was collected after 48 h of transfection, pooled, and filtered using a 0.45-μm filter. The filtered supernatant was used along with 1 μg/ml Polybrene for infecting HCT116 cells. 24 h postinfection, cells were grown in medium containing 1 μg/ml puromycin.

Tumorigenicity in vivo

A total of 12 female nude mice, between 6 and 8 weeks of age were randomly divided into two groups. Five million HCT116 cells stably expressing either pLKO.1 control or pLKO.1 miR-874 were suspended in 100 μl of PBS along with 10% Matrigel and injected subcutaneously into the right flank of each nude mouse. Tumor growth was measured every alternate day using a vernier caliper, and the mice were euthanized after 17 days. The tumor volume was calculated using the formula, tumor volume = (width² × length)/2. Next, the tumors were extracted and weighed. For Western blotting, lysis buffer was added to the tumors in a 1:10 (w/v) ratio, followed by homogenization and sonication until a clear lysate was formed. Next, the lysates were centrifuged at 13,000 rpm for 30 min, and the clear supernatant was collected. The protein amount for each sample was estimated, and 20 μg of each sample was mixed with 3× Laemmli buffer and processed for SDS-PAGE.

Statistical analysis

The results are presented as mean ± S.D. and were analyzed with Student’s t test. A p value of <0.05 was considered significant unless noted otherwise.

Author contributions—T. G. and A. V. conceived the project, conducted the experiments, analyzed the data, and wrote the manuscript. P. K., M. K., V. K., R. S., R. D., P. P., M. M. K., and S. S. contributed to the experiments of this work.

Acknowledgments—We thank Dr. P. Nagarajan, Aparna Sharma, Ananya Kar, and Sunder Bisht for helping with various parts of the paper.
miR-874 regulates CCNE1

References
1. Hanahan, D., and Coussens, L. M. (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21, 309–322
2. Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324, 1029–1033
3. Levin, V. A., Panchabhai, S. C., Shen, L., Kornblau, S. M., Qiu, Y., and Baggerly, K. A. (2010) Different changes in protein and phosphoprotein levels result from serum starvation of high-grade glioma and adenoacarcinoma cell lines. J. Proteome Res. 9, 179–191
4. Golpou, M., Akhavan Niaki, H., Khorasani, H. R., Hajan, A., Mehrasa, R., and Mostafazadeh, A. (2014) Human fibroblast switches to anaerobic metabolic pathway in response to serum starvation: a mimic of Warburg effect. Int. J. Mol. Cell Med. 3, 74–80
5. Chen, M., Huang, J., Yang, X., Liu, B., Zhang, W., Huang, L., Deng, F., Ma, J., Bai, Y., Lu, R., Huang, B., Gao, Q., Zhuo, Y., and Ge, J. (2012) Serum starvation induced cell cycle synchronization facilitates human somatic cells reprogramming. PLoS One 7, e28203
6. Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. (1983) Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33, 389–396
7. Ekholm, S. V., Zickert, P., Reed, S. I., and Zetterberg, A. (2001) Accumulation of cyclin E is not a prerequisite for passage through the restriction point. Mol. Cell. Biol. 21, 3256–3265
8. Minella, A. C., Loeb, K. R., Knecht, A., Welcker, M., Varnum-Finney, B. J., Bernstein, I. D., Roberts, J. M., and Clurman, B. E. (2008) Cyclin E phosphorylation regulates cell proliferation in hematopoietic and epithelial lineages in vivo. Genes Dev. 22, 1677–1689
9. Shuttman, M., Hershko, T., Maisel, A., Fima, E., and Livneh, E. (2003) PKCβ associates with cyclin E/Cdk2 complex in serum-starved MCF-7 and NIH-3T3 cells. Exp. Cell Res. 286, 22–29
10. Leeper, N. J., Rasesdana, A., Kojima, Y., Chun, H. J., Azuma, J., Maegdfessel, L., Kundu, K. R., Quertermous, T., Tsao, P. S., and Spin, J. M. (2011) MicroRNA-26a is a novel regulator of vascular smooth muscle cell function. J. Cell Physiol. 226, 1035–1043
11. Russland, O. S., Hong, S. J., and Bartel, D. P. (2011) MicroRNA destabilization enables dynamic regulation of the miR-16 family in response to cell-cycle changes. Mol. Cell 43, 993–1004
12. Zhang, F., Cui, J., Liu, X., Lv, B., Liu, X., Xie, Z., and Yu, B. (2015) Roles of microRNA-34a targeting SIRT1 in mesenchymal stem cells. Stem Cells Transl. Med. 4, 165
13. Suh, E. J., Remillard, M. Y., Legesse-Miller, A., Johnson, E. L., Lemons, J. M., Chapman, T. R., Forman, J. J., Kojima, M., Silberman, E. S., and Laptch, B. D., and Simon, I. (2008) Genome-wide transcriptional analysis of the human cell cycle identifies genes differentially regulated in normal and cancer cells. Proc. Natl. Acad. Sci. U.S.A. 105, 955–960
14. Kozomara, A., and Griffiths-Jones, S. (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 42, D68–D73
15. D’Abo, G. M., Hooper, S., Paterson, H., and Marshall, C. J. (2002) Loss of Rb overrides the requirement for ERK activity for cell proliferation. J. Cell Sci. 115, 4607–4616
16. Shattuck, M. J., Dai, J., Huang, X., Tao, Y., Deng, A. I., and Imoto, M. (2003) Overexpression of cyclin D1 contributes to malignancy by up-regulation of fibroblast growth factor receptor 1 via the pRB/E2F pathway. Cancer Res. 63, 424–431
17. Tasori, E., Maruki, H., Minato, Y., Doki, Y., Weinstein, I. B., and Imoto, M. (2003) Overexpression of cyclin D1 contributes to malignancy by up-regulation of fibroblast growth factor receptor 1 via the pRB/E2F pathway. Cancer Res. 63, 424–431
18. Yang, R., Li, P., Zhang, G., Lu, C., Wang, H., and Zhao, G. (2017) Cyclin E1 is amplified and overexpressed in osteosarcoma. J. Mol. Diagn. 13, 289–296
19. Ohtani, K., DeGregori, J., and Nevins, J. R. (1995) Regulation of the cyclin E gene by transcription factor E2F1. Proc. Natl. Acad. Sci. U.S.A. 92, 12146–12150
20. Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R. S., and Nevins, J. R. (1998) E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev. 12, 2120–2130
21. Paukte, C., Schieker, M., Tisch, T., Kolk, A., Neth, P., Mutschler, W., and Milz, S. (2004) Characterization of osteosarcoma cell lines and identification of miRNAs and miRNAs associated with aggressive cancer phenotypes. Br. J. Cancer 109, 2229–2236
22. Ohtani, K., DeGregori, J., and Nevins, J. R. (1995) Regulation of the cyclin E gene by transcription factor E2F1. Proc. Natl. Acad. Sci. U.S.A. 92, 12146–12150
23. Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R. S., and Nevins, J. R. (1998) E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev. 12, 2120–2130
24. Paukte, C., Schieker, M., Tisch, T., Kolk, A., Neth, P., Mutschler, W., and Milz, S. (2004) Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res. 24, 3743–3748
25. Billiau, A., Edy, V. G., Heremans, V., Van Damme, J., Desmyter, J., Georgetades, J. A., and De Somer, P. (1977) Human interferon: mass production in a newly established cell line, cell MG-63. Antimicrob. Agents Chemother. 12, 11–15
26. McAllister, R. M., Gardner, M. B., Greene, A. E., Bradt, C., Nichols, W. W., and Landing, B. H. (1971) Cultivation in vitro of cells derived from a human osteosarcoma. Cancer 27, 397–402
27. Jiang, B., Li, Z., Zhang, W., Wang, H., Zhi, X., Feng, J., Chen, Z., Zhu, Y., Yang, L., Xu, H., and Xu, Z. (2014) miR-874 inhibits cell proliferation, migration and invasion through targeting aquaporin-3 in gastric cancer. J. Gastroenterol. 49, 1011–1025
28. Nohata, N., Hanazawa, T., Kinosita, T., Inamine, A., Kikkawa, N., Iesako, T., Yoshino, H., Enokida, H., Nakagawa, M., Okamoto, Y., and Seki, N. (2013) Tumour-suppressive microRNA-874 contributes to cell proliferation through targeting of histone deacetylase 1 in head and neck squamous cell carcinoma. Br. J. Cancer 108, 1648–1658
36. Wang, L., Gao, W., Hu, F., Xu, Z., and Wang, F. (2014) MicroRNA-874 inhibits cell proliferation and induces apoptosis in human breast cancer by targeting CDK9. FEBS Lett. 588, 4527–4535
37. Zhang, X., Tang, J., Zhi, X., Xie, K., Wang, W., Li, Z., Zhu, Y., Yang, L., Xu, H., and Xu, Z. (2015) miR-874 functions as a tumor suppressor by inhibiting angiogenesis through STAT3/VEGF-A pathway in gastric cancer. Oncotarget 6, 1605–1617
38. Wolowiec, D., Mekki, Y., Ffrench, P., Manel, A. M., Bertrand, Y., Rimokh, R., Philippe, N., Bryon, P. A., and Ffrench, M. (1996) Differential expression of cell proliferation regulatory proteins in B- and T-lineage acute lymphoblastic leukaemias. Br. J. Haematol. 95, 518–523
39. Yasui, W., Kuniyasu, H., Yokozaki, H., Semb, S., Shimamoto, F., and Tahara, E. (1996) Expression of cyclin E in colorectal adenomas and adenocarcinomas: correlation with expression of Ki-67 antigen and p53 protein. Virchows Arch. 429, 13–19
40. Iida, H., Towatari, M., Tanimoto, M., Morishita, Y., Kodera, Y., and Saito, H. (1997) Overexpression of cyclin E in acute myelogenous leukemia. Blood 90, 3707–3713
41. Tissier, F., Louvel, A., Grabar, S., Hagnéré, A. M., Bertherat, J., Vacher-Lavenu, M. C., Dousset, B., Chapuis, Y., Bertagna, X., and Gicquel, C. (2004) Cyclin E correlates with malignancy and adverse prognosis in adrenocortical tumors. Eur. J. Endocrinol. 150, 809–817
42. Yen, M. L., Chien, C. C., Chiu, I. M., Huang, H. I., Chen, Y. C., Hu, H. I., and Yen, B. L. (2007) Multilineage differentiation and characterization of the human fetal osteoblastic 1.19 cell line: a possible in vitro model of human mesenchymal progenitors. Stem Cells 25, 125–131
43. Liu, Q., Fu, H., Sun, F., Zhang, H., Tie, Y., Zhu, J., Xing, R., Sun, Z., and Zheng, X. (2008) miR-16 family induces cell cycle arrest by regulating multiple cell cycle genes. Nucleic Acids Res. 36, 5391–5404
44. Han, J., Liu, Z., Wang, N., and Pan, W. (2016) MicroRNA-874 inhibits growth, induces apoptosis and reverses chemoresistance in colorectal cancer by targeting X-linked inhibitor of apoptosis protein. Oncology Rep. 36, 542–550
45. Anaya, J. (2016) OncoLnc: linking TCGA survival data to mRNAs, miRNAs, and lncRNAs. PeerJ Comput. Sci. 2, e67
46. Tatsumi, Y., Sugimoto, N., Yugawa, T., Narisawa-Saito, M., Kiyono, T., and Fujita, M. (2006) Deregulation of Cdt1 induces chromosomal damage without rereplication and leads to chromosomal instability. J. Cell Sci. 119, 3128–3140
47. Sheinerman, K. S., Tsivinsky, V. G., Abdullah, L., Crawford, F., and Umanisky, S. R. (2013) Plasma microRNA biomarkers for detection of mild cognitive impairment: biomarker validation study. Aging 5, 925–938
48. Balcells, I., Cirera, S., and Busk, R. K. (2011) Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. BMC Biotechnol. 11, 70