Cell Cycle 12:24, 3781–3790; December 15, 2013; © 2013 Landes Bioscience

Cyclin D1 is a major target of miR-206 in cell differentiation and transformation

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Keywords: miR-206, cyclin D1, myogenic differentiation, cell proliferation, cell transformation

Abbreviations: AP1, activator protein 1; DM, differentiation medium; ER-α, estrogen receptor-α; ERR-γ, estrogen-related receptor-γ; GM, growth medium; HD, high density; HPV, human papillomavirus; LD, low density; MyHC, myosin heavy chain; NRF2, nuclear factor erythroid-2-related factor 2; NSCLC, non-small cell lung carcinoma; SHP, small heterodimer partner; YY1, ying yang 1

miR-206, a member of the so-called myomiR family, is largely acknowledged as a specific, positive regulator of skeletal muscle differentiation. A growing body of evidence also suggests a tumor suppressor function for miR-206, as it is frequently downregulated in various types of cancers. In this study, we show that miR-206 directly targets cyclin D1 and contributes to the regulation of *CCND1* gene expression in both myogenic and non-muscle, transformed cells. We demonstrate that miR-206, either exogenous or endogenous, reduces cyclin D1 levels and proliferation rate in C2C12 cells without promoting differentiation, and that miR-206 knockdown in terminally differentiated C2C12 cells leads to cyclin D1 accumulation in myotubes, indicating that miR-206 might be involved in the maintenance of the post-mitotic state. Targeting of cyclin D1 might also account, at least in part, for the tumor-suppressor activity suggested for miR-206 in previous studies. Accordingly, the analysis of neoplastic and matched normal lung tissues reveals that miR-206 downregulation in lung tumors correlates, in most cases, with higher cyclin D1 levels. Moreover, gain-of-function experiments with cancer-derived cell lines and with in vitro transformed cells indicate that miR-206-mediated cyclin D1 repression is directly coupled to growth inhibition. Altogether, our data highlight a novel activity for miR-206 in skeletal muscle differentiation and identify cyclin D1 as a major target that further strengthens the tumor suppressor function proposed for miR-206.

Introduction

microRNAs (miRNAs) are a class of 20–23 nucleotide noncoding RNAs that regulate gene expression in multiple biological and pathological processes, including cell differentiation, proliferation, apoptosis, heart disease, neurological disorders, and human cancers.[1-3] Interestingly, some miRNAs appear to be tissue-specific and/or restricted to definite developmental stages, implicating important functions in differentiation.[4] Among them, the so-called myomiRs, miR-1, miR-133 and miR-206, represent a well-defined family (for a review see ref. 5), primarily involved in heart and skeletal muscle development.

miR-206 is the only myomiR specific to skeletal muscle. Its activity positively regulates myogenic differentiation. Transfection of miR-206 in C2C12 myoblasts promotes differentiation with no need for serum depletion,[6] suggesting that this miRNA is able to contrast mitogenic signals from serum growth factors. The effects of miR-206 are partially mediated by repression of the p180 subunit of DNA polymerase-α.[6] Additional miR-206 direct targets involved in muscle differentiation, include the gap-junction protein connexin 43,[7] follistatin-1, and utrophin.[8] Subsequent studies have better dissected the function of miR-206, outlining an important role during the process of muscle regeneration. Indeed, it has been suggested that, in satellite cells, miR-206 participates in a regulatory circuitry that controls the transition to the differentiated state by repressing Pax7.[9-12] miR-206 has been also proposed to mediate the pro-apoptotic activity of MyoD by inhibiting Pax3.[13] More recently, Goljanek-Whysall et al.[14] have reported the identification of several novel target mRNAs of miR-206, whose downregulation in C2C12 cells is critical not only for myogenic differentiation, but also for preventing the activation of alternative differentiation programs.

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Submitted: 05/31/2013; Revised: 09/13/2013; Accepted: 10/02/2013
http://dx.doi.org/10.4161/cc.26674

According to its pivotal role in the regulation of myogenesis, miR-206 has been found downregulated in skeletal muscle-derived tumors such as rhabdomyosarcomas. Re-expression of miR-206 in these tumors promotes myogenic differentiation and blocks tumor growth by inhibiting c-*met* expression.[15,16]

In addition to the anti-cancer activity strictly linked to muscle-derived tumor tissues, it has been suggested that miR-206 might have a broader role in neoplastic growth inhibition. A possible role for miR-206 in breast carcinogenesis draws upon the observation that it is differentially expressed in normal and cancer tissues.[17] Subsequently, it was shown that the expression of miR-206 and the estrogen receptor-α (ER-α) in breast cancers and in endometrial endometrioid adenocarcinomas are mutually exclusive, and that miR-206 targets ERα in both cancer cells, implicating its involvement in the inhibition of estrogen-dependent growth.[18-20] It has been also reported that miR-206 may function as a pro-apoptotic factor by inhibiting Notch3 signaling in HeLa cells.[21] More recently, miR-206 has been associated to invasion and metastasis of lung[22] and laryngeal[23] cancers, as its expression was inversely related to the metastatic phenotype and to gastric cancer proliferation.[24] These findings have led many authors to consider miR-206 a true tumor suppressor miRNA.

The data we report here further reinforce the role of miR-206 as a potential tumor suppressor miRNA and, at the same time, add new insight into the well-known promyogenic activity of miR-206. Indeed, we find that miR-206 directly regulates the expression of cyclin D1 by binding the 3′ UTR in normal and transformed cells. In non-transformed cells, cyclin D1 gene is regulated by coordinated signaling from the extracellular matrix and soluble growth factors. These controls can be lost during cell transformation, and cyclin D1 is correspondingly deregulated and overexpressed in several cancers. Conversely, repression of cyclin D1 gene expression is a hallmark of cell differentiation. We provide evidence that miR-206 participates in cyclin D1 repression in C2C12 myogenic cells, contributing to maintaining low levels of the protein in terminally differentiated myotubes. Moreover, we demonstrate that, by the same mechanism, forced expression of miR-206 is able to counteract the mitogenic signals from activated Ras in NIH3T3 cells. We also show that under-expressed miR-206 in lung tumors nicely correlates with higher cyclin D1 levels, and that miR-206 is able to suppress cyclin D1 in lung tumor cells resulting in decreased cell proliferation.

Results

miR-206 targets cyclin D1

Using PicTar[25] and TargetScan[26] algorithms, we identified cyclin D1 as a candidate miR-206 target gene. Indeed, both mouse and human cyclin D1 3′ untranslated regions (UTRs) comprise a binding site for miR-206, base-pairing with nucleotides 1–7 of the microRNA (**Fig. 1A**). Alignment of the cyclin D1 3′ UTRs of different species with the miR-206 "seed" region revealed a high degree of evolutionary conservation (**Fig. 1A**).

To determine whether miR-206 could decrease cyclin D1 expression through the predicted binding site, we inserted the cyclin D1 3′ UTR into pGL3 control plasmid, downstream of the firefly luciferase coding region. A mutant of the putative binding site was also prepared (**Fig. 1B**). Co-transfection of the miR-206 expression vector along with the mouse cyclin D1 3′ UTR luciferase construct, containing the miR-206 target

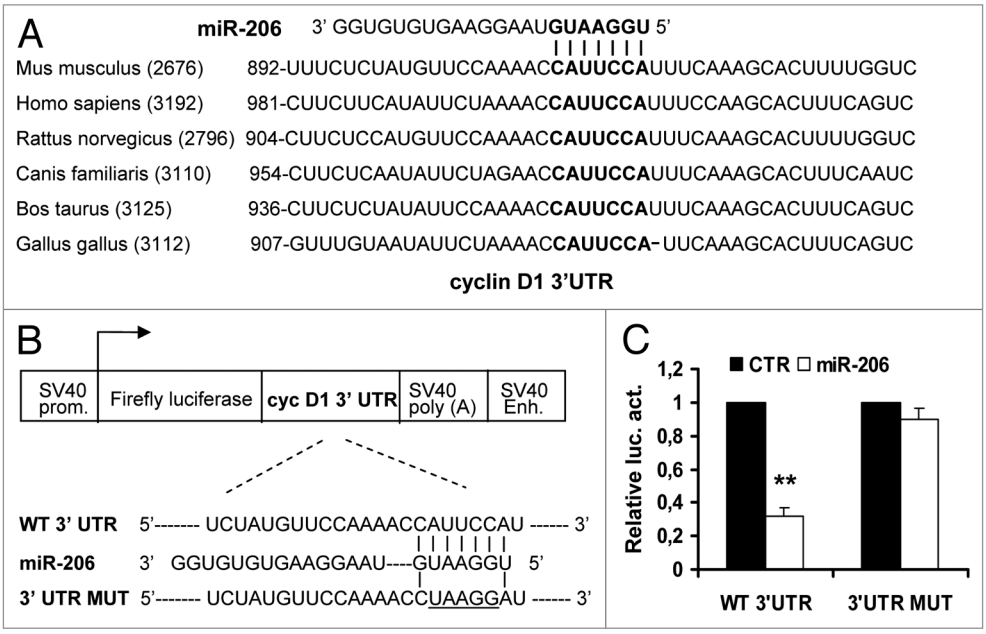

Figure 1. miR-206 targets cyclin D1. (**A**) Sequence alignment between miR-206 and the 3′UTRs of cyclin D1 from different species. In brackets the 3′UTR size. (**B**) Diagram of the luciferase reporter construct with the putative miR-206 binding site (WT 3′UTR) and mutations (3′UTR MUT). (**C**) Relative luciferase activity was measured in HeLa cells after transfection of reporter constructs along with pSP65-U1 (CTR) or pSP65–206 (miR-206). Relative Firefly luciferase values were determined by a ratio of Firefly to *Renilla* luciferase with the control set to 1.00. Values are the means ± SD of 3 separate experiments. **A Student *t* test performed between control and miR-206 transfected cells yielded *P* values < 0.01.

site, caused a remarkable reduction of luciferase activity (70%) as compared with the empty vector (**Fig. 1C**). The inhibitory effect of miR-206 on the expression of the reporter gene was abolished by the mutations introduced at the miR-206 binding site (**Fig. 1C**). These results clearly show that miR-206 directly targets the cyclin D1 3′ UTR.

miR-206 contributes to the control of cyclin D1 in C2C12 cells

In the light of the results from the luciferase experiments, we compared the expression of cyclin D1 and miR-206 at different time points during C2C12 differentiation. As shown in **Figure 2**, cyclin D1 and miR-206 are expressed with opposite kinetics. Indeed, cyclin D1 is abundant in proliferating myoblasts, where miR-206 is absent, and strongly decreases up to 12 h after serum deprivation; beyond that time point reduction slows down. In contrast, miR-206 is detectable only 24 h after serum deprivation, and its levels increase steadily up to 72 h. Thus, in terminally differentiated C2C12 cells, increased miR-206 accumulation coincides with cyclin D1 reduction, suggesting that miR-206 might contribute to the post-transcriptional regulation of *Ccnd1* gene expression during muscle differentiation.

In order to test this hypothesis and assess whether miR-206 is capable of regulating cyclin D1 expression in the context of its native mRNA sequence, miR-206 was transiently expressed in C2C12 myoblasts. To distinguish the decrease of cyclin D1 due to the direct activity of miR-206 from that associated with the induction of differentiation, transfections were performed on sparse cultures maintained in growth medium throughout the experiment, preventing myoblasts from undergoing differentiation. Cyclin D1 expression, as well as proliferation state and differentiation ability, was determined 48 h after transfection. **Figure 3A and C** show that the expression of cyclin D1 is repressed by forced expression of miR-206 in C2C12 cells. The decrease of cyclin D1 corresponds to a significant reduction of the percentage of cycling cells (more than 20% inhibition), which, however, does not necessarily mirror an improvement of the differentiation potential (**Fig. 3B and C**). Indeed, enhancement of differentiated cells by miR-206 overexpression was only evident when C2C12 cells were seeded at higher density, a condition that favors the entry of myoblasts into the differentiation program also in growth medium (not shown, and ref. 6). Consistent with this observation, we found that induction of endogenous miR-206 in C2C12 cells is not always linked to the achievement of the differentiated state. As previously described in vitro myogenic differentiation strictly depends on an appropriate cell density.[27] In particular, upon serum deprivation, low-density (LD) C2C12 cells, although growth arrested, fail to undergo terminal differentiation. **Figure 3D** illustrates expression of miR-206 vs. cyclin D1 and differentiation marker levels in LD and high-density (HD) C2C12 cells in growing medium or after 3 d in differentiation medium. miR-206 is strongly and equally upregulated upon shift to differentiation medium at both culture conditions (LD and HD) despite the opposite differentiation phenotype. Increase of miR-206 also coincides with cyclin D1 decrease, which, as expected, was more evident in HD differentiated C2C12 cells. Overall, these data associate miR-206 more specifically to induction/maintenance of myogenic cell quiescence, a function that appears to be independent of induction of differentiative traits.

It is well known that the expression of miR-206 in C2C12 cells is associated with late stages of differentiation (see ref. 6 and **Fig. 2A**), while cyclin D1 expression is promptly and efficiently downregulated upon serum deprivation (see **Fig. 2B**). This behavior excludes the possibility that the targeting of cyclin D1 by miR-206 might promote cell cycle withdrawal of myogenic cells. Rather, it can be hypothesized that miR-206 might attenuate the level of cyclin D1 associated with residual transcription in terminally differentiated C2C12 cells. In order to assess this issue, the expression of miR-206 was knocked down in purified C2C12 myotubes by LNA antisense oligonucleotide transfection (**Fig. 4A**). Three × 10^4/cm^2 C2C12 cells were induced to differentiate in DM for 72 h in the presence of 50 μM cytosine β-D-arabinofuranoside (AraC) to eliminate undifferentiated, proliferating cells. After washing out AraC, cells were left to recover for 24 h in DM and then transfected with LNA against miR-206. The expression of cyclin D1 was analyzed after further 48 h. **Figure 4B** shows that suppression of miR-206 results in a significant increase of cyclin D1 protein in terminally differentiated, multinucleated myotubes. **Figure 4B** also shows that, despite the high similarity with miR-206, miR-1

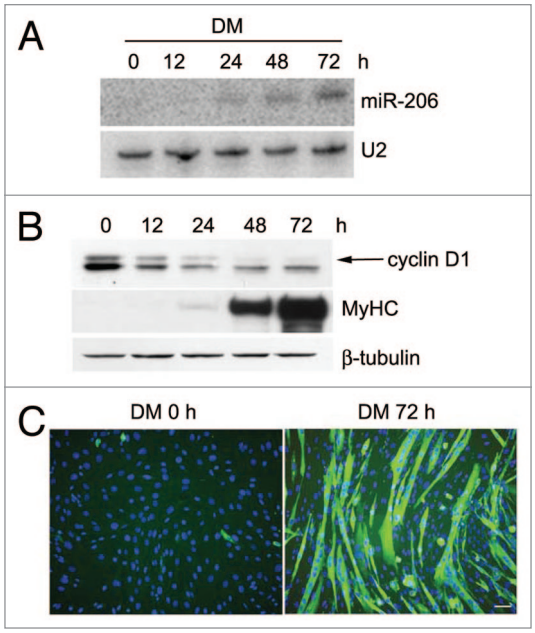

Figure 2. Expression kinetics of miR-206 and cyclin D1 in differentiating C2C12 cells. C2C12 myoblasts were seeded in GM at 1.5 × 10^4/cm^2. Cells were shifted in DM 24 h after plating and left to differentiate for further 72 h. (**A**) Northern blot analysis of miR-206 expression in C2C12 cells after 24 h in GM (0) and at different time points upon shift to DM. (**B**) Western blot analysis of cyclin D1 and MyHC expression in C2C12 cells cultured as in (**A**). Equal RNA and protein loading was confirmed by detecting, snRNA U2 and β-tubulin, respectively. (**C**) MyHC immunofluorescence staining (green) of C2C12 cells after 24 h in GM (DM 0 h) and after 72 h in DM (DM 72 h). Nuclei were counterstained in blue (DAPI) and individual pictures of the same field, taken with a DC camera, were merged using a LEICA Microsystems Imaging Equipment. Bar = 20 μm.

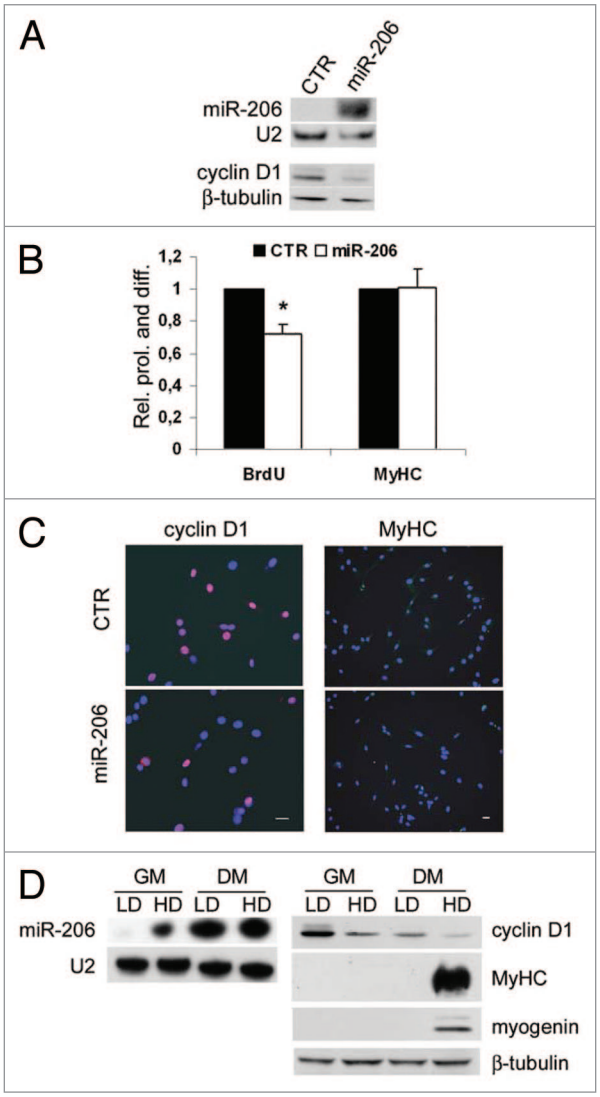

Figure 3. miR-206 controls cyclin D1 accumulation in C2C12 cells. C2C12 myoblasts were seeded in GM at 2.5 × 10^3/cm^2. Cells were transfected 24 h after plaiting. (**A**) Northern blot analysis of miR-206 expression (upper) and western blot analysis of cyclin D1 expression (lower) in C2C12 cells 48 h after transfection with a control vector (CTR) or with a miR-206 expression vector (miR-206). Cells were kept in GM throughout the experiment. (**B**) The effect of miR-206 overexpression on C2C12 cell proliferation and differentiation was evaluated 48 h after transfection by 1 h BrdU incorporation and MyHC staining, respectively. Results are represented relative to the $BrdU^+$ nuclei or nuclei in $MyHC^+$ cells in CTR (set to 1.00), as individually assessed in each independent experiment. Values are the means ± SD of 3 separate experiments. *A Student *t* test performed between control and miR-206 transfected cells yielded *P* values < 0.05. (**C**) Immunofluorescence staining of cyclin D1 (pink) and MyHC (green) 48 h after transfection. Nuclei were counterstained in blue with DAPI. Individual pictures of the same field, taken with a DC camera, were merged using a LEICA Microsystems Imaging Equipment. To obtain cyclin D1 images, before merging, individual pictures were pseudocolored using a LEICA Microsystems Imaging software. Bar = 10 μm. (**D**) C2C12 myoblasts were seeded at low (LD) and high (HD) density in GM. Cells were shifted to DM the day after plating and analyzed after further 3 d. The panels show a northern blot analysis of miR-206 expression (left panel) and a western blot analysis of cyclin D1 and differentiation associated marker expression (right panel) after 24 h in GM and 72 h after shifting to DM. Equal RNA and protein loading was confirmed by detecting, snRNA U2 and β-tubulin, respectively.

expression is only poorly affected by LNA transfection, and its levels remain very high, supporting a prominent role for miR-206 in the control of cyclin D1 accumulation. The repressive function of miR-206 on cyclin D1 in terminally differentiated C2C12 cells is further confirmed by the double positive staining, MyHC/cyclin D1, upon miR-206 inhibition in myotubes (**Fig. 4C**).

Based on these findings, it is reasonable to envisage an additional and novel role for miR-206 in myogenic differentiation, placing it among key factors that contribute to preserve the post-mitotic state in terminally differentiated cells.

miR-206 inhibits transformed cell proliferation

In addition to its involvement in cell differentiation, downregulation of cyclin D1 is crucial for maintaining cellular quiescence and preventing uncontrolled cell proliferation.

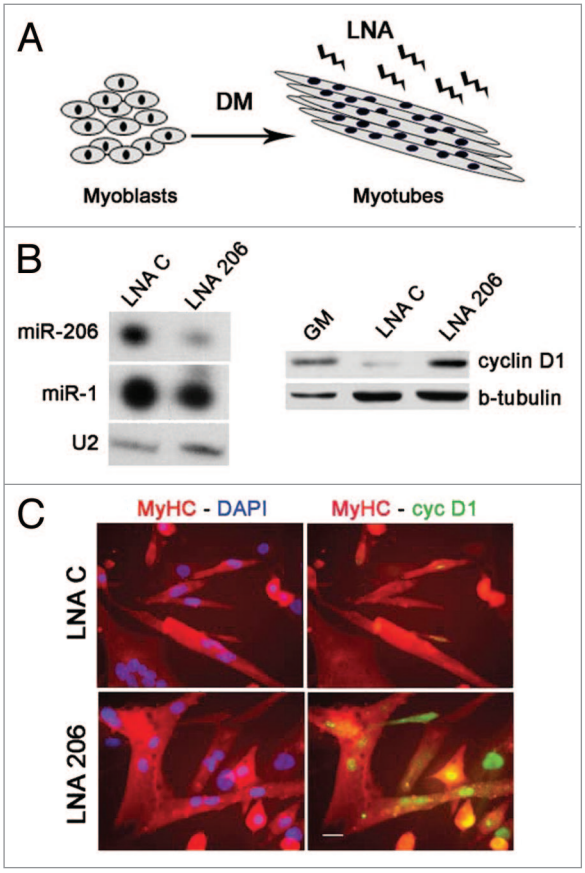

Figure 4. Inhibition of miR-206 rescues cyclin D1 in myotubes (**A**) Experimental scheme. C2C12 myoblasts were induced to differentiate in DM in the presence of AraC. After 3 d, AraC was washed out and cells left to recover in DM for further 24 h. Finally, pure myotubes were transfected with LNA against miR-206 and analyzed 48 h later. (**B**) Northern blot analysis of miR-206 and miR-1 expression (left panel) and western blot analysis of cyclin D1 expression (right panel) in pure myotubes transfected with a control LNA (LNA C) or anti-miR-206 LNA (LNA 206). Cyclin D1 expression in proliferating myoblasts is also shown (GM). Equal RNA and protein loading was confirmed by detecting, snRNA U2 and β-tubulin, respectively. (**C**) Double immunofluorescence staining of MyHC and cyclin D1 of pure myotubes transfected with a control LNA (LNA C) or anti-miR-206 LNA (LNA 206). Individual pictures of the same field, taken with a DC camera, were merged using a LEICA Microsystems Imaging Equipment. Bar = 10 μm.

Consequently, cyclin D1 is often deregulated and overexpressed in many different transformed cells. For example, it has long been known that transformation of immortalized mouse cells by the Ras oncogene results in high levels of cyclin D1, which are responsible for a shortened G_1 phase. Inhibition of cyclin D1 accumulation by antisense RNA results in a slower G_1 progression.[28] On this basis, we decided to investigate whether miR-206 could play any role in activated Ras-induced cyclin D1 dysregulation.

NIH3T3 cells transformed by v-H-Ras, referred to as NIH3T3(Ras), were generated as described in "Materials and Methods". The transformed population displayed growth factor independent proliferation and loss of contact inhibition, as assessed by BrdU labeling of cells cultured in 0.5% FBS-containing medium and focus formation assay, respectively (not shown). As expected, western blot analysis revealed that cyclin D1 expression in NIH3T3(Ras) was higher compared with untransformed NIH3T3(BN) control cells (**Fig. 5A**). As the expression of miR-206 in NIH3T3 cells has been previously reported in a microRNA expression profiling study,[29] we asked whether miR-206 expression was modulated by Ras-induced transformation. We found that miR-206 was actually detectable in untransformed NIH3T3(BN) cells, and that its levels were significantly decreased in NIH3T3(Ras) cells (**Fig. 5B**), showing an opposite trend with respect to cyclin D1 (**Fig. 5A**). Prompted by this observation, we forced the expression of miR-206 in transformed cells. NIH3T3(Ras) were transiently transfected with the miR-206 expression vector and analyzed 24 h later. **Figure 5C** shows that miR-206 was able to repress cyclin D1 accumulation in NIH3T3(Ras) cells. Moreover, the analysis of cell proliferation by BrdU labeling revealed that the percentage of cells in S phase was significantly reduced in miR-206-expressing NIH3T3(Ras) cells (**Fig. 5D**).

Together, these data suggest that miR-206 may contribute to the control of cyclin D1 expression in different context, and reveal a more general growth-inhibitory function associated with it, which, according to the results collected with myogenic cells, we hypothesize might concern the maintenance of the quiescent status.

Control of cyclin D1 is functional to the tumor suppressor activity proposed for miR-206

Since miR-206 is known as a differentiation-associated microRNA, it is not surprising that one of its activities is the control of the cell proliferation status. The discovery that this activity is based on the regulation of cyclin D1 raises the question whether, beyond the involvement in myogenesis, this function can be also significant in tissues other than skeletal muscle.

Several studies have focused on the role of miR-206 in human cancers and revealed that miR-206 is frequently downregulated in various types of neoplasia.[17,22-24,30] However, concerning the function of miR-206 in tissues different from the skeletal muscle, little information is available, and most of the data concerns the repression of ERα and the inhibition of estrogen-dependent growth of breast and endometrial endometrioid carcinomas.[18-20] On the contrary, the physiological role of cyclin D1 in the cell cycle progression is well known, and the notion that cyclin D1 is the most frequently altered cell cycle regulator in cancers is largely acknowledged. Thus, we asked whether: (1) a relationship exists between dysregulation of miR-206 and aberrant cyclin D1 expression in neoplastic tissues; (2) miR-206 might be able to reduce cancer cell growth via negative regulation of cyclin D1.

We analyzed the expression of miR-206 in murine skeletal muscle, heart, and some non-muscle tissues by northern blot. **Figure 6A** illustrates that, in addition to skeletal muscle, appreciable levels of miR-206 are present in cardiac and lung tissues. On the basis of these results, to assess the first issue, we focused on the analysis of normal and neoplastic tissues derived from human lungs. RT real-time PCR experiments were performed on RNA from 8 primary human non-small cell lung cancers (NSCLCs) and pair-matched normal lung tissues. **Figure 6B** illustrates that miR-206 expression was significantly reduced in 7 out of 8 tumor tissues. Six NSCLC tissues with decreased miR-206 also displayed altered expression of cyclin D1, as compared with the surrounding normal tissue (**Fig. 6C**). Interestingly, in 1 of the 8 patients tested (15131) the expression

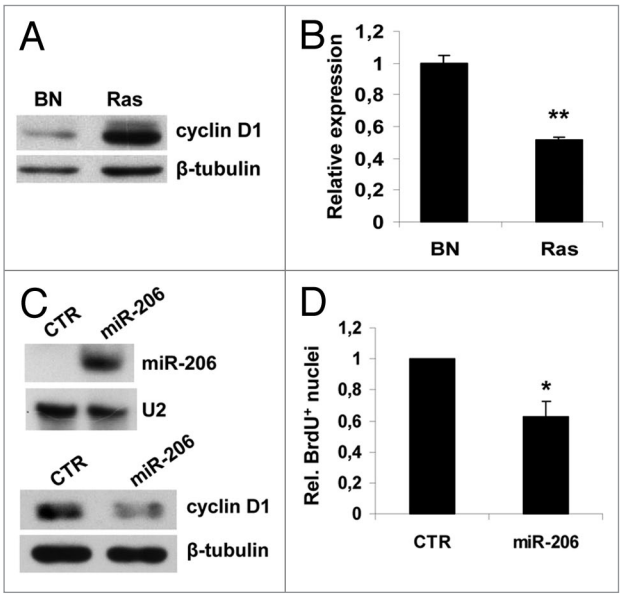

Figure 5. miR-206 inhibits cell proliferation in Ras-transformed fibroblasts. (**A**) Expression levels of cyclin D1 in NIH3T3(Ras) cells as compared with NIH3T3(BN) cells. (**B**) Real-time PCR analysis of miR-206 expression in NIH3T3(Ras) cells. Results are shown relative to untransformed NIH3T3(BN) cells set to value 1.00. Each sample was analyzed in triplicate, and values are the means ± SD of 3 independent experiments. **A Student *t* test performed between untransformed and transformed cells yielded *P* values < 0.01. (**C**) NIH3T3(Ras) cells were transfected with a control vector (CTR) or with a miR-206 expression vector (miR-206) and analyzed 24 h later. Upper, northern blot analysis of miR-206 expression; lower, western blot analysis of cyclin D1 expression. (**D**) Effect of miR-206 forced expression on cell proliferation as determined by 1 h BrdU incorporation. Data are reported relative to $BrdU^+$ nuclei in CTR (set to 1.00), as individually assessed in each independent experiment. Values are the means ± SD of 3 separate experiments. *A Student *t* test performed between control and miR-206 transfected cells yielded *P* values < 0.05. Equal RNA and protein loading was confirmed by detecting, snRNA U2, and β-tubulin, respectively.

of miR-206 in normal and neoplastic tissue attained closely comparable levels (**Fig. 6B**), and the accumulation of cyclin D1 barely differed between the 2 tissue types (**Fig. 6C**).

Functional experiments were performed by transiently expressing miR-206 in the lung adenocarcinoma cell line, A549, and the HeLa cells. The latter were used as a control, as they are known to derive from an HPV-positive cervix tumor expressing the E6 and E7 oncogenes. Given the inhibitory function of HPV E7 on pRb, it is reasonable to expect that the E7 oncogene overcomes the cyclin D1 inhibition, if any, imposed by miR-206 expression in HeLa cells. Cyclin D1 levels and proliferation rate were evaluated 72 h after transfection. **Figure 7A** shows that miR-206 is able to reduce cyclin D1 in both cell lines. Results from the proliferation assay demonstrate that cyclin D1 decrease is accompanied by a reduced percentage of A549 cells in S phase (**Fig. 7B**). Conversely, proliferation of HeLa cells is, as expected, unaffected by miR-206 expression (**Fig. 7B**). This observation further highlights the direct relationship between cyclin D1 targeting by miR-206 and growth inhibition.

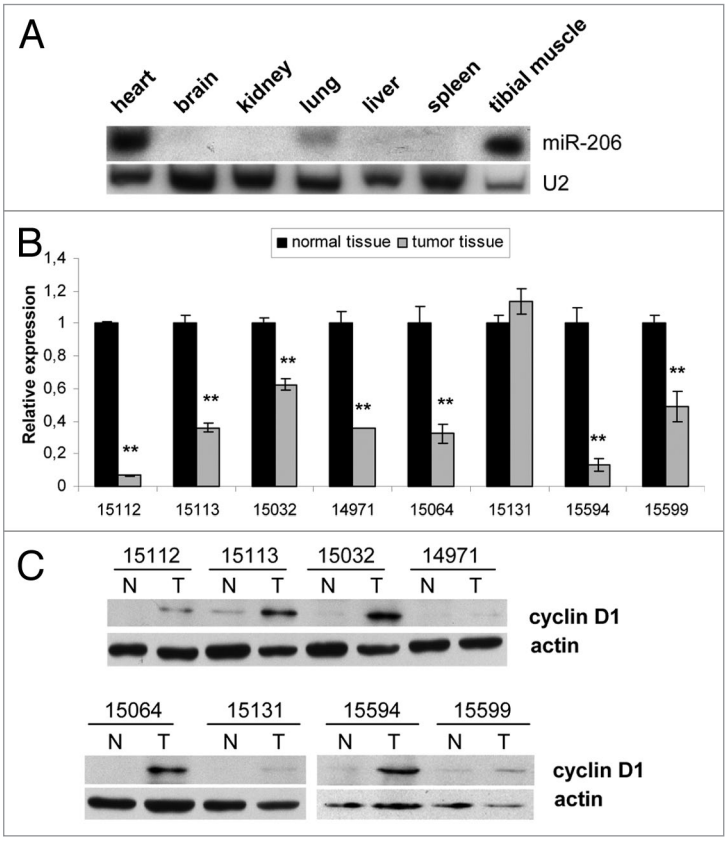

Figure 6. Relationship between miR-206 downregulation and cyclin D1 expression in NSCLCs. (**A**) Northern blot analysis of miR-206 in different murine tissues. snRNA U2 levels were used as a loading control. (**B**) Real-time PCR analysis of miR-206 expression in human NSCLC tissues. Results are shown relative to the matched normal lung tissues set to value 1.00. Each sample was analyzed in triplicate, and values are the means ± SD of three independent experiments. **A Student *t* test performed between normal and tumor tissues yielded *P* values < 0.01. (**C**) Western blot analysis of cyclin D1 expression in normal and neoplastic lung tissues. Equal protein loading was confirmed by detecting actin. n, normal tissue; t = tumor tissue

Discussion

In this work we provide strong evidence of cyclin D1 regulation by miR-206, adding new insights into the role of miR-206 in skeletal muscle differentiation. Moreover, our observations identify cyclin D1 as a valuable target that greatly supports the function of tumor suppressor proposed for miR-206, stressing a broader role of this myomiR in the maintenance of cell quiescence and tissue homeostasis.

Banking on results from many different laboratories it is well established that miR-206, a skeletal muscle-specific miRNA, promotes myogenic differentiation.[31] Loss-of-function experiments indicate that miR-206 is needed for C2C12 myoblasts to enter the differentiation program, while transfection of physiological levels of miR-206 can force C2C12 myoblasts to undergo differentiation, with no need for serum depletion, suggesting that miR-206 is particularly important for myoblast growth arrest.[6] Our findings indicate that miR-206 directly controls cyclin D1 accumulation also in the absence of myogenic differentiation, and that induction of endogenous miR-206 in C2C12 cells is not always linked to the differentiated state, reinforcing the hypothesis that miR-206 plays a prominent role in the control of myoblast proliferation. As widely reported, however, miR-206 accumulates during late C2C12 differentiation, indicating that it is likely not involved in myoblast cell cycle exit upon differentiation stimuli. Rather, it might be envisaged that miR-206 exerts its cyclin D1 repressor activity in terminally differentiated myotubes. Accordingly, we find that miR-206 knockdown in a purified myotube population results in significant cyclin D1 accumulation. As it is well known, terminal myoblast differentiation entails definitive withdrawal from the cell cycle and molecular mechanisms exist that preserve the post-mitotic state in terminally differentiated cells. In fact, it has been demonstrated that, with the exception of DNA tumor virus oncogenes, neither activated proto-oncogenes nor mitogenic stimuli from growth factors or other positive cell cycle regulators are able to induce myotube S-phase entry, although they cause a typical immediate-early response.[32] More recently, it has been reported that terminally differentiated skeletal muscle cells can be induced to re-enter the cell cycle by knocking down cyclin-dependent kinase inhibitor(s).[33] Although myotubes can be forced to re-enter the cell cycle, they are unable to complete DNA replication and sustain heavy DNA damage, which triggers apoptosis or results in mitotic catastrophe.[34] Altogether, these observations suggest that myotubes are characterized by intrinsic attributes that efficiently protect them from inappropriate DNA replication. The results we present in this study strongly suggest that control of cyclin D1 levels by miR-206 in myotubes might well represent one of those intrinsic mechanisms contributing to maintain cell cycle exit permanently and underline a novel, additional function for miR-206 in skeletal muscle differentiation.

Given the central role of *CCND1* gene regulation in cell cycle control, we have extended our investigations on

the activity of miR-206 also to cells other than myoblasts. We show that miR-206 is downregulated by activated Ras induced-transformation in NIH3T3 cells, suggesting a relationship with aberrant cyclin D1 expression observed in NIH3T3(Ras) cells. We also show that, by targeting cyclin D1, miR-206 counteracts activated Ras-induced transformation, at least in terms of proliferation rate. These findings are suggestive of a broader activity associated with miR-206 as a crucial factor for the maintenance of cellular quiescence and for preventing unwanted cell proliferation.

Although described as a skeletal muscle-specific microRNA, the expression of miR-206 is not solely restricted to skeletal muscle, but is detectable also in several other organs, albeit at much lower levels (see ref. 31 and present work). Nevertheless, very little is known concerning the role in tissues other than skeletal muscle, and most studies have focused on the regulation of ER-α and on the relevance in ER-α-dependent tumor growth.[18-20] Further evidence suggests that miR-206 may function as a pro-apoptotic factor by inhibiting Notch3 signaling in HeLa cells,[21] but no involvement in specific cancers has been reported so far. We detect appreciable levels of miR-206 in mouse lung and demonstrate that miR-206 is downregulated in human lung cancers compared with matched normal tissues. The latter result is in agreement with a recent report showing a connection between lung cancer metastasis and miR-206-negative regulation.[22] In this study we were able to assign a possible role to miR-206 downregulation in lung cancers, and we propose that one of the activities of miR-206 in the lung concerns the control of cyclin D1 levels. Indeed, we observe an inverse relationship between miR-206 and cyclin D1 expression, as, in most cases, lower levels of miR-206 inversely correlate with accumulation of cyclin D1. Thus, downregulation of miR-206 in tumor tissues may partially account for abnormal expression of *CCND1* gene in affected cancers with a significant impact on neoplastic growth. This hypothesis is further supported by the demonstration that miR-206-mediated cyclin D1 repression specifically leads to growth inhibition in the lung adenocarcinoma A549 cells.

Cyclin D1, along with cyclin D2 and cyclin D3 (D-cyclins), is a G_1-specific cyclin that associates with CDK4 or CDK6 and promotes restriction point progression during G_1 phase.[35] Molecular analyses have revealed that, among the D-cyclins, cyclin D1 is more frequently dysregulated and overexpressed in human tumors as compared with cyclin D2 and D3. Abnormal expression of cyclin D1 in human tumors is guided by multiple mechanisms comprising genetic alterations, aberrant post-transcriptional regulation, and post-translational protein stabilization. Regulation by miRNAs is one of the mechanisms responsible for post-transcriptional regulation of *CCND1* gene. The results reported here add miR-206 to the growing list of validated miRNAs controlling cyclin D1 expression, supporting its role as an anti-oncogene miRNA implicated in cell cycle control. Indeed, although the functions as regulator of *Notch3*[21] and *CCND1* all converge toward the pro-myogenic activities of miR-206 (see refs. 12, 36, 37, and present work), the data from experiments with transformed cells and lung cancer analysis lead to hypothesize that miR-206 might be also involved in the process of cellular transformation in cells other than myogenic cells.

Concerning the transcriptional regulation of miR-206 in tissue different from skeletal muscle limited information is available. To date, 2 main reports are present in literature. The first depicts an interesting "dual inhibitory" mechanism responsible for the activation of miR-206 gene transcription governed by SHP and involving ERRγ, YY1, and AP1.[38] More recently, a relationship between the repression of miR-1 and miR-206 by the transcription factor NRF2 and tumorigenesis in lung has been described.[39] Establishment of a connection between the 2 models, if any, will be of importance for a better understanding of miR-206 in tumor development.

Materials and Methods

Cells, culture conditions, and human primary lung samples

Cells were grown in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (referred to as growth medium, GM). NIH3T3(BN) and NIH3T3(Ras) cells were generated by infecting NIH3T3 cells with BabeNeo and BabeNeo-v-H-*ras* viruses, respectively. pBabeNeo-H-*ras* was generated by subcloning the BamH1

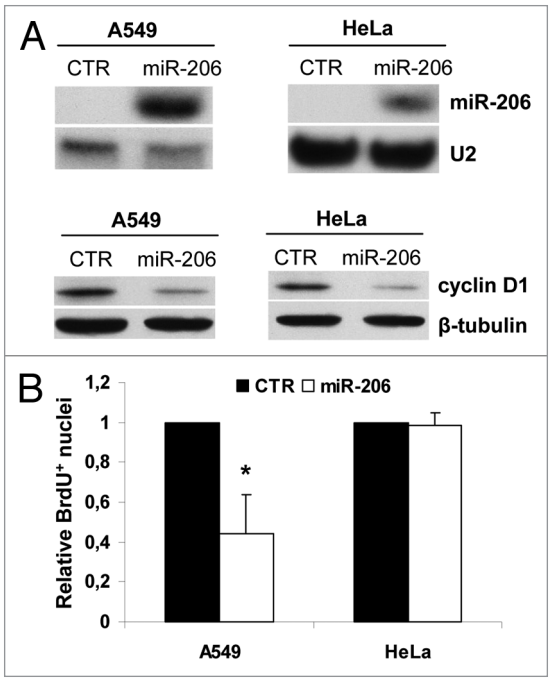

Figure 7. miR-206 inhibits cancer cell proliferation through repression of cyclin D1. (**A**) A549 and HeLa cells were transfected with a control vector (CTR) or with a miR-206 expression vector (miR-206) and analyzed 72 h later. Top panel: northern blot analysis of miR-206 expression; lower panel, western blot analysis of cyclin D1 expression. Equal RNA and protein loading was confirmed by detecting, snRNA U2 and β-tubulin, respectively. (**B**) Effect of miR-206 forced expression on cell proliferation as determined by 1 h BrdU incorporation and immunofluorescence staining. Data are reported relative to $BrdU^+$ nuclei in CTR (set to 1.00), as individually assessed in each independent experiment. Values are the means ± SD of 3 separate experiments. *A Student *t* test performed between control and miR-206 transfected cells yielded *P* values <0.05.

v-H-*ras* fragment from the pZip-v-H-*ras* into the replication defective retroviral vector pBabeNeo (provided by H Land, University of Rochester). High titer viral stocks were obtained from the ecotropic Phoenix packaging cell line (kindly provided by G Nolan, Stanford University) transiently transfected with the retroviral vectors pBabeNeo and pBabeNeo-v-H-*ras*. NIH3T3(BN) and NIH3T3(Ras) polyclonal populations were obtained by selection with G418 antibiotic (GIBCO/Life Technologies). C2C12 low and high density conditions were achieved by seeding isolated myoblasts at 5×10^2/cm^2 and confluent myoblasts at 2×10^4/cm^2, respectively.[27] Myogenic differentiation was induced, 24 h after plating in GM, by incubating C2C12 cells in D-MEM supplemented with 0.5% FBS (referred to as differentiation medium, DM). Cell proliferation was assessed by Bromodeoxyuridine (BrdU) (Sigma Chemical Co.) incorporation by adding 50 μM BrdU for 1 h. Frozen primary non-small cell lung cancers (NSCLC) and matched normal lung tissues, sampled at least at 5 cm from the autologous NSCLC, were obtained from patients surgically treated at the Regina Elena National Cancer Institute with approval by institutional ethic committee. Both neoplastic and paired normal lung tissues were reviewed by a specialized lung consultant pathologist (PV) to confirm histology in each specimens used throughout the study. Informed consent was obtained from all subjects.

Immunofluorescence

A mouse monoclonal antibody (MF20) that recognizes the skeletal muscle myosin heavy chain (MyHC)[40] was used to assess terminal differentiation in C2C12 cells. Cells were fixed in cold methanol:acetone (1:1) for 10 min and treated with 0.25% Triton X-100 in PBS. Cells were then incubated with the MF20 supernatant, followed by incubation with a FITC-conjugate goat anti-mouse antibody (Cappel/MP Biomedicals). The mouse monoclonal antibody 72–13G (Santa Cruz Biotechnology) was used to stain cyclin D1-positive cells. Cells were fixed in 4% paraformaldehyde for 10 min and treated 30 min at room temperature (r.t.) with 1% BSA in PBS/0.2% Triton-X100 to block aspecific interaction and to get permeabilized cells. Cells were then incubated 30 min at r.t. with 1% normal goat serum in PBS to further minimize aspecific antibody binding. Primary antibody was added for 1 h at r.t. After extensive washings with 1% BSA in PBS/0.2% Triton-X100, cells were incubated with the secondary antibody FITC-conjugated affinity purified goat anti-mouse IgG (Cappel/MP Biomedicals). Double staining cyclin D1/MyHC was achieved by using a rabbit polyclonal antibody specific for all sarcomeric MyHCs[41] followed by TRITC-conjugated goat anti-rabbit IgG (Cappel/MP Biomedicals). BrdU-labeled cells were processed as described.[42] Nuclei were stained with 0.2 μg/ml DAPI (Sigma Chemical Co). Stained cells were examined using the Leica DMRE microscope equipped with 20×, 40×, and 100× lenses. Single images were recorded on Leica DC250 camera and processed using the Qwin software (Leica Microsystems).

miRNA overexpression and knockdown

Transient transfections were performed by DNA-calcium-phosphate co-precipitation method [C2C12, NIH3T3, NIH3T3(BN), and NIH3T3(Ras) cells] or by Lipofectamine 2000 (Invitrogen) (A549 and HeLa cells) according to the manufacturer's instructions. Cells were transfected with the control empty vector, pSP65-U1, or the pSP65–206 expression vector, the pSP65-U1 plasmid containing the miR-206 precursor[9] (kindly provided by I Bozzoni, Sapienza-University of Rome). LNA oligonucleotides against miR-206 (Exiqon) were transfected into C2C12 cells at a final concentration of 25 nM by using the Hyperfect reagent (Qiagen).

Luciferase reporter assay

The 3′-UTR segment, either wild-type or mutated (see **Fig. 1B**), of mouse cyclin D1 gene encompassing the miR-206 seed sequence (*Ccnd1*, nucleotides 1991–2070 of NM_007631) was subcloned into the XbaI site downstream of the Firefly luciferase stop codon of pGL3 Control Vector (Promega). Twenty-four hours before transfection, 1.5×10^5 HeLa cells were plated per well in a 12-well plate. Seven hundred ng of pGL3 reporter plasmids containing the wild-type or mutated 3′-UTR of cyclin D1, 700 ng of pSP65–206 or control vector, and 70 ng of *Renilla* luciferase plasmid, pRL-TK (Promega) were co-transfected using the Lipofectamine 2000 reagent (Invitrogen). Cells were harvested 48 h post-transfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions.

RNA isolation, northern blot, and RT real-time PCR analyses

Total RNA from cultured cells and frozen mouse and human tissues was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Ten μg of total RNA were separated on a 10% denaturing urea-PAGE gel and transferred onto positively charged nylon membrane (Amersham/GE Healthcare Life Sciences). Hybridization was performed with terminally ^{32}P-labeled DNA oligonucleotides. Probes were as follows:

miR-206, 5′-CCACACACTT CCTTACATTC CA-3′;
miR-1, 5′ -ATACATACTT CTTTACATTC CA-3′;
U2, 5′-TTAGCCAAAA GGCCGAGAAG C-3′.

Real-time reverse transcription PCR quantification of miR-206 was performed on 40 ng of total RNA using the miR-206 TaqMan miRNA assay (Applied Biosystems) and the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The ubiquitously expressed U6b snRNA was also quantified and used as an internal control. Relative quantification was performed using the comparative cycle threshold ($2^{-\Delta\Delta C}_t$) method.

Protein extraction and western blot

Cells were lysed as described.[27] Twenty–30 μg of protein extracts were resolved on 8% or 15% SDS-PAGE (according to the different molecular weight) and transferred onto nitrocellulose. After saturation in 5% milk in Tris-buffered saline plus 0.02% Tween-20 (TBS-T), filters were incubated with the following antibodies: mouse monoclonal anti-cyclin D1 (72–13G, Santa Cruz Biotechnology); rabbit polyclonal anti-cyclin D1 (M20, Santa Cruz Biotechnology); goat polyclonal anti-actin (I-19, Santa Cruz Biotechnology); mouse monoclonal anti-MyHC, MF20;[40] mouse monoclonal anti-Myogenin, IF5D;[40] mouse monoclonal anti-β-tubulin (ICN). After washing, membranes

were incubated with horseradish-peroxidase-conjugated species-specific secondary antibodies (Bio-Rad Laboratories) followed by enhanced chemiluminescence system (Amersham/GE Healthcare Life Sciences).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We wish to thank I Bozzoni, D Cacchiarelli, M Crescenzi, D Fischmann, H Land, G Micheli, R Negri, G Nolan, and all our colleagues who generously provided many reagents and helpful comments. This work was partially supported by grants from Sapienza-University of Rome to MG and to CP (C26F09HPNN and C26A11R95L, respectively).

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