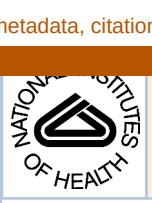

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HuR involvement in mitotic clonal expansion during acquisition of the adipocyte phenotype

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Abstract

In the nucleus HuR binds to mRNAs containing adenylate-uridylate rich elements in the 3′-untranslated region. HuR may influence expression of its ligand mRNA through regulation of polyadenylation, translocation of the message to the cytosol, stabilization of the mRNA and/or altering its translational efficiency. Suppression of HuR using siRNA resulted in an attenuation of the 3T3-L1 differentiation program, consistent with HuR control of the expression of mRNA ligand (s) critical to the differentiation process. In the current study we begin to identify mRNA ligands of HuR whose regulated expression is necessary for adipogenesis.

Keywords

adipogenesis; differentiation; 3T3-L1; HuR; elav; siRNA; β-actin; p53

Introduction

Fundamental to obesity is adipocyte hyperplasia that occurs through recruitment and proliferation of preadipose cells present in the vascular stroma of adipose tissue [1]. This hyperplasia is mimicked in culture by 3T3-L1 preadipocytes which when induced to differentiate, synchronously re-enter the cell cycle and undergo several rounds of mitotic clonal expansion prior to growth arrest and expression of the adipocyte phenotype [2]. Critical/essential to the differentiation process is the RNA binding protein, HuR [3]. HuR is a 32-kDa protein containing 3 RNA recognition motifs (RRMs) and belongs to the Hu/ELAV family (embryonic lethal, abnormal vision) of RNA binding proteins [4]. Unlike the other three family members, HuB, HuC and HuD, which are exclusively neuronal, HuR is ubiquitously expressed, localized predominantly to the nucleus and demonstrated to shuttle between the nucleus and cytoplasm [5]. In the nucleus, recent data have supported roles for HuR in the splicing as well as in the regulation of polyadenylation by competitively inhibiting the binding of the cleavage and polyadenylation specificity factor, thereby attenuating polyadenylation and nuclear export [4,6]. In the cytosol there is compelling evidence to suggest that HuR functions to control the stability and translational efficiency of its ligand mRNAs [4].

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Our previous work demonstrated that siRNA mediated suppression of HuR resulted in an inhibition of 3T3-L1 preadipocyte differentiation [3]. The inhibition was transient, as the effect of the siRNA was lost after several days and the cells began to express the adipocyte phenotype [3]. Even with that consideration, those data suggest that during the differentiation process, there is formation of a HuR-mRNA complex that is essential for the differentiation program to progress. Whether the function of the complex is export from the nucleus or stabilization of the message, disruption of the interaction leads to alteration of expression and attenuation of the differentiation program. In terms of timing, formation of the HuR-mRNA complex has to be early in the process, because as we have previously demonstrated disruption of this interaction must precede expression of C/EBPβ [7]. In addition, more than one ligand mRNA may be involved in the process. Our interest is in identifying these HuR ligands and characterizing their interaction with HuR in the context of mitotic clonal expansion and the differentiation program.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco/Invitrogen (Grand Island, New York). Bovine calf serum and fetal calf serum were purchased from Hyclone Laboratories (Logan, Utah). The 3T3-L1 cells used in this work were obtained from Howard Green (Harvard University, Boston, MA). Antibodies directed against: Cyclin A and HuR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); Cyclin D1 and p53 were from Cell Signaling (Danvers, MA); Cyclin E were from Upstate (Charlottesville, VA); β-Actin were from Sigma (St. Louis, MO); p21 were from Calbiochem (LaJolla, CA). The β-tubulin antibody was the generous gift of Ted Bertrand, Department of Medicine, Brody School of Medicine (Greenville, NC). The antibody used as a control, anti-Armenian-Syrian hamster monoclonal antibody was obtained from BD Pharmingen (San Diego, CA). All other chemicals were of reagent grade and purchased from Sigma-Aldrich Biochemical (St Louis, MO).

Methods-3T3-L1 cell culture

3T3-L1 preadipocytes were cultured, maintained and differentiated as previously described [3,7].

SiRNA treatment of the 3T3-L1 preadipocytes

transfection of the cells was performed using Dharmacon siGENOME SMARTpool^{tm} reagent (catalog number M-0538-00-0020) as we have previously described [3]. Briefly, cells were transfected twice, the first time at 60% confluence, and the second 24h later while the cells remained pre-confluent. At 24h after the second transfection, two monolayers were combined to generate immediate confluency, 24h after the second transfection differentiation was initiated as we have previously described [3,7]. The 0 time point represents the time of exposure of the cells to the differentiation inducers.

Western blot analysis

Protein extracts were prepared as previously described [3,7] and Western blot analysis performed as previously detailed [3,7].

Results and Discussion

Results from previous studies indicated that the β-actin mRNA was a major ligand for HuR on induction of differentiation (V. A. Karschner and P.H. Pekala, unpublished results). These data led us to examine the effect of decreased HuR expression on β-actin levels during the early

phase of the differentiation process. Cells were transfected with siRNA directed against HuR as described in Materials and Methods and induced to differentiate. As shown in Fig. 1, an 86% suppression of HuR expression was observed 48 h after induction of differentiation (Lane 1) relative to mock transfected control (Lane 2). β-actin expression decreased by 47% (Lane 1) relative to control (Lane 2). At 72h (Lane 3) HuR suppression maintained at 78% of control (Lane 4) while β-actin levels were down by 38%. Quantification was by scanning densitometry. β-Tubulin content was used for normalization as its levels do not change during the course of the experiment, moreover, it does not bind HuR. While β-actin expression is known to decrease as the cells attain the adipocyte phenotype, this early decrease prior to mitotic clonal expansion might be expected to slow cell division. Indeed recent observations in HeLa cells by Dormoy-Raclet et al [8] found that siRNA suppression of HuR led to decreased actin expression resulting in an increased proliferation time. In a parallel experiment, we inhibited actin polymerization by exposure of the cells to cytochalasin D and examined the effect on differentiation and used C/EBPα as marker for terminal differentiation. The cytochalasin D treated cells maintained an altered morphology and never accumulated lipid, in addition, on day 8 post induction of differentiation, C/EBPα protein levels were only 30% of that found in the untreated cells (Karschner and Pekala, unpublished results). These data were similar to what we observed for C/EBPα expression in cells treated with siRNA directed against HuR [3], providing further confirmation of the importance of actin expression (polymerization) for appropriate induction of differentiation.

In their study, Doemoy-Raclet et al [8] observed that key β-actin mediated functions including cell adhesion, migration and invasion, were markedly decreased and these defects correlated with the loss of the actin stress fiber network. In addition there is significant evidence to link down regulation of β-actin expression to signaling processes that control transcription [9,10].

To examine the influence of HuR on the cell cycle, we used siRNA to suppress HuR expression, induced differentiation and examined 3 cell cycle components. As shown in Fig. 2, (panel A.), suppression of HuR was in excess of 85% at 0, 8, and 12h after induction of differentiation and 70% for the 16, 20 and 24h time points. With this degree of HuR depletion, cyclins A, E and D1 were expressed at apparent lower levels and on a slightly delayed (4 to 8h) time course (Fig. 13, panel C.) than that observed for the mock transfected cells (Fig. 2, panel B.). The data are similar to those reported by Dormoy-Raclet et al. [8], a study in which depletion of HuR lead to an attenuation rather than an inhibition of movement through the cell cycle. However, there are differences. One must consider that Dormoy-Raclet et al. [8] were working with cells that had lost contact inhibition of growth and were constantly moving through the cell cycle, while in our study, the 3T3-L1 cells had attained contact inhibition of growth and on induction of differentiation entered the mitoic clonal expansion phase of the differentiation program.

Using the same extracts we examined for alterations in the expression of p53, reported to be activated as a consequence of down regulation of actin expression and/or loss of cytosolic actin filaments [10]. As shown in Fig. 3, we observed a transient, near 60% decrease in p53 expression at the time of induction of differentiation. The p53 mRNA has a HuR binding site in the 5′-UTR and the interaction is thought to control translational efficiency, thus, loss of HuR led to decreased p53. We also examined for expression of the cell cycle inhibitor, p21/waf1/cip1 (p21), a known target for p53 transactivation and a HuR ligand. As shown in Fig. 4, p21 expression is decreased by 65% at 0 time, when the cells are induced to differentiate.

When p21 expression was decreased using siRNAs, the differentiation program in the 3T3-L1 cells was attenuated in proportion to the extent of the suppression [11]. Similarly, embryonic fibroblasts derived from p21 knock out mice have an impaired ability to differentiate to adipose tissue. Thus the transient suppression of p21 shown in Fig. 4 could contribute to the observed lag in the expression of the adipocyte phenotype. We should note that both p53 and p21 are

expressed at significant levels throughout the differentiation program [11]. While the level of expression does not address activation there is ample evidence consistent with the a requirement for both proteins for a productive differentiation program. Interestingly, in a recent analysis we examined for potential mRNA ligands of HuR using an RNA Immunoprecipitation – Chip assay [12]. In this analysis both β-actin and p53 were demonstrated to be ligands of HuR, while p21 was not. This would suggest that when HuR protein content was decreased, the resulting mRNAs coding for β-actin and p53 were also decreased leading to decreased β-actin and p53 protein. It appears likely that the decrease in p21 protein may be mediated by loss of p53 and thus decreased transactivation of the p21 gene.

Thus, our data are consistent with HuR controlling the expression of a number of gene products that individually could influence/attenuate the progress of the differentiation program. Taken together down-regulation of β-actin, p53 and p21 expression most certainly leads to a decreased ability of the cells to undergo mitotic clonal expansion. We are currently developing a shRNA strategy that would permit long term suppression of HuR such that the exact stage at which the differentiation program is stopped can be identified.

Acknowledgments

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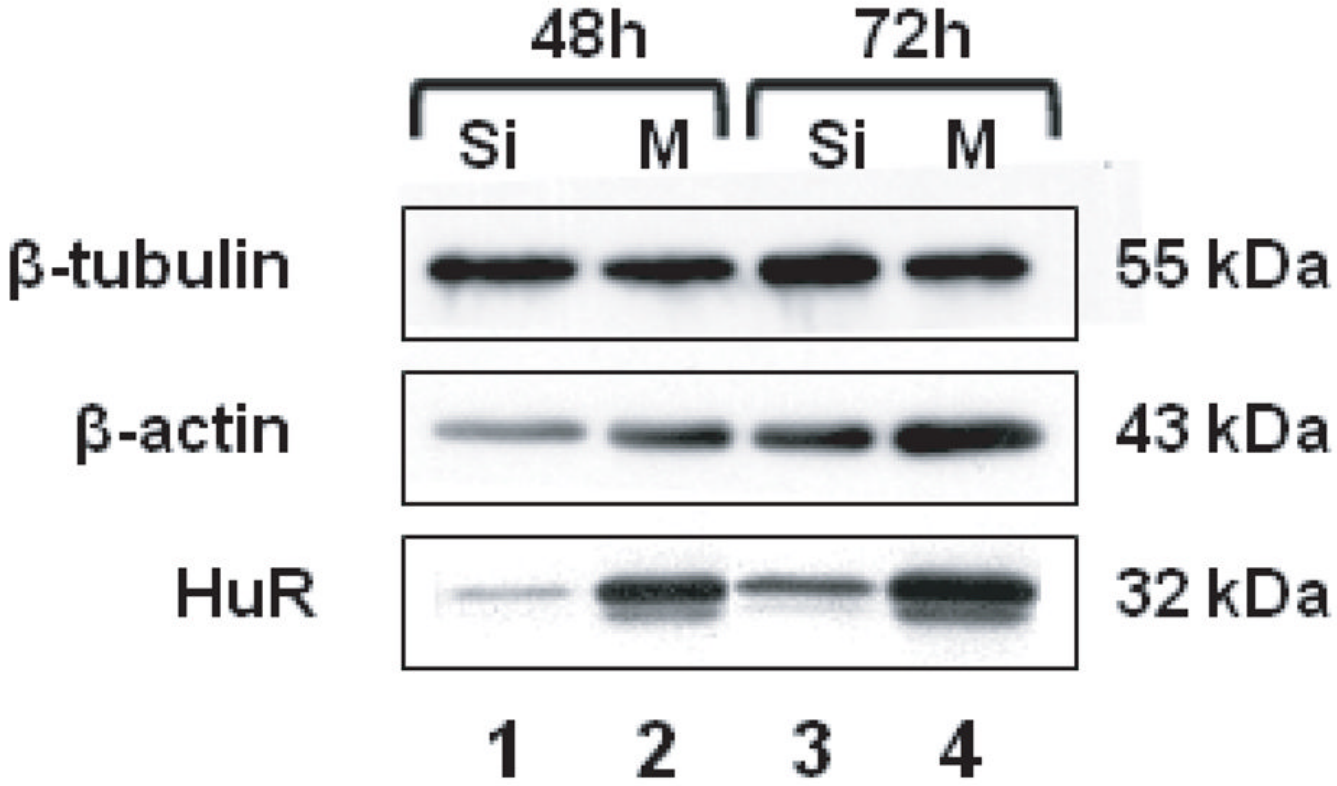

Fig. 1. Suppression of HuR expression leads to attenuation of β-actin expression

Cells were transfected with siRNA directed against HuR and induced to differentiate as described in Materials and Methods. Extracts were prepared at 48 and 72h post induction of differentiation and Western blot analysis of β-tubulin, β-actin and HuR performed. Quantification of protein levels was performed using scanning densitometry.

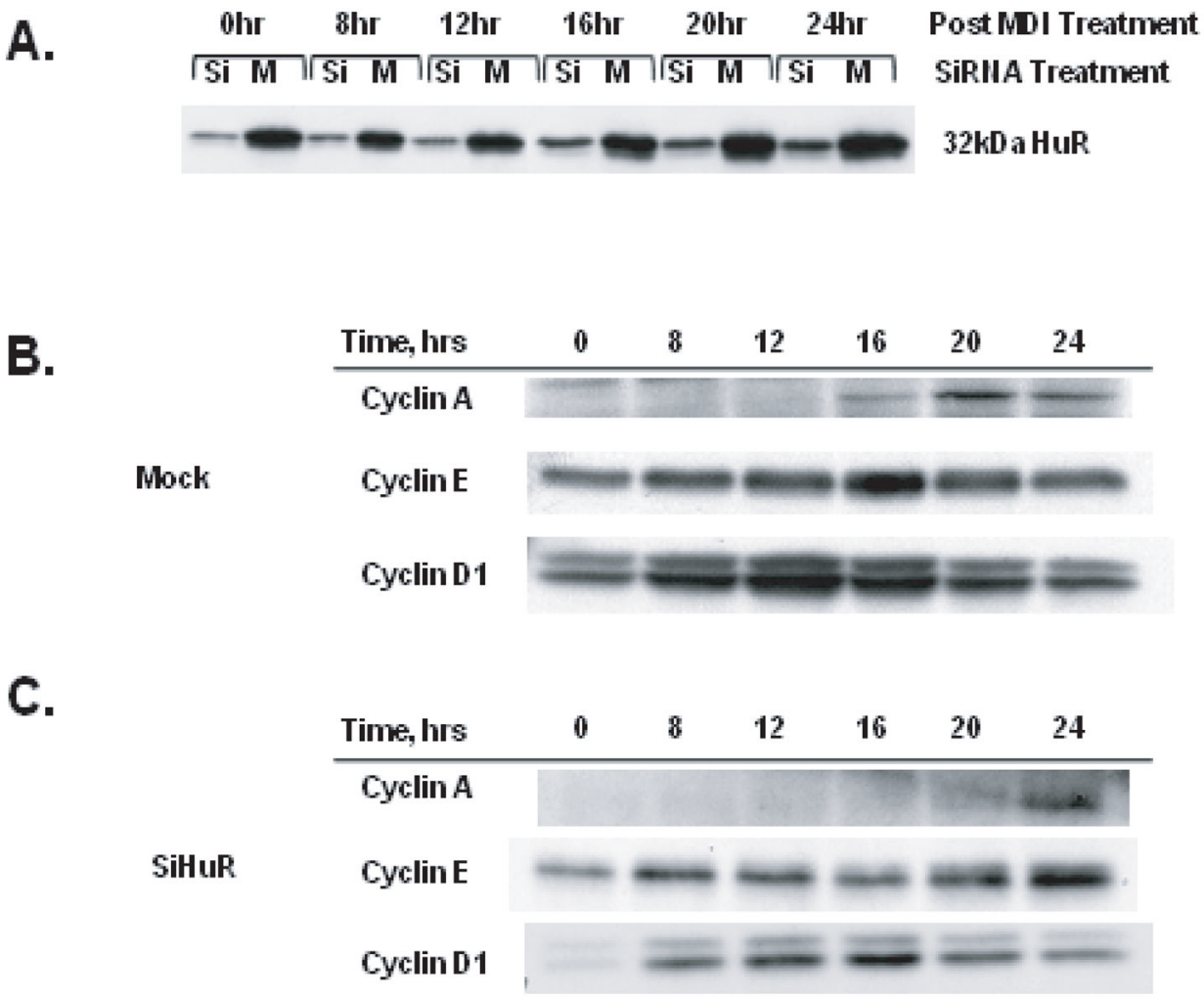

Fig. 2. Effect of decreased HuR expression on the expression of Cyclins A, E and D1

siRNA mediated suppression of HuR expression was performed as detailed in Materials and Methods. Cells were induced to differentiate and extracts prepared over a 24h period. Western blot analysis was performed to determine HuR content (Panel A.) as well as that of Cyclins A, E and D1 in mock (Panel B.) and siRNA (Panel C.) treated cells. Quantification was by scanning densitometry.

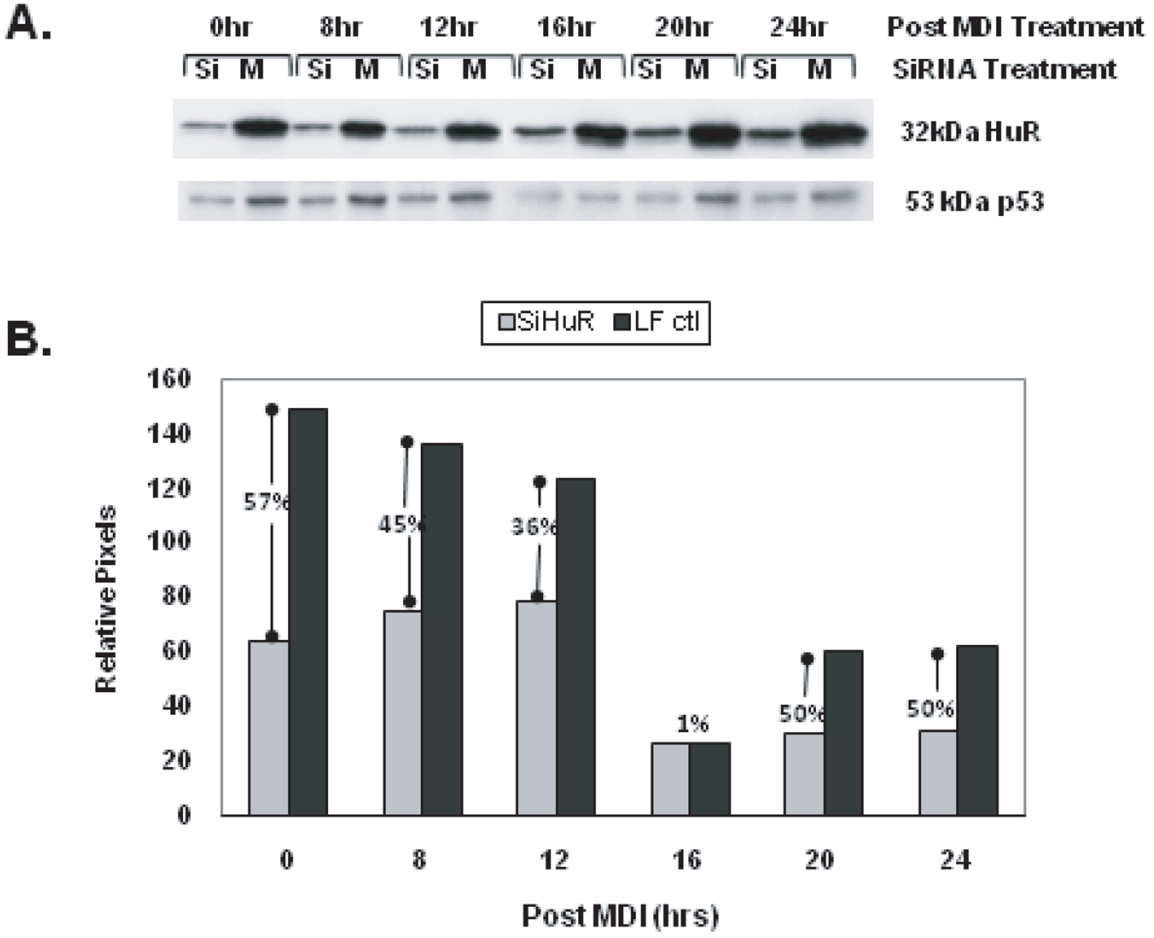

Fig. 3. siRNA mediated suppression of HuR leads to a transient suppression of p53

Cells were prepared for experimentation as detailed in the legend to Fig. 2. Extracts were prepared from siRNA and mock transfected cells and Western Blot anlaysis performed for p53 (Panel A.). Quantification was by scanning densitometry (Panel B).

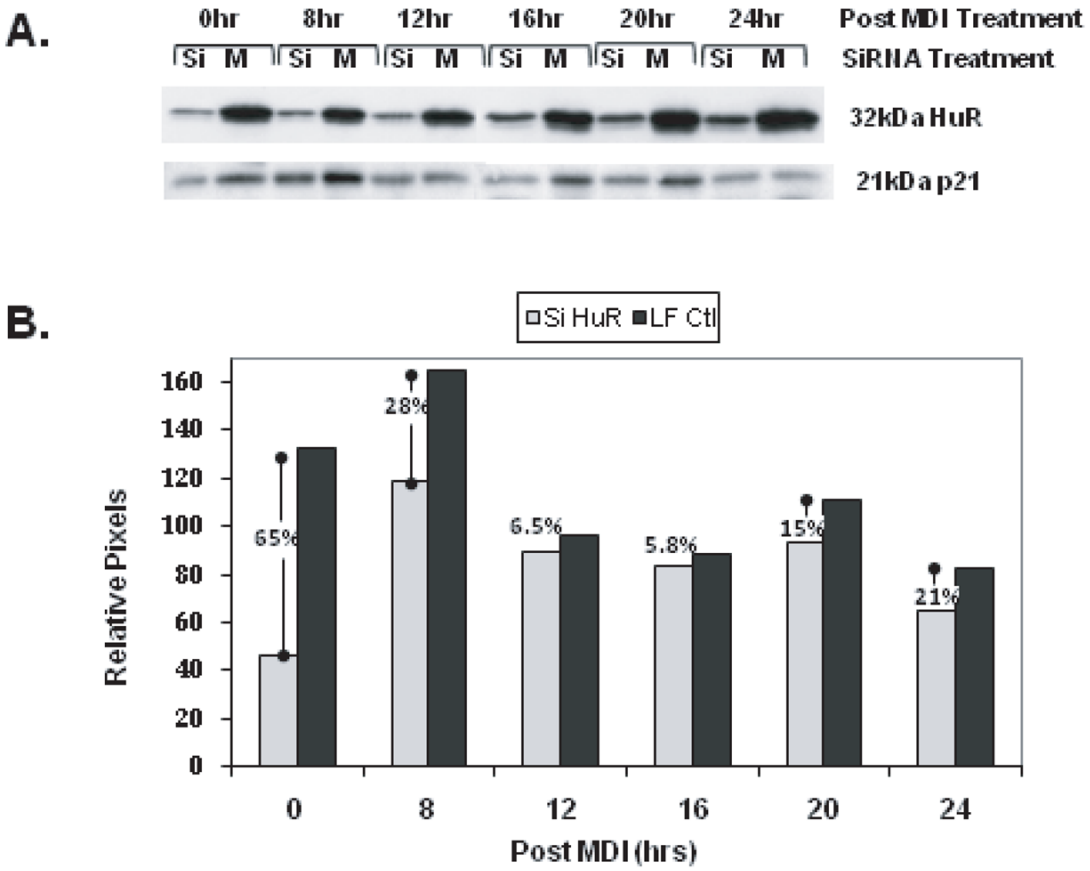

Fig. 4. siRNA mediated suppression of HuR leads to a transient suppression of p21

Cells were prepared for experimentation as detailed in the legend to Fig. 2. Extracts were prepared from siRNA and mock transfected cells and Western blot analysis performed for p21 (panel A.). Quantification was by scanning densitometry (Panel B.).