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HuR and miR-1192 regulate myogenesis by modulating the translation of *HMGB1* mRNA

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Abstract

Upon muscle injury the high mobility group box 1 (HMGB1) protein is up-regulated and secreted to initiate reparative responses. Here we show that HMGB1 controls myogenesis both *in vitro* and *in vivo,* during development and after adult muscle injury. HMGB1 expression in muscle cells is

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Author Contributions

V.D. and A.C. designed, carried out, analyzed and interpreted all of the *in vitro* experiments in the manuscript. B.C., S.B. and M.B. designed and carried out the *in vivo* experiments described in Figures 2, 3. X.J.L., K.V. and M.Z. assisted with some of the *in vitro* experiments. F.R., G.S. and R.D. R.D. designed and carried out the experiments described in Supplementary Figure S2 c,d as well as Supplementary Figure S10. R.D. helped writing part of the manuscript. B.W. provided technical expertise and helped in the analysis of microarray and sequencing experiments. S.D. helped in the design of several experiments in the manuscript. M.B. participated in writing and editing the manuscript. I.E.G. conceived and helped design all experiments in the manuscript, aided in the analysis of the data and wrote the manuscript.

Competing Financial Interests

M.E.B. is part owner of HMGBiotech, a company that provides reagents and services for HMGB1 research. The remaining authors declare no competing financial interests.

Accession codes

Microarray data has been deposited in Gene Expression Omnibus under accession code XXX.

regulated at the translational level: the miRNA miR-1192 inhibits HMGB1 translation and the RNA-binding protein HuR promotes it. HuR binds to a *cis*-element, HuRBS, located in the 3′UTR of the *HMGB1* transcript, and at the same time miR-1192 is recruited to an adjacent seed element. The binding of HuR to the HuRBS prevents the recruitment of Argonaute 2 (Ago2), overriding miR-1192-mediated translation inhibition. Depleting HuR reduces myoblast fusion and silencing miR-1192 re-establishes the fusion potential of HuR-depleted cells. We propose that HuR promotes the commitment of myoblasts to myogenesis by enhancing the translation of HMGB1 and suppressing the translation inhibition mediated by miR-1192.

Introduction

The process leading to muscle fiber formation during embryonic development, also known as myogenesis, involves the fusion of mononucleated myoblasts to form multinucleated myofibers [1]. Likewise, upon injury adult muscle tissues are repaired by satellite cells, which are quiescent mononucleated cells that coexist with myofibers [2]. In response to injuries, satellite cells are activated; they first proliferate and then exit the cell cycle to fuse and form muscle fiber [3–5]. During both embryonic and injury-induced myogenesis a cohort of intra- and extra-cellular factors act in concert.

HMGB1 (the high mobility group box 1) is a cytokine that is secreted by damaged muscle fibers and by infiltrating inflammatory cells after muscle injury. One of its main functions is to promote myogenesis by associating with the receptor for advanced glycation end products (RAGE), which is expressed on the surface of myoblasts, resulting in the activation of a signal transduction cascade that induces the expression of promyogenic factors such as MyoD and Myogenin [6–12]. It is also known that while HMGB1 is highly expressed in myoblasts or satellite cells, its level in muscle fibers is significantly reduced [3,9]. This suggests that maintaining a high expression level of HMGB1 during the early steps of myogenesis is required for the formation of functional myotubes. However, the mechanism controlling HMGB1 levels during myogenesis have never been investigated.

It has been shown that the 3′ untranslated region (3′UTR) of *HMGB1* mRNA is very long and contains elements that are uridyl(U)-rich [13]. U-rich elements in the 3′UTR are known to modulate posttranscriptional events such as the cellular movement, the turnover and the translation of many mRNAs [14,15]. The expression of mRNAs encoding MyoD and Myogenin is regulated posttranscriptionally. These mRNAs harbour AU-rich elements (AREs) located in their 3′UTRs that mediate their association with RNA-binding proteins (RBPs) such as HuR. This association is crucial for the stability and the expression of these messages during myogenesis [16,17]. Since HuR binds to *MyoD* and *Myogenin* mRNAs only during the transition state from myoblasts to myotubes but not at earlier stages [17], we concluded that HuR promotes myogenesis by stabilizing these mRNAs specifically at this later step during the myogenic process. However, knocking down the expression of HuR in undifferentiated muscle cells prevented their entry into the differentiation process [17]. Thus, HuR-dependent promyogenic activities could also involve modulating the expression of mRNA targets during the early steps of myogenesis.

In this study, we show that HMGB1 is required for myogenesis and that its expression in muscle cells is controlled at the translational level. Both miR-1192 and HuR associate with a U-rich element in the 3′UTR of the *HMGB1* mRNA. miR-1192 inhibits HMGB1 translation, but HuR promotes the translation of *HMGB1* mRNA by preventing the formation of Ago2/miR-1192 complex. We propose that HuR promotes the commitment of myoblasts to myogenesis by enhancing the translation of HMGB1 and suppressing the translation inhibition mediated by miR-1192.

Results

The HuR-mediated expression of HMGB1 promotes myogenesis

HuR modulates the expression of *MyoD* and *Myogenin* mRNAs in an ARE-dependent manner during the transition state from myoblasts to myotubes, but not at earlier stages [16–18]. To identify potential HuR mRNA targets during the early steps of myogenesis, we performed an immunoprecipitation (IP) experiment combined with cDNA microarray analysis on total extracts from undifferentiated C2C12 cells, a well-established murine myogenic cell line [19].

C2C12 cell extracts were immunoprecipitated with an anti-HuR or -IgG antibody. The RNAs associated with HuR were isolated and hybridized to mouse arrays. We revealed that HuR bound to 64 mRNAs in undifferentiated myoblasts (Supplementary Table S1). Among these messages, *HMGB1* and the *β-actin* mRNAs are known to encode proteins that directly affect muscle cell differentiation [9,10,20]. Since HuR associates with *MyoD* and *Myogenin* mRNAs only at later stages of the myogenic process [17,21] these messages were not on this list. While *β-actin* mRNA expression is known to depend on HuR[22], nothing is known regarding the link between HMGB1 expression, its promyogenic function and HuR protein. Using IP coupled with quantitative (q) RT-PCR (RT-qPCR) we validated the association between HuR and *HMGB1* mRNA in these cells (Supplementary Figs. S1a–b). Therefore, it is possible that HuR regulates HMGB1 expression during the early steps of myogenesis.

Several studies have suggested that the high expression level of HMGB1 in myoblasts is important for myogenesis [3,9]. Indeed, we observed that while HMGB1 mRNA and protein are highly expressed during the early steps of muscle cell differentiation, their expression decreases at later steps (Supplementary Fig. S2). Despite this, the role of HMGB1 in the commitment of muscle cells to myogenesis is still elusive. To address this question, we depleted the expression of HMGB1 or HuR in C2C12 cells and the efficiency of myogenesis was determined by assessing cell morphology by phase contrast, the expression of the Myosin heavy chain (My-HC) by immunofluorescence (IF), and by determining the fusion index [17] (Figs. 1a–e). We observed that HMGB1 depletion reduced the efficiency of muscle fiber formation by >85% (Figs. 1c–e). As expected [17] a similar reduction in myogenesis was also observed in C2C12 depleted of HuR. Western blot analysis showed that the levels of My-HC and to a lesser extent myoglobin were reduced due to the knockdown of HMGB1 or HuR (Fig. 1f). Interestingly, exposing C2C12 cells depleted of endogenous HMGB1 to 400nM of recombinant HMGB1 (rHMGB1) re-established their ability to enter the myogenic process (Supplementary Figs. S2c–d). These observations demonstrate that

HMGB1 promotes the early steps of myogenesis via its extracellular association with the RAGE receptor.

Next, we assessed the impact of HMGB1 on muscle development and growth *in vivo* using *Hmgb1* wild type (+/+), +/− and −/− mice. During vertebrate embryogenesis, skeletal muscle in the limb develops from progenitor cells originating in the somites [23]. Mice where the *Hmgb1* gene has been deleted die perinatally [24]. To assess the role of HMGB1 in mouse myogenesis, we therefore crossed *Hmgb1*$^{+/-}$ mice with homozygous MLC1/3F-nlacZ transgenic mice (wt for *Hmgb1*) [25] and collected the embryos at E10.5. As expected, half of the embryos were *Hmgb1*$^{+/+}$, whereas the other half was *Hmgb1*$^{+/-}$; all of them carried the MLC1/3F-nlacZ transgene and showed blue myonuclei when stained for X-gal (Fig. 2). We observed a significant reduction in the number of somites in *Hmgb1*$^{+/-}$ embryos when compared to wt controls (Fig. 2a). We then collected explants from wt, *Hmgb1*$^{+/-}$ and *Hmgb1*$^{-/-}$ embryos (E9.5), cultured them for 4 days [26] and determined the differentiation efficiency of myoblasts. We observed that *Hmgb1*−/− and +/− myoblasts formed significantly fewer myotubes than wt myoblasts (Fig. 2b). We next analyzed one-year old *Hmgb1*$^{+/-}$ mice to score for any reduction in muscle mass and cellularity when compared to wt mice. *Hmgb1*$^{+/-}$ mice showed a significant reduction in both total body weight and the mass of the Tibialis Anterior (TA) muscle (Fig. 2c). The myofibers in the TA muscle of *Hmgb1*$^{+/-}$ mice had a smaller cross-sectional area (XSA) than those of control mice (Figs. 2d–e). We also confirmed that mouse embryonic fibroblasts and muscle tissue from *Hmgb1*$^{+/-}$ *mice* express 50% less HMGB1 than their *Hmgb1*$^{+/+}$counterparts (Fig. 2f). Additionally, a muscle injury experiment showed a marked delay in the *Hmgb1*$^{+/-}$ mice in the regeneration process after injury as evidenced by the significant reduction in the XSA of regenerating fibers at both 7 and 14 days after injury (Fig. 3). Together, these data demonstrate that maintaining a high expression level of HMGB1 in myoblasts is required for embryonal myogenesis and muscle regeneration after acute injury because of its release from myoblasts and/or damaged myofibers[10,12,27].

HuR promotes the translation of the HMGB1 mRNA

The HuR protein is known to modulate the export, stability and/or the translation of its target mRNAs [14,28]. The fact that HuR plays a key role during the early steps of myogenesis [17] and associates with *HMGB1* mRNA in muscle cells (Supplementary Fig. S1) suggested that HuR promotes muscle differentiation by regulating *HMGB1* expression posttranscriptionally. Hence, we first determined whether HuR is required for the expression of HMGB1 in C2C12 cells. We observed that knocking down HuR in myoblasts reduced the levels of the HMGB1 protein (by >65%), but not its three mRNA isoforms (Figs. 4a–d). Pulse-chase mRNA stability experiments [22], RNA fluorescence *in situ* hybridization (FISH) [29] and cellular fractionation, indicated that HuR does not affect the cellular movement or the stability of *HMGB1* messages (Supplementary Figs. S3–4). These observations therefore suggest that HuR regulates the translation of the HMGB1 mRNA. Sucrose fractionation experiments were performed to assess this possibility. We observed that while the depletion of HuR had no significant effect on the general distribution profile of polysomes (Fig. 4e), knocking down HuR resulted in a shift in the distribution of *HMGB1* mRNA towards lighter polysome fractions when compared to siCtr-treated cells. These results indicated that the

HMGB1 transcript is less translated in the absence of HuR (Fig. 4f). Since HuR-mediated effects on target mRNAs in muscle cells have been linked to its ability to accumulate in the cytoplasm [30], we tested whether this could also be the case for *HMGB1* mRNA. To do this, we treated undifferentiated C2C12 cells with the HuR cleavage product 1 (HuR-CP1), which promotes the cytoplasmic accumulation of HuR during myogenesis [30]. We observed that HuR-CP1 increased HuR level in the cytoplasm of muscle cells and promoted the expression of the HMGB1 protein (Figs. 4g–h). Taken together, these results show that HuR maintains the high expression level of HMGB1 in muscle cells by promoting the translation of *HMGB1* mRNA.

HuR prevents miR-1192-mediated repression of HMGB1 translation

HuR affects the translation of target messages by associating with HuR binding sites (HuRBS) located at either the 5′ or 3′UTRs [14]. Sequence analysis revealed the presence of a U-rich element in the 3′UTR of the *HMGB1* mRNA that is similar to a HuRBS in the *β-actin* mRNA [22] (Fig. 5a). To assess whether this and/or other elements could mediate HuR binding, we performed RNA electromobility shift assays (REMSAs) using total extracts from C2C12 cells and thirteen radiolabeled cRNA probes that covered the entire 5′ and 3′ UTRs of *HMGB1* mRNA (Fig. 5a). All the probes except P6 and P8 formed RNA-protein complexes when incubated with total cell extract (Fig. 5b). However, an anti-HuR antibody only shifted RNA-complexes (HuR-C) containing the P4 but not the other probes (Fig. 5b). A pull-down experiment confirmed the specificity of HuR binding: biotinylated P4 associated with HuR but not with CUGBP1, an RNA-binding protein known to modulate muscle differentiation [31] (Supplementary Fig. S5a).

To delineate the HuRBS within P4, we divided this element into radiolabeled cRNA probes P4-1, -2 and -3 (Fig. 5c). A supershift containing the HuR-C was only generated with P4-1. A P4-1 mutant probe (mut-P4-1) in which every second U in the U-rich element (U_{15}) was changed to a C (Fig. 5d) also failed to form a complex when incubated with extracts in the presence of the anti-HuR-antibody. P4-HuR complexes were gradually competed away in the presence of an excess of unlabeled P-4 or P4-1 probes but not in the presence of an excess of unlabeled P4-3 or mut-P4-1 probes (Figs. 5e–f). These observations, together with the fact that recombinant GST-HuR but not GST alone was able to form an HuR-C (Supplementary Fig. S5b), demonstrate that the U_{15} element located between nucleotides (nt) 1218 and 1233 comprises the HuRBS in the *HMGB1*-3′UTR.

Previous studies have indicated that HuR either competes or collaborates with miRNAs in order to regulate the translation of some of its mRNA targets [32,33]. We investigated whether this could also be the case for HMGB1 expression in muscle cells. As a first step, we identified miRNAs that associate with HuR in muscle cells. C2C12 cell extracts were immunoprecipitated with an anti-HuR antibody as described above and the miRNAs associated with HuR were isolated and identified using miRNA arrays (Exiqon, USA). We identified 20 miRNAs that were immunoprecipitated two folds or more with the anti-HuR antibody when compared to the IgG control (Supplementary Table S2). Using the MSKCC (http://www.microrna.org/microrna/releaseNotes.do), the BiBiSer (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and the miRmap (http://mirmap.ezlab.org/app/)

websites, we identified that among these 20 HuR-miRNAs only miR-1192 is predicted to target the HMGB1 mRNA. miR-1192 stood out since the first seven Us (nt 1218-1225) of its "seed" sequence (miRNA binding site, miRBS) are part of the U-rich element containing the HuRBS (Figs. 6a–b). Additionally, our screening (RT-qPCR and sequencing) indicated that miR-1192 is expressed in C2C12 cells as well as in muscle and heart tissues, but not in lung, spleen, MEFs, or HeLa cells (Supplementary Fig. S6 and Table S3). Therefore, it was possible that this miRNA could contribute to the modulation of HMGB1 expression in muscle cells.

We first determined the impact of the miRBS and HuRBS on the translation of a reporter mRNA containing the HMGB1 3′UTR. For these experiments we used a *Renilla* luciferase (R Luc) reporter containing either a wt (R Luc-3′HMGB1) or a mutated 3′UTR with mutations in the seed element of miR-1192 (R Luc-3′HMGB1-mut-miRBS), or the HuRBS (R Luc-3′HMGB1-mut-HuRBS) (Fig. 6c). Of note, since all the 15 Us of HuRBS were mutated in R Luc-3′HMGB1-mut-HuRBS, this reporter should be unable to recruit *both* HuR and miR-1192 to the *HMGB1*-3′UTR. We transfected each one of the constructs in C2C12 cells depleted or not of HuR and measured luciferase activity [34] in comparison to the baseline activity of R Luc alone (Fig. 6d). In cells expressing endogenous HuR the luciferase activity of R Luc-3′HMGB1 was >60% higher than R Luc alone (Fig. 6d). Mutating the HuRBS (R Luc-3′HMGB1-mut-HuRBS) prevented this increase, confirming that the binding of HuR to the *HMGB1*-3′UTR actively promotes translation. Mutating the seed element of miR-1192 (R Luc-3′HMGB1-mut-miRBS) however, did not prevent the translation increase mediated by the *HMGB1*-3′UTR. Indeed, REMSA experiments demonstrated that HuR can bind to the HuRBS despite mutations in miR-1192 seed element (Supplementary Fig. S7). These results clearly indicate that the recruitment of HuR to the HuRBS is sufficient to promote the translation of HMGB1 in muscle cells.

Since the inhibition of HMGB1 translation occurs only in cells depleted of HuR (Fig. 4), we repeated the experiments described above in HuR-knockdown C2C12 cells. We observed that the R Luc-3′HMGB1 reporter, unlike the R Luc-3′HMGB1-mut-miRBS or R Luc-3′HMGB1-mut-HuRBS constructs, displayed a ~50% decrease in luciferase activity when compared to its control R Luc counterpart (Fig. 6d). Additionally, silencing miR-1192 by a miRNA silencer (antagomir anti-miR-1192) abrogated the decrease in the luciferase activity mediated by the *HMGB1*-3′UTR. No such effect was however seen in cells treated with a control antagomir against miR-16 (Anti-miR-16) (Fig. 6e and Supplementary Fig. S8a). Importantly, the concentration of antagomir used in these experiments did not affect the viability of C2C12 cells and the steady state levels of endogenous *HMGB1* mRNA (Supplementary Fig. S8b–d). We next generated mimics of miR-1192 with or without an A-to-C mutation in its 5th nucleotide (Fig. 6f). In HeLa cells, which do not express endogenous miR-1192 (Supplementary Fig. S6), the mimic miR-1192 reduced the luciferase activity of R Luc-3′HMGB1 by ~50%. The mutant miR-1192 mimic however, had no effect on this activity (Fig. 6f). The luciferase activity of the R Luc-3′HMGB1-mut-miRBS was not affected by the Mimic miR-1192, indicating that inhibition of R Luc-3′HMGB1 translation occurs via a direct binding to the miRBS. Collectively, these results demonstrate that in muscle cells miR-1192 directly inhibits the translation of *HMGB1* mRNA via the miRBS element, but this effect is overcome by the binding of HuR to its HuRBS.

Our IP combined to miRNA microarray data (Supplementary Table S2), which were validated by performing IP/RT-qPCR experiments (Fig. 6g) indicated that HuR and miR-1192 can coexist in the same complex in C2C12 cells. To determine whether this complex is indeed assembled on the *HMGB1*-3′UTR, we performed the same IP/RT-qPCR experiment on C2C12 cells expressing either *R Luc* alone, *R Luc-3′HMGB1* or the *R Luc-3′HMGB1-mut-miRBS* mRNAs. We observed that the level of miR-1192 in the HuR complex was five fold greater in cells expressing the *R Luc-3′HMGB1* mRNA when compared to cells expressing mRNAs deficient in their ability to bind miR-1192 (*R Luc* or *R Luc-3′HMGB1-mut-miRBS*) (Figs. 6h–I). Therefore, these observations clearly indicate that HuR and miR-1192 can bind simultaneously the 3′UTR of *HMGB1* mRNA. We next verified mechanistically how HuR could negate the ability of miR-1192 to repress the translation of the *HMGB1* mRNA. miRNAs are known to translationally repress mRNAs via the recruitment of the Ago2, a component of the RNA-induced silencing complex (RISC) [35]. We thus verified if HuR prevents the miR-1192 mediated recruitment of Ago2 to the HMGB1-miRBS. Our data indicate that although both HuR and miR-1192 simultaneously bind to the HMGB1 mRNA 3′UTR, the association of Ago2 to both miR-1192 and the *HMGB1* mRNA is increased when HuR is depleted from C2C12 cells (Fig. 7). These experiments, therefore, indicate that HuR prevents the miR-1192 mediated translational repression of the HMGB1 mRNA by interfering with the recruitment of Ago2.

Our data show that HMGB1 is one of the mRNA targets through which HuR promotes myogenesis. Specifically, the results outlined in Figure 6 suggest that HuR-depleted muscle cells fail to enter myogenesis because in the absence of HuR miR-1192 inhibits HMGB1 translation. Hence, silencing miR-1192 should rescue HMGB1 expression in HuR-depleted muscle cells and should also re-establish their myogenic potential. Our experiments showed that silencing miR-1192 but not miR-16 prevented the inhibition of HMGB1 translation in HuR-depleted C2C12 cells (Figs. 8a–b and Supplementary Figs. S9a). In the presence of endogenous HuR, however, Anti-miR-1192 had no effect on HMGB1 translation (Figs. 8a–b and Supplementary Figs. S9a). This result was further confirmed by the fact that while the depletion of HuR shifted the distribution of *HMGB1* mRNA towards lighter polysome fractions (Fig. 4f), silencing miR-1192 reversed this effect (Supplementary Fig. S9b). Next, we assessed whether the Anti-miR-1192-mediated rescue of HMGB1 translation is sufficient to promote the myogenic potential of HuR-knockdown cells. Indeed, silencing miR-1192 but not miR-16 reestablished the myogenic potential of HuR-depleted cells (Figs. 8c–d) and the expression levels of My-HC, myoglobin and myogenin (Fig. 8e).

It was previously shown that miRNAs such as miR-519 and miR-16 inhibit HuR expression in various cell lines [36–38]. However, since HuR levels does not significantly change during the early steps of myogenesis *in vitro* [16,17,39] and increase during muscle regeneration *in vivo* [30] (Supplementary Fig. S10), miRNA-mediated modulation of HuR expression in muscle cells seems to be ineffective in these conditions. Our experiments show that while the expression level of miR-16 increases during myogenesis both *in vitro* and *in vivo* (Supplementary Figs. S11a–b), it is the depletion but not the increased expression of miR-16 that modestly reduces the expression level of HuR, with limited effects on HMGB1 as well as My-HC and Myoglobin (Fig. 8e). In fact, a marked reduction of HMGB1 expression and the inhibition of myogenesis is observed only when HuR level is further reduced by siRNA

(Fig. 8e). These observations indicate that miR-1192 is active only when HuR level is below a critical threshold. All together, our data support a model whereby HuR helps undifferentiated muscle cells to enter myogenesis by supporting the translation of HMGB1 transcripts and preventing the action of Ago2/miR-1192 (Fig. 8f).

Discussion

In this study we show that HMGB1 is required for muscle fiber formation both *in vitro* and *in vivo* and uncover a novel mechanism by which muscle cells modulate HMGB1 expression. A general reduction in skeletal muscle tissues was previously observed in *Hmgb1*$^{-/-}$ mice, among several other defects [24]. Here, we show that a precise level of HMGB1 expression is required for proper muscle fiber formation, since a 50% reduction in HMGB1 expression in embryos significantly decreases the efficiency of myogenesis. Thus, a tight control of HMGB1 expression levels appears essential for myogenesis.

More than a decade ago it was discovered that *HMGB1* mRNA harbours a long 3′UTR with U-rich elements [40–42] suggesting that posttranscriptional events could regulate HMGB1 expression. Surprisingly, this possibility was never explored. Our data clearly establish that a posttranscriptional mechanism, via HuR, plays a key role in promoting HMGB1 expression in muscle cells. HuR, one of the well known posttranscriptional regulators, affects the expression of its target mRNAs by binding to specific U-rich-elements in their 3′UTRs [14,15]. We show that the 3′UTR of *HMGB1* mRNA harbours such an element, the HuRBS, through which HuR promotes the translation of *HMGB1* mRNA. In the absence of HuR, however, the microRNA miR-1192 is able to inhibit the expression of HMGB1. To our knowledge, this is the first report of the impact of a miRNA on HMGB1 expression. Incidentally, this is also the first report on the biological activity of miR-1192. Using several different techniques including miRNA microarray analysis, RT-qPCR with specific primers for miR-1192, and the sequencing of the PCR product that was generated by these primers, our data indicate that miR-1192 is expressed in muscle cells. Previous reports using RNA sequencing (RNA-seq), however, have failed to detect miR-1192 in the whole mouse embryo and muscle tissues[43,44]. As demonstrated by several recent studies [45,46], technical biases during capture and preparation of the small RNA library could impact the detection of small RNA molecules. Despite potential technical differences in methods used to precisely quantify the expression level of miR-1192 in muscle cells, our data clearly show that this miRNA is expressed in muscle cells and is functionally relevant in modulating HMGB1 expression.

Since HuR, HMGB1 and miR-1192 are highly expressed in muscle cells and during muscle regeneration [30] (Supplementary Figures S10, S11) and both HuR and miR-1192 can simultaneously associate with the *HMGB1*-3′UTR, our data support a model whereby the binding of HuR to its HuRBS is sufficient to prevent miR-1192-mediated inhibition. Interestingly, the association of miR-1192 with Ago2, a key player in the RISC complex [35,47], is dramatically enhanced in muscle cells depleted of HuR. This suggests that HuR prevents the action of miR-1192 by interfering with the recruitment of Ago2 to the *HMGB1* 3′UTR. These data are consistent with previous observations showing that HuR promotes the translation of target messages by preventing the assembly of an active RISC complex on

the Let-7 miRNA seed element [48] even if its binding site is far away from the miRNA seed element. Therefore, these observations with our data suggest that HuR promotes the translation of target mRNAs by interfering with the formation of an active RISC complex regardless of the distance of its binding site from the seed element. This mechanism of action is different from the one described for the dead end 1 protein (Dnd1), another RBP, which promotes translation of the the cyclin-kinase inhibitor $p27^{cip}$ by preventing the recruitment of miR-221/222 to their seed elements [49]. Therefore, our findings not only add another example to the few studies on the cross talk between RBPs and miRNAs, but also uncover a novel way by which cells, via proteins such as HuR, control miRNA-mediated effects. Our data demonstrate that via such a mechanism HuR induces the translation of HMGB1, which in turn promotes the entry of muscle cells into myogenesis.

By linking the interplay between HuR and miR-1192 to the translation modulation of HMGB1, we found a posttranscriptional mechanism that controls the expression of the main "alarmin" in the organism [50,51]. HMGB1 is a damage-associated molecular pattern (DAMP) molecule that upon severe injury activates the innate immune system to recognize tissue damage and initiate reparative responses [52,53]. Typically, the increase in HMGB1 levels in stressed or activated inflammatory cells precedes the upregulation of HMGB1 transcript levels by several hours [53]. Our results suggest that posttranscriptional events might be responsible for this increase since the *HMGB1* mRNAs are both abundant [40,41] and stable (Supplementary Figure S2). Therefore, our study may open the door to new strategies to manipulate HMGB1 levels under various conditions. Such a strategy could help promote the beneficial effects of HMGB1 (e.g. the activation of muscle regeneration and wound healing) while limiting/preventing the deleterious outcomes of its overproduction during life-threatening assaults (e.g. ischemia, burn, infection, or sepsis).

Methods

Plasmid construction

The pCMV-SPORT6 plasmid containing the full-length HMGB1 cDNA (Accession Number: BC008565) was purchased from Open Biosystems (Catalogue Number: MMM1013-64094). pRL-luc-3′HMGB1-mut-miRBS was generated by Norclone Biotech Laboratories, Kingston, ON, Canada. The full-length 3′UTR of mouse HMGB1 was subcloned into a pRL-SV40 vector (Promega) by performing PCR amplification using the following primers: forward 5′-TTG GTT CTA GCG CAG TTT TT-3′ and reverse 5′-TCA TCC AGG ACT CAT GTT CAG-3′. The pRL-SV40 vector was digested by Xba1 restriction enzyme (New England Biolabs), followed by a treatment with the T4 DNA polymerase, and then dephosphorylated. The PCR insert was ligated into the plasmid using the Quick Ligase enzyme (Promega) according to the manufacturer's instructions. pRL-luc-3′HMGB1-mutHuRBS was generated using a specific primer containing the mutations and Quickchange site-directed mutagenesis kit (Stratagene). The MISSION shRNA plasmid (siHMGB1; Sigma-Aldrich) was used to knock down HMGB1. MISSION pLKO.1-puro vector encoding scrambled shRNA (siCtr; SHC001) was used as a control.

Cell culture and transfection

C2C12 muscle cells (ATCC) were grown in media containing 20% fetal bovine serum (FBS, Invitrogen) in DMEM (Dulbecco's modified eagle medium from Invitrogen). In order to induce muscle cell differentiation, cells were switched to a media containing DMEM, 2% horse serum, penicillin/streptomycin antibiotics (Invitrogen), and 50mM HEPES, pH 7.4 (Invitrogen) when their confluency reached 100%[17,39]. *Hmgb1*$^{+/+}$ and *Hmgb1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (HMGBiotech, Milan, Italy) were cultured in DMEM supplemented with Penicillin/streptomycin and 10% FBS (Sigma-Aldrich). Transfections with siRNAs specific for HuR or plasmids were performed using Lipofectamine and Plus reagents (Invitrogen) or jetPEI (Polyplus Transfection) according to the manufacturer's instructions. The same transfection protocol was used to treat C2C12 cells with 200 nM miR-1192 or miR-16 antagomirs (Dharmacon).

HMGB1 rescue experiments

Transient transfection of C2C12 cells was carried out using jetPEI™ (Polyplus Transfection), as recommended by the manufacturer. Briefly, C2C12 cells were transfected with the MISSION shRNA plasmid (Sigma-Aldrich) to knock down HMGB1 or with the MISSION pLKO.1-puro vector encoding a scrambled shRNA (SHC001) as a control. 48 h post-transfection, the cells were switched to differentiation media and cultured for additional 48 h in the absence or presence of recombinant HMGB1 (400nM).

Preparation of cell extracts and immunoblotting

Total cell extracts were prepared by incubating undifferentiated or differentiated C2C12 cells on ice for 15 min with lysis buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton, 10 mM pyrophosphate sodium, 100 mM NaF, 1 mM EGTA, 1,5 mM $MgCl_2$, 1X protease inhibitor (Roche), 0,1 M orthovanadate, 0,2 M PMSF), then centrifuge at 12 000 rpm at 4°C and the supernatant was kept[17]. The extracts were run on an SDS-PAGE and transferred to nitrocellulose membranes (BIORAD). The samples were analyzed by western blotting [54] with antibodies against HuR (3A2,1:10000) [54], HMGB1 (Abcam, 1:1000), My-HC (MY32;Sigma,1:1000), Myogenin (F5D, Developmental studies Hybridoma Bank, 1:250) α-tubulin (Developmental studies Hybridoma Bank, 1:1000), hnRNPA1 (Cell Signalling, 1:1000) [22], β-actin (Sigma,1:500), myoglobin (DAKO, 1:500) and Ago2 (Cell Signalling,1:1000). Full blots are provided in Supplementary Figure S12.

Immunofluorescence

Immunofluorescence was performed using undifferentiated or differentiated C2C12 cells that were grown to sub-confluency in DMEM (Dulbecco's modified eagle medium from Invitrogen)[17]. After the appropriate experimental treatments, cells were rinsed twice in PBS, fixed in 3% phosphate-buffered paraformaldehyde (Sigma), and permeabilized in 0.5% PBS-goat serum with Triton. After permeabilization, cells were incubated with primary antibodies [against My-HC (MF-20, Developmental studies Hybridoma Bank, 1:1000), HuR (3A2,1:1000) or HMGB1 (Abcam,1:1000)] for 1 h at room temperature and then incubated with goat anti-mouse secondary antibodies conjugated with Rhodamine (*red*) or FITC (*green*) from Molecular Probes (Eugene, OR). To visualize the nucleus, cells were stained

with DAPI (Molecular Probes). Microscopic analyses were performed using an AXIOVERT 200M (Zeiss).

β-Galactosidase Staining

MLC1/3F-nlacZ homozygous transgenic mice [25] were mated with *Hmgb1*$^{+/-}$ heterozygotes. Pregnant females were sacrified by cervical dislocation at E10.5. Embryos were isolated in phosphate-buffered saline (PBS), fixed for 2 hours in 4% paraformaldehyde pH 7.4 at 4°C, washed in PBS for 20 min and equilibrated in 20% sucrose PBS. Fixed embryos were then stained for 1 hr at 37°C in 1mg/ml X-gal solution of PBS, also containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.2% NP40 PBS. *Hmgb1*$^{+/-}$ embryos were identified by PCR analysis of yolk sac DNA using the following primers: HMGB1for GCA GGC TTC GTT GTT TTC ATA CAG and HMGB1rev TCA AAG AGT AAT ACT GCC ACC TTC for the wt allele; NEOfor TGG TTT GCA GTG TTC TGC CTA GC and NEOrev CCC AGT CAT AGC CGA ATA GCC for the targeted allele. These animal studies were approved by the San Raffaele University Animal Care Committee.

Histology and morphometric analysis

Tibialis anterior (TA) muscles were dissected from 1-year old wild type and *Hmgb1*$^{+/-}$ mice and frozen in liquid-nitrogen-cooled isopentane. Serial sections (8 μm thick) were stained with hematoxylin and eosin (H&E). Morphometric analyses were performed on sections of TA using ImageJ to determine the cross-sectional area of 900 fibers for each group.

Fusion index

Explants of presomitic mesoderm (PSM)[55] were dissected from wt, *Hmgb1*$^{+/-}$ and *Hmgb1*$^{-/-}$ embryos at E9.5, plated on gelatin-coated dishes and cultured for four days in DMEM supplemented with 20% FCS and 50μg/ml gentamycin. On day 4, cultures were processed for immunoflorescence with an antibody specific for MyHC (MF20, Developmental Studies Hybridoma Bank, 1:20 dilution). Fusion index was determined as the number of nuclei in sarcomeric myosin-expressing cells with more than two nuclei versus the total number of nuclei.

Regeneration assay

Muscle injury was performed on the TA of 3-mo-old wt and *Hmgb1+/−* mice by injecting 50 μl of 10 μM CTX (three animals per group). Mice were sacrificed 3, 7 or 14 d after CTX injection, and the TA muscles were dissected and frozen in liquid N_2–cooled isopentane. Ten-μm serial muscle sections were stained with H&E. Alternatively, injury of C57BL/6 (Charles River) mouse muscles was performed by $BaCl_2$ injection in the TA muscle of 8-wk old mice, under zolazepam/tiletamine anesthesia [56]. These animal studies were approved by the San Raffaele University Animal Care Committee.

RNA electromobility shift assays

The *HMGB1* cRNA probes were produced by *in vitro* transcription using a T7 RNA polymerase [57]. All *HMGB1* probes (5′UTR and P1 to P12) were generated by PCR amplification using a forward primer fused to the T7 promoter as well as pCMV-HMGB1

expression vector as the template (see Supplementary Table S4). For smaller probes (P4-1 to P4-3), oligonucleotide sense and anti-sense were directly annealed and used for *in vitro* transcription. The RNA binding assays were performed[22] by incubating either 10 μg total cell extracts (TCE) or 300 ng purified recombinant protein (GST or GST-HuR) with 50 000 cpm of ^{32}P-labelled cRNAs in a total volume of 20 μl EBMK buffer (25 mM HEPES pH 7.6, 1.5 mM KCl, 5 mM $MgCl_2$, 75 mM NaCl, 6% sucrose and protease inhibitors) at RT for 15 minutes. For competition assays, 0.01X, 0.1X, 1X, 10X and 100X excess unlabeled specific or unspecific transcripts were incubated with the TCE for 15 minutes at RT before the ^{32}P-labelled probes were added to the reaction. 2 μl of a 50 mg/ml heparin sulphate stock solution were then added to the reaction for an additional 15 minutes at RT. In supershift experiments, 5 μg of a purified monoclonal anti-HuR antibody were then added to the reaction for an additional 15 minutes at RT. Samples were then loaded on a 4% polyacrylamide gel containing 0.05% NP-40.

Quantitative RT-PCR

One μg of total RNA was reverse transcribed using the M-MuLV Reverse Transcriptase (New England Biolabs) according to the manufacturer's protocol. 1/80 dilution of cDNA was used to detect *HMGB1, GAPDH* and *R luc* mRNAs using SsoFast™ EvaGreen® Supermix (Bio-Rad). Expression of *HMGB1* and *R luc* was standardized using *GAPDH* as a reference, and relative levels of expression were quantified by calculating $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is the difference in C_T between target and reference. For miRNA detection, 200 ng of total RNA was reverse transcribed using the Universal cDNA synthesis kit (Exiqon) and the presence of miR-1192, miR-16 and U6 was assessed by qPCR using the SyBr Green Master Mix (Exiqon).

Northern blot analysis and Actinomycin D pulse-chase experiments

The extraction of total RNA was performed using Trizol reagent (Invitrogen) [22]. For the isolation of miRNAs, twice as much isopropanol was used in the RNA purification protocol. Northern blot analysis was performed using 10 μg total RNA [22]. After transferring to a Hybond-N membrane (Amersham) and UV-cross-linking, the blot was hybridized with probes specific for *MyoD* [17], *GAPDH* [17], *18S* [58], *5.8S* [34] and *HMGB1* mRNAs were generated using the PCR Purification Kit (GE Healthcare) with primers described below. The probes were radiolabeled with α–^{32}P dCTP using Ready-to-Go DNA labeling beads (GE Healthcare) according to the manufacturer's instructions. The stability of *HMGB1* mRNA was assessed by the addition of the general transcriptional inhibitor actinomycin D (5 μg/ml)[58] for the indicated periods of time. Total RNA was isolated from the cells after 0, 4, 6, 8, 10 and 12 hours following ActD treatment using TRIzol reagent (Invitrogen), and analyzed by Northern blotting.

For miRNA detection, 25 μg of total RNA was separated on a 12% denaturating urea polyacrylamide gel. RNA was subsequently transferred to a Hybond-N^+ membrane (Amersham Biosciences) and cross-linked to the membrane. Hybridization was carried out by using ULTRAHybOligo solution according to the manufacturer's instructions (Ambion). The probe sequences were complementary to the mature forms of miR-16 or U6 RNA and

were labeled using StarFire system (Integrated DNA Technologies) according to the manufacturer's protocol.

Polysome fractionation

Forty million myoblasts were grown and treated with siRNAs as described above and polysome fractionation experiments were performed. Briefly, the cytoplasmic extracts obtained from lysed myoblast cells were centrifuged at 130,000 x *g* for 2 h on a sucrose gradient (10–50% w/v)[59,60]. Polysomal (P) or non-polysomal (NP) fractions were pooled and RNA was extracted using Trizol LS (Invitrogen). RNA samples were then analyzed on an agarose gel. The levels of *HMGB1* and *GAPDH* mRNAs were determined using quantitative RT-PCR.

Luciferase activity

The activity of *Renilla* luciferase was measured using a Renilla luciferase assay system (Promega) with a luminometer following the manufacturer's instructions.

Fluorescence in situ Hybridization

The fluorescence *in situ* hybridization experiments were performed[29] using a DNA fragment of ~500 bp corresponding to the coding region of mouse HMGB1. The fragment was amplified by PCR using the following primers fused to either a T7 or T3 minimal promoter sequence: HMGB1 forward, 5′-AAA AAG CCG AGA GGC AAA AT-3′, and HMGB1 reverse, 5′-CTT TTT CGC TGC ATC AGG TT-3′. The PCR product was used as the template for *in vitro* transcription of the *HMGB1* probe needed for fluorescence *in situ* hybridization. The antisense (T3) and sense (T7) probes were prepared using digoxigenin-RNA labeling mix from Roche Diagnostics. The RNA probes were quantified, denatured and incubated with permeabilized cells at 37°C overnight in the hybridization buffer (50% formamide, 5X SSC, 50 mM phosphate buffer, pH=7.4, 5X Denhardt's, 1 mM EDTA and 250 ng/μl of salmon sperm DNA). After the hybridization, the cells were used for immunofluorescence to detect the HMGB1 mRNA and HuR protein[29]. Finally, the cells were incubated with secondary goat anti-rabbit antibody and anti-DIG antibody for immunofluorescence.

Preparation of mRNA complexes and analysis with RT-PCR

Total cell extracts were prepared in lysis buffer (50 mM Tris, pH 8; 0.5% Triton X-100; 150 mM NaCl; complete protease inhibitor from Roche). 25 μl of the anti-HuR (3A2) or IgG (Jackson Immunoresearch Laboratories) antibodies were incubated with 200 μl of pre-swelled proteinA-Sepharose beads for 4 h at 4°C. Alternatively, C2C12 cells depleted or not of HuR were incubated with an anti-Ago2 (Cell Signalling) or IgG control antibody. After three washes in lysis buffer, 1.5 mg of cell extract was added overnight at 4°C. The final dilution of the antibodies during the incubation with lysates is 1/18. Beads were washed three times with cell lysis buffer, incubated with Proteinase K and the RNA isolated by phenol/cloroform extraction followed by precipitation overnight at −20°C with isopropanol [17,61]. Purified RNA was resuspended in 12 μl of water, and 2 μl was reverse transcribed using the M-MuLV Reverse Transcriptase (New England Biolabs) according to the

manufacturer's protocol. Association of *HMGB1* and *β-actin* mRNAs with HuR as well as *HMGB1* with Ago2 was defined using quantitative RT-PCR. Ten μl of purified RNA was reverse transcribed using the Universal cDNA synthesis kit (Exiqon) and the presence of miR-1192, miR-16 and U6 was assessed by qPCR.

cDNA array analysis

Microarray experiments were performed using mouse array, which contain 17,000 probe sets of known and unknown expressed sequence tags [62]. HuR was immunoprecipitated from exponentially growing C2C12 cells using an anti-HuR monoclonal antibody (3A2) and an anti-IgG monoclonal antibody as a negative control. The final dilution of the antibodies during the overnight incubation with lysates is 1/18. Then the samples were spun 5min at 3000 RPM and washed with TSE1 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH: 8.1, 150mM NaCl), TSE2 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH: 8.1, 500mM NaCl) and TSE3 (0.25M, LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH: 8.1) buffers. The immunoprecipitate were then incubated for 30 min at 55°C in 100ml NT2 buffer (50mM Tris pH:7.4, 150mM NaCl, 1mM $MgCl_2$, 0.05% NP-40) with0.1% SDS and 30mg proteinase K. The associated RNAs were extracted by adding ~15ml of 2M NaOAc pH: 4.0, 150 μl of water saturated phenol and ~30 μl chloroform followed by 15 min incubation on ice. After spinning (12000g for 20min) the aqueous phase is mixed with glycogen and isopropanol and incubated overnight at −20°C. The isolated RNAs were then resuspended in water and hybridized on cDNA arrays. The data were processed using the Array Pro software (Media Cybernetics, Inc.), then normalized by Z-score transformation [63] and used to calculate differences in signal intensities. Significant values were tested using a two-tailed Z-test and a P of ≤0.01. The data were calculated from two independent experiments.

Subcellular Fractionation

Subcellular fractionation was performed using the PARIS kit (Ambion) (Austin, TX) according the manufacturer's instructions. An equal number of cells per sample were used.

Biotin pull-down assay

The P4 fragment of the 3′UTR of mouse HMGB1 was subcloned into a pGEM-Teasy vector (Promega) according to the manufacturer's instructions by performing PCR amplification using the following primers: forward 5′-GCC ACT AAC CTT GCC TGG TA-3′ and reverse 5′-TCG TAT AAG CTG CAT CAG AGA CA-3′. The transcript is transcribed using the T7RiboMAX™ Express large scale RNA production system (Promega) according to the manufacturer's and the NotI digested PGEM-Teasy-P3P4 vector as a template. For preparing protein extracts, 30-40 millions of cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in 1mL of EBMK buffer (25mM Hepes pH 7,6; 5mM $MgCl_2$; 1,5mM KCl; 75mM NaCl; 175mM Sucrose) containing 0,5% NP-40, complete protease inhibitor without EDTA (Roche). Lysates were sonicated 10s 3 times at 200W and cleared by centrifugation at 12,000 rpm for 15 min. The protein concentration was determined (Bio-Rad microassay) and 1mg were used for each assay. Biotin pull-down assay is performed using Miltenyi Biotech mMACS streptavidin kit according to the manufacturer's instructions.

Primers used to prepare probes for Northern blot analysis

mHMGB1: For: 5′-GCA TCC TGG CTT ATC CAT TG-3′; Rev: 5′-TGC TCT TTT CAG CCT TGA CC-3′

mGAPDH: For: 5′-AAG GTC ATC CCA GAG CTG AA-3′; rev: 5′-AGG AGA CAA CCT GGT CCT CA-3′

Statistical analyses

The Statistical analyses in this study were performed using the Graphpad Prism5 software to determine significance (two tailed, Student's *t*-test).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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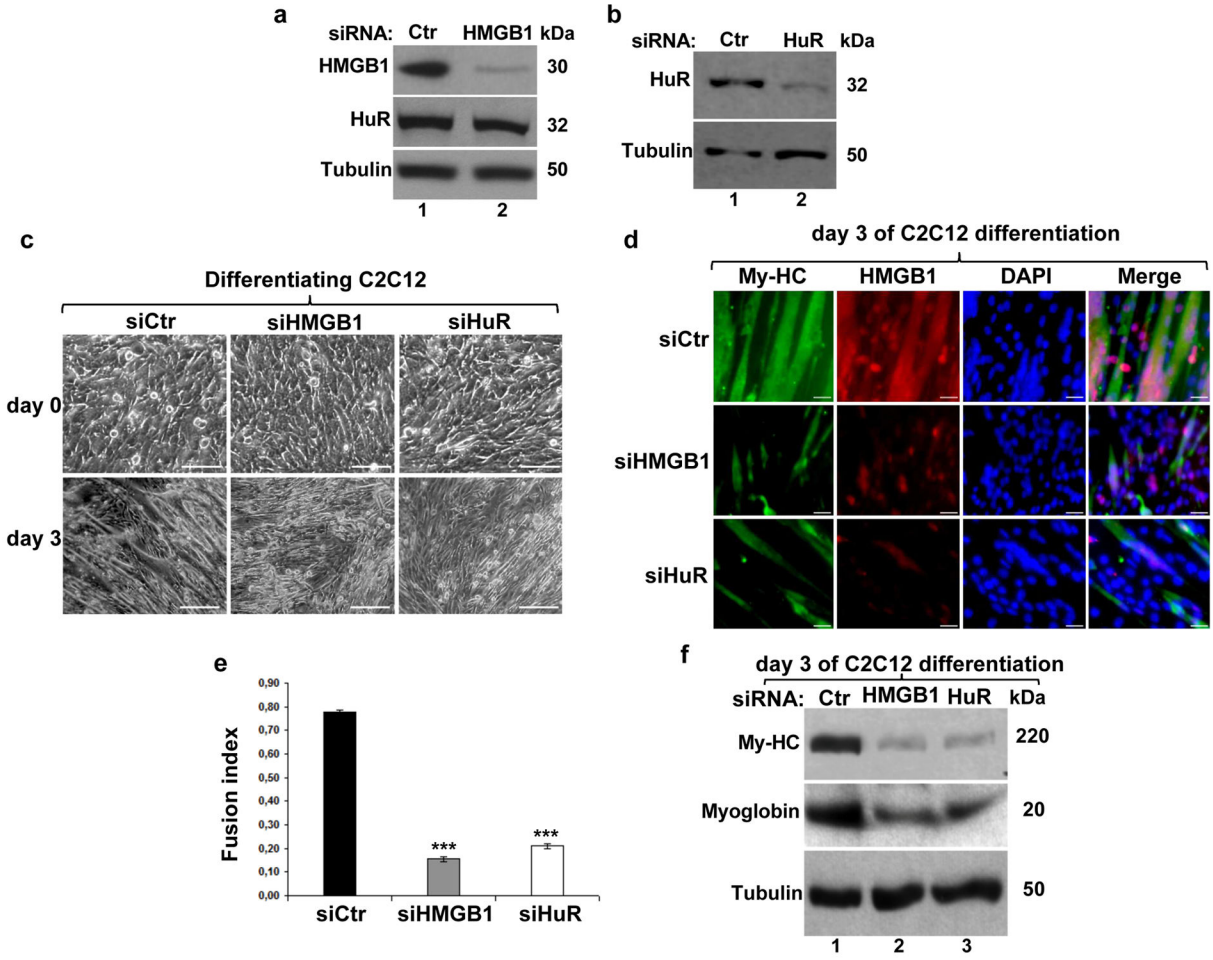

Figure 1. Knocking down HMGB1 expression in myoblasts prevents their entry into the myogenic process

(a–b) HuR and HMGB1 knockdown were performed in C2C12 cells and total cell lysates were prepared 48 hours post-transfection. Western blotting was performed using antibodies against HMGB1, HuR and α-tubulin as a loading control. **(c)** Phase contrast pictures showing the morphology of C2C12 cells transfected with control (siCtr), siHMGB1 or siHuR, at the time of differentiation induction (day 0) and at day 3. *Scale Bars, 50μm* **(d)** Immunofluorescence with anti-My-HC, anti-HMGB1 antibodies and DAPI was performed to determine the differentiation status of the C2C12 cells treated with the indicated siRNAs. *Scale Bars*, 20 μm. Representative images from three independent experiments are shown. **(e)** The fusion index indicating the efficiency of C2C12 differentiation was determined by calculating the number of nuclei in cells with more than 2 nuclei (myotubes) in relation to the total number of nuclei in each microscopic field. Data are presented as +/− S.E.M. of three independent experiments. ***$P<0.0001$ (t test). **(f)** Total cell extracts were prepared at day 3 of differentiation. Western blotting was performed using antibodies against My-HC, myoglobin, and α-tubulin as a loading control. Blots shown in a-b-f are representative of three independent experiments.

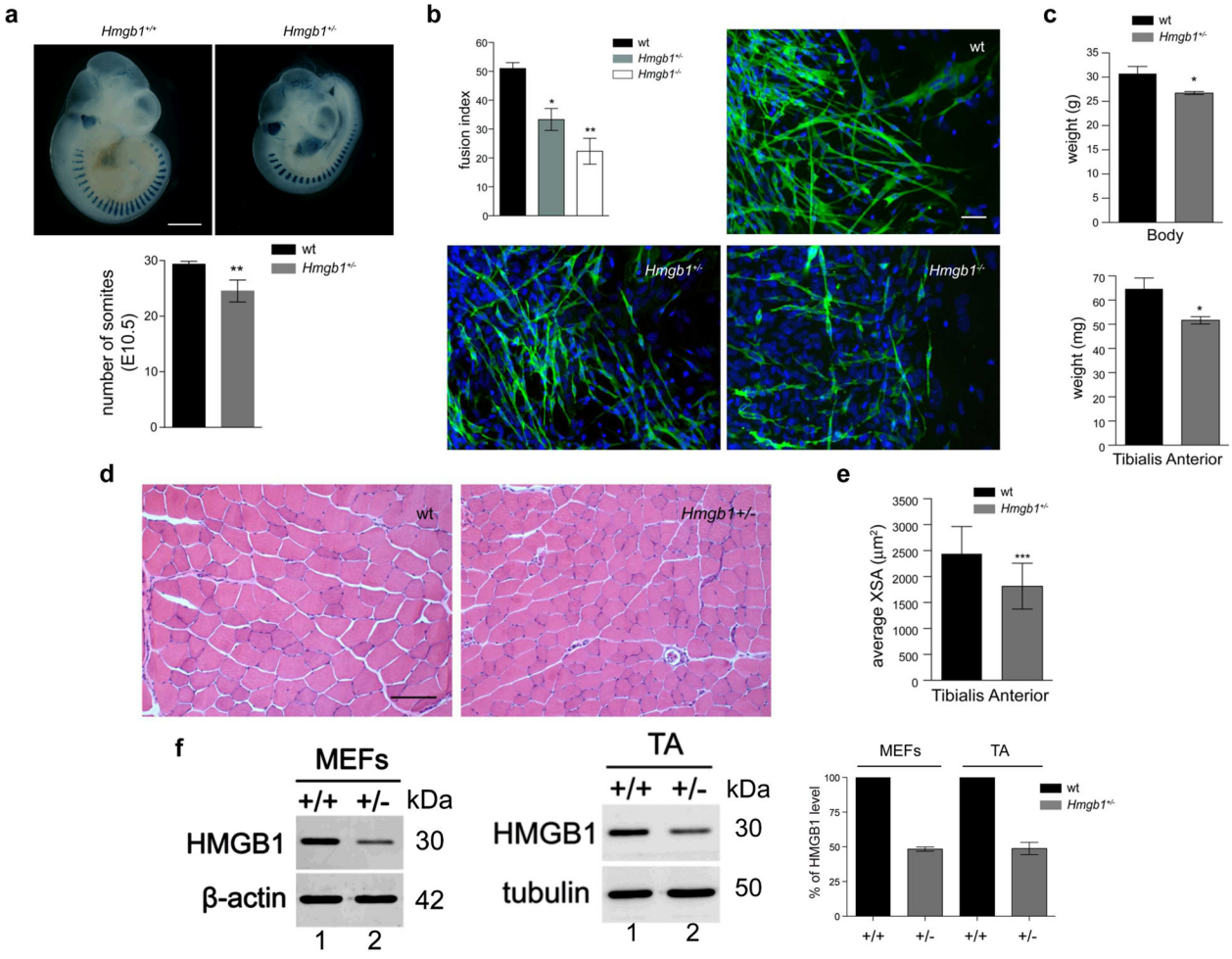

Figure 2. HMGB1 is required for proper muscle fiber formation in vivo

(a) *Top panel*: whole-mount X-Gal staining of control heterozygote MLC1/3F-nlacZ and double heterozygote MLC1/3F-nlacZ-*Hmgb1+/−* embryos collected at E10.5. *Scale Bars, 1mm. Bottom panel*: Histogram showing the average number of somites expressing MLC1/3F-nlacZ in control and double heterozygotes embryos at E10.5. ***P* <0.01 indicates a significant reduction in the number of transgene-expressing somites in *Hmgb1+/−* embryos compared to *Hmgb1+/+* (Data are presented as +/− SD, t test, n=6). **(b)** Presomitic mesoderm (PSM) explants were derived from wt, *Hmgb1+/−* and *Hmgb1−/−* embryos (E9.5), cultured for 4 days and analyzed by immunofluorescence with a specific antibody against My-HC (green) and DAPI counterstaining (blue). *Scale bar, 20 µm*. Fusion index was calculated as the number of nuclei in My-HC positive cells with more than 2 nuclei (myotubes) in relation to the total number of nuclei in each microscopic field. Data are presented as +/− S.E.M. of three independent experiments. **P*<0.05 and ***P*<0.01 (t test, n=3). **(c)** Body and Tibialis Anterior weights of 1-year old wt and *Hmgb1+/−* mice. Three animals were analyzed per group. Error bars represent SD. ***P*<0.01 (t test). **(d)** Histology of TA muscle. Representative images of H&E stained sections of TA muscles of 3-month old wt and *Hmgb1+/−* mice. *Scale bar, 100 µm*. **(e)** Mean cross-section area (XSA) of TA muscle fibers from 1-year old wt and *Hmgb1+/−* mice. Error bars represent SD. Nine

hundred fibers were analyzed for each group. ***P<0.001 vs wt (ANOVA). **(f)** Western blots for HMGB1 were performed on equal amounts of total extracts from wt (+/+) and heterozygous (+/−) Mouse Embryonic Fibroblasts (MEFs) and adult Tibialis Anterior (TA). β-actin and tubulin are shown as loading controls. The blots shown are representative of three independent experiments. Western blot signals were quantified with ImageQuant software (GE Healthcare) and plotted +/− SD from three independent experiments.

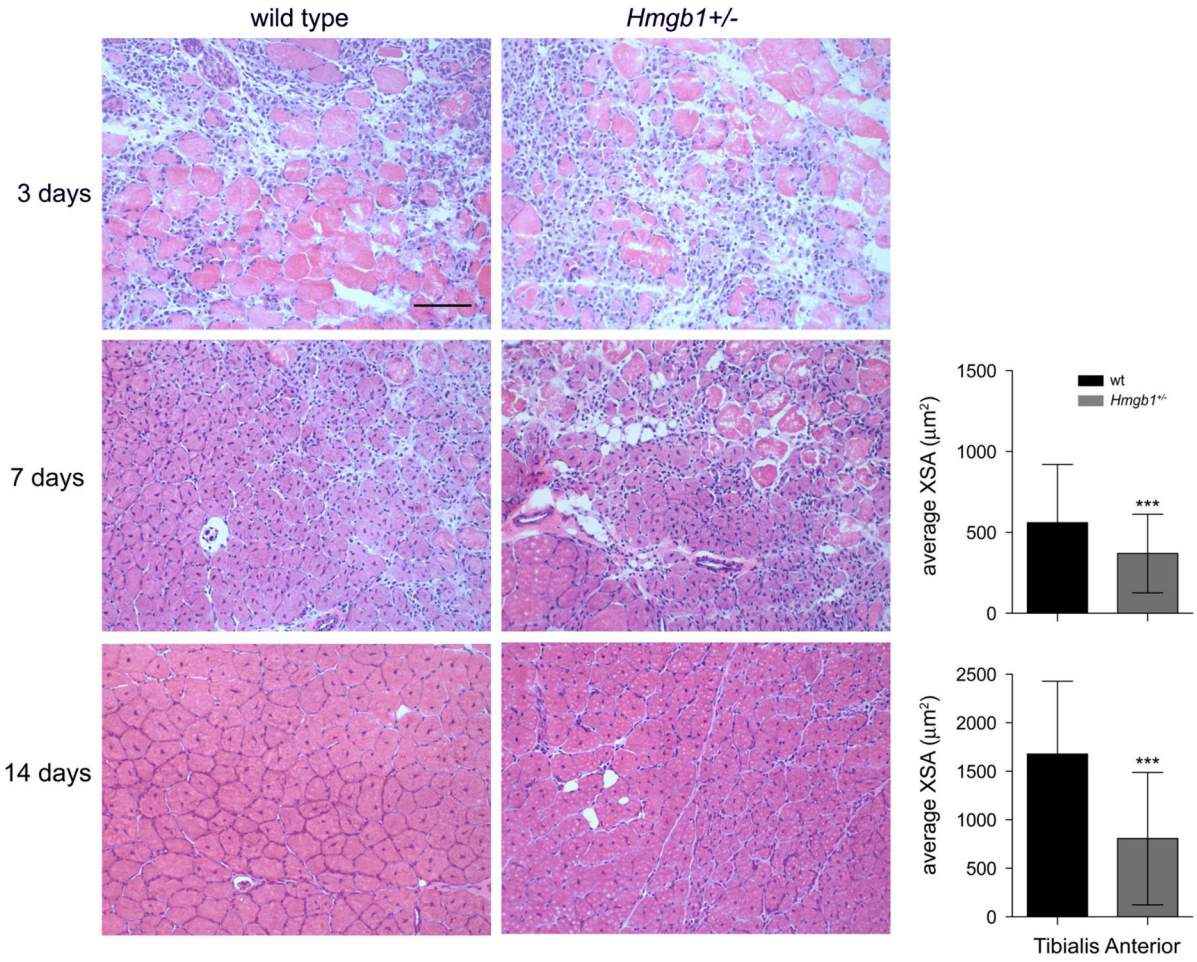

Figure 3. HMGB1 is required for normal muscle regeneration after injury

Representative sections of TA muscles from wt and *Hmgb1+/−* mice stained with H&E 3, 7 and 14 days after CTX injury. *Scale bar, 100 µm*. The cross-sectional area (XSA) of regenerating TA myofibers was decreased in *Hmgb1+/−* mice at both 7 and 14 days following injury. Nine hundred fibers were analyzed for each group (3 animals per group). Error bars in the histograms represent SD ***P<0.001 vs wt (ANOVA).

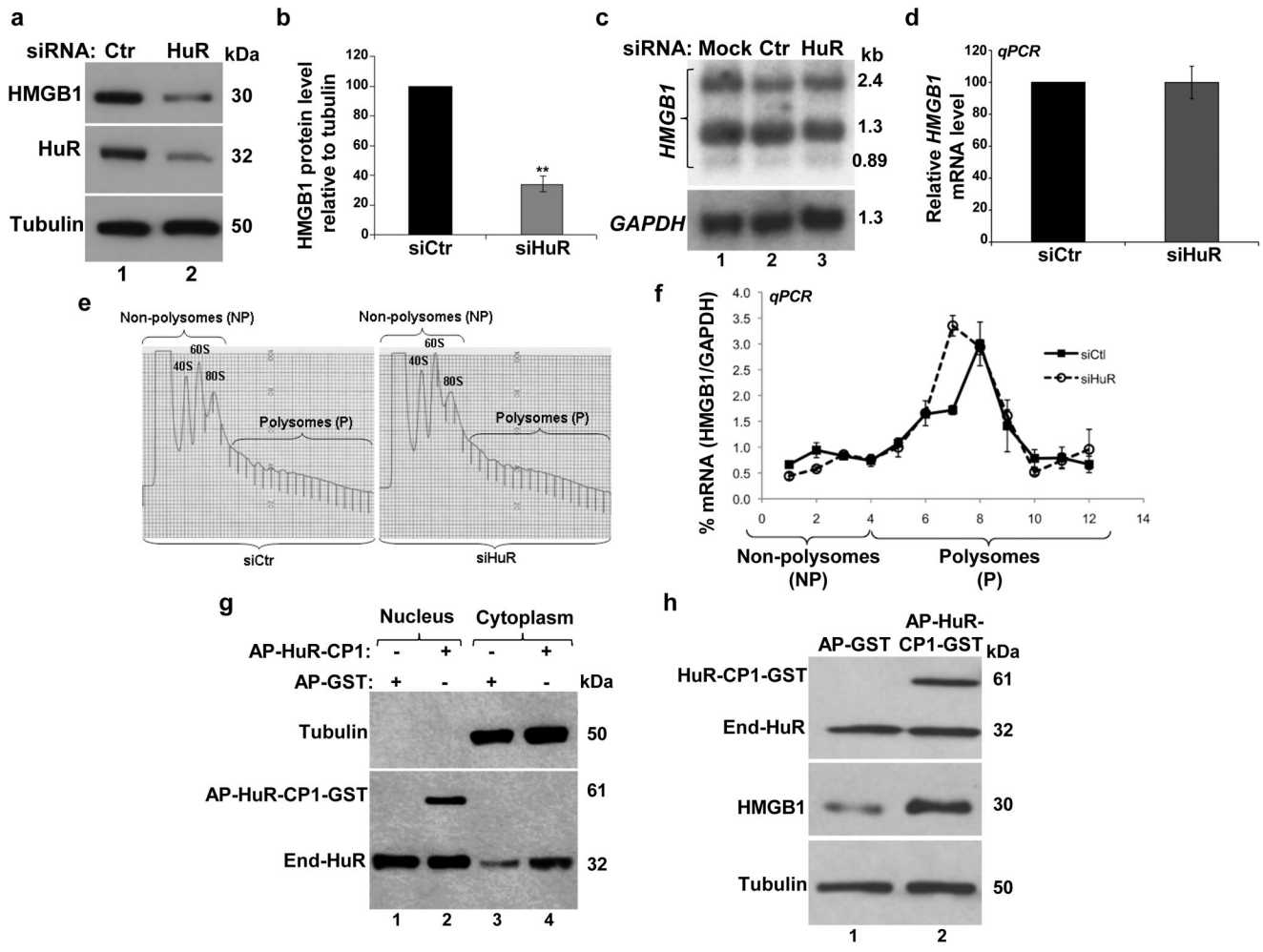

Figure 4. HuR promotes the translation of HMGB1 in C2C12 cells

(a) Total extracts from C2C12 cells treated with siHuR or siCtr were used for western blots with antibodies against HMGB1, HuR and α-tubulin as a loading control. **(b)** The HMGB1 protein level relative to α-tubulin for each treatment was plotted as the percentage relative to siCtr-treated sample +/− the S.E.M. of three independent experiments. ** $P<0.001$ (t test). **(c)** Northern blot of total RNA from C2C12 cells treated with siHuR or siCtr. A representative blot of three independent experiments is shown. **(d)** Total RNAs from siHuR or siCtr treated C2C12 cells were subjected to RT-qPCR analysis using specific primers for *HMGB1* and *GAPDH* mRNAs and plotted +/− the S.E.M from three independent experiments. **(e)** Sucrose gradient (15–50%) polysome fractionation of extracts from exponentially growing C2C12 cells that were treated with siHuR or siCtr. The profile of polysome distribution did not differ between C2C12 cells treated with siHuR or siCtr. **(f)** Quantitative RT-PCR was performed on the sucrose fractions using specific primers for *HMGB1* and *GAPDH* mRNAs. Error bars represent S.E.M. from four independent experiments. **(g–h)** C2C12 cells were treated with HuR-CP1 as described [30]. The nuclear and cytoplasmic fractions (g) or total extracts (h) were prepared and used for western blot analysis with anti-HuR, -HMGB1

and α-tubulin antibodies. All blots shown in g and h are representative of three independent experiments.

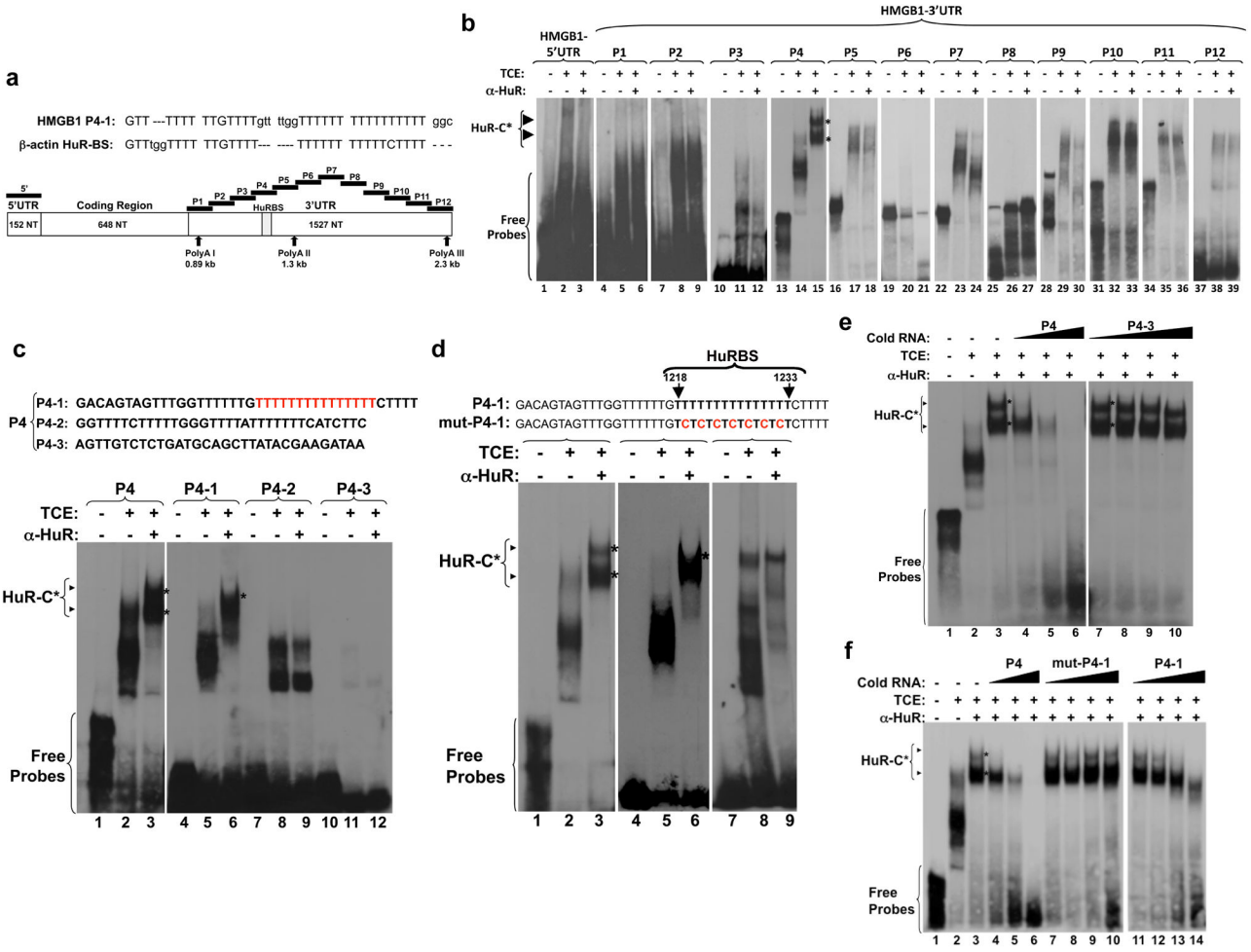

Figure 5. HuR specifically binds to a U-rich element within the HMGB1 3′UTR

(a) Upper panel: alignment of the U-rich HuR binding sites (HuRBS) within the *HMGB1* and *β-actin* 3′UTRs. Lower panel: schematic representation of the *HMGB1* mRNA sequence. The elements covering the 5′ and the 3′ (P1-P12) UTRs of *HMGB1* used to generate radiolabeled RNA probes for RNA electromobility shift assays are indicated. The accession number in the NCBI database of the *HMGB1* mRNA sequence used to generate these probes is NM_010439. **(b–f)** Representative gels of supershifts carried out by incubating total C2C12 cell extracts (TCE) with radiolabelled cRNA probes and anti-HuR antibody. Supershifted complexes (HuR-C) contain HuR protein. (b) Gel-shift assay performed using radiolabeled 5′UTR and the 12 probes (P1 to P12) depicted in (a). (c) Nucleotide sequence of the probes P4-1, P4-2 and P4-3 that were generated to localize HuR binding site (upper panel). These probes were used for gel-shift assays (lower panel). (d) Nucleotide sequences of probe P4-1 (HuRBS) and mut-P4-1, showing the T->C changes in mut-P4-1 (upper panel). The arrowhead shows HuR-C (lower panel). (e–f) Gel-shift competition was performed with radiolabeled probe P4 and 1X, 10X and 100X excess of the indicated unlabeled probes. All blots shown in b-f are representative of three independent experiments.

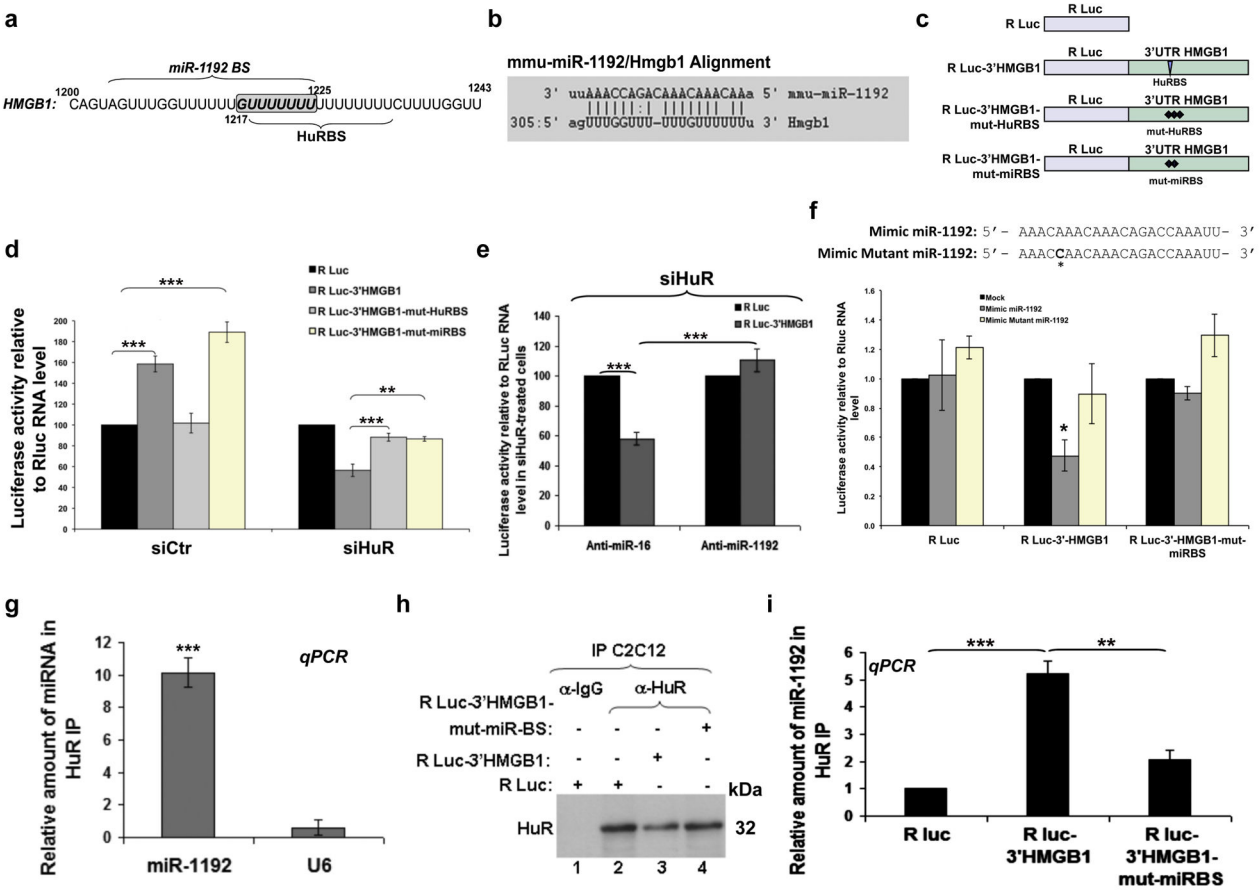

Figure 6. HuR binding to a U_8 element within HuRBS is sufficient to prevent the miR-1192-mediated inhibition of HMGB1 translation

(a) Schematic representation of the HuRBS and the seed element of miR-1192 (miR-1192 BS). **(b)** Diagram illustrating the bioinformatic approach used to predict miR-1192 as a putative miRNA targeting *HMGB1* 3′UTR. Shown here are complementarities of miR-1192 with *HMGB1* 3′UTR. **(c)** Schematic diagrams of luciferase constructs. (**d-g**) Effects of HuR, miRNAs, antagomirs, and mimics. Error bars represent S.E.M of three independent experiments. ***P<0.0001, **P<0.001, *P<0.05 (Student's t test). The amount of reporter RNA expressed in cells was determined by qPCR and used to normalize Renilla luciferase activities for each treatment. (d) Exponentially growing C2C12 cells were treated with siCtr or siHuR and 24 h later luciferase constructs were introduced. Luciferase activity of R Luc construct was considered as 100%. (e) miR-1192 and miR-16 antagomirs were transfected in siHuR-treated C2C12 cells previously transfected either with R luc alone or R luc-3′HMGB1. (f) Upper panel: Schematic representation of the Mimic miR-1192 and Mimic mutant miR-1192. The asterisk (*) indicates the A to C mutation in the Mimic mutant miR-1192. Lower panel: HeLa cells were transfected with Mimic miR-1192 or Mimic mutant miR-1192 and 24 h later luciferase constructs were transfected. The luciferase activity of the mock treated cells was set as reference. (g) Immunoprecipitation experiments were performed using a monoclonal HuR antibody, or IgG as a control, on total cell lysates from exponentially growing C2C12 cells. RNA was isolated from the immunoprecipitate,

and quantitative RT-PCR was performed using primers specific to miR-1192, miR-16 and U6 RNAs. The levels of miR-1192 and U6 RNAs in each IP were normalized against the level of miR-16. Error bars represent S.E.M of three independent experiments. t test was used for statistical analysis**. (h–i)** Immunoprecipitation experiments were performed as in (g) on total cell lysates from C2C12 cells transfected with either R-luc alone, R-luc-3′HMGB1 or R-luc-3′HMGB1-mut-miRBS. (h) Western blot was performed using an HuR antibody. This blot is a representation of three independent experiments. (i) The levels of miR-1192 in each IP were determined and are plotted with the S.E.M. of three independent experiments. ***P<0.0001, **P<0.001 (Student's t test).

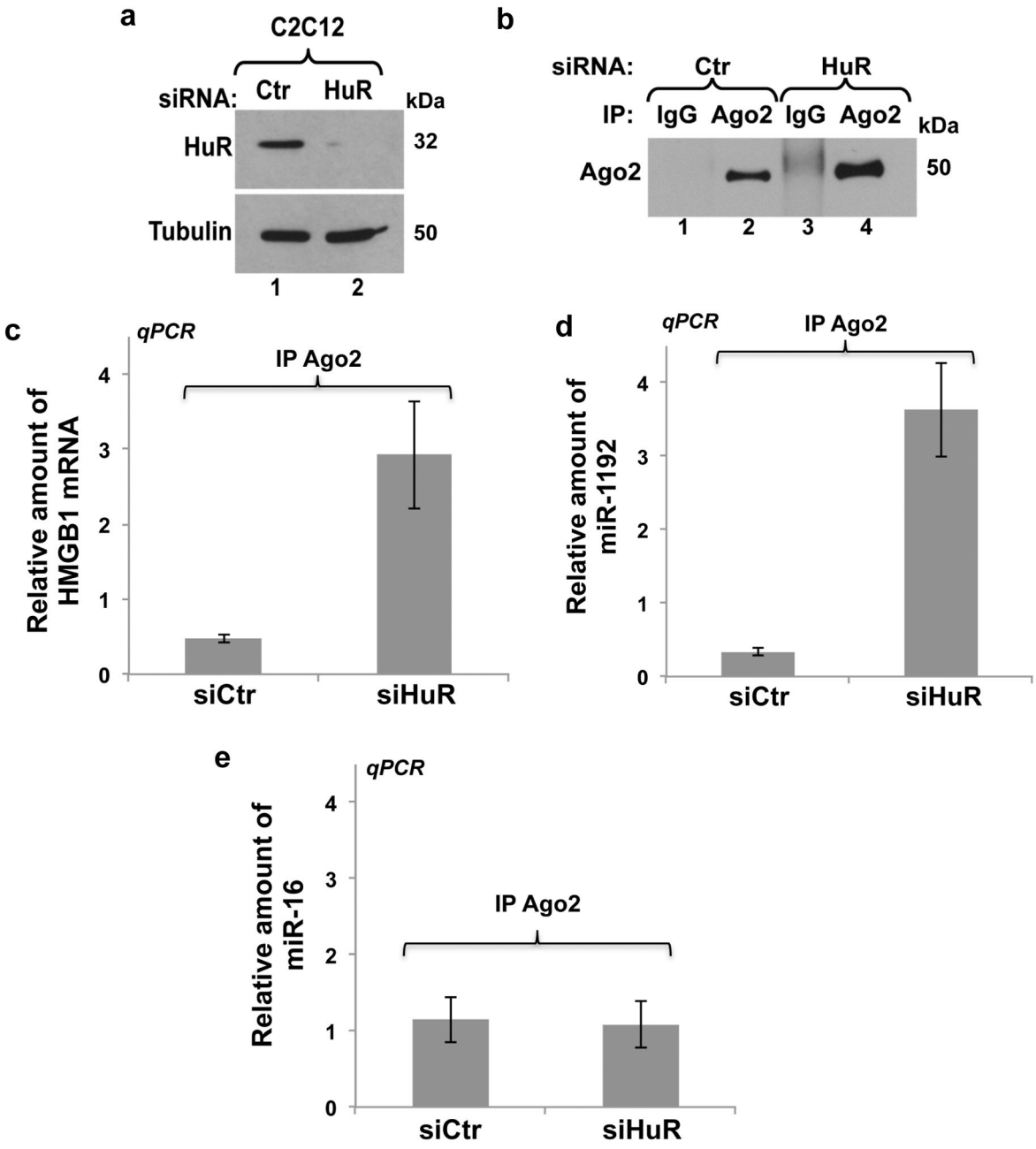

Figure 7. HuR binding to the HuRBS prevents the recruitment of Ago2 to the HMGB1 mRNA

(a) Western blot analysis was performed using antibodies against HuR and α-tubulin as a loading control on total cell extracts obtained from exponentially growing C2C12 cells treated with a control (Ctr) or HuR specific (siHuR) siRNA. **(b)** Immunoprecipitation experiments were performed using a monoclonal Ago2 antibody, or anti-IgG antibody as a control, on the total cell lysates described in (a). The immunoprecipitation of Ago2 was then assessed by western blotting using an anti-Ago2 antibody. **(c–e)** RNA was isolated from the immunoprecipitate described above, and quantitative RT-PCR was performed using primers specific to (c) HMGB1 (d) miR-1192 (e) miR-16. The levels of *HMGB1* mRNA, miR-1192

and miR-16 in each IP, relative to those in the IgG IP, were respectively normalized against the *GAPDH* mRNA and U6 levels. Error bars represent S.E.M of three independent experiments.

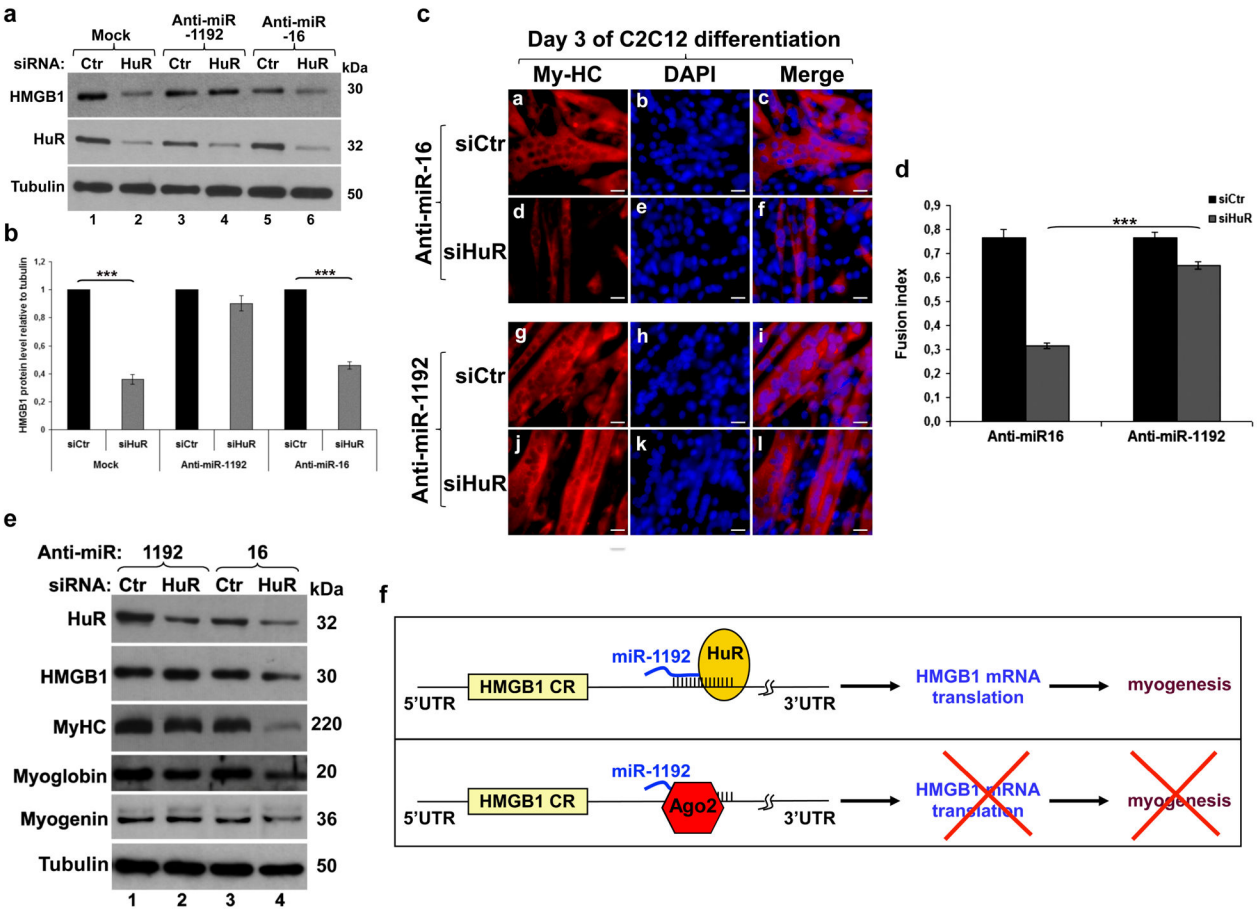

Figure 8. Silencing miR-1192 reestablishes HMGB1 translation and rescues the myogenic potential of HuR depleted muscle cells

(a) Exponentially growing C2C12 were treated with siCtr or siHuR and were transfected 5 h later with antagomirs to miR-1192 or miR-16. Extracts from these cells were harvested and used for western blotting with anti-HMGB1, -HuR and -α-tubulin antibodies. **(b)** The expression level of HMGB1 protein relative to α-tubulin. Error bars represent S.E.M. of three independent experiments. ***P<0.0001 (student's t test). **(c)** C2C12 treated as in (A) were grown to confluency, induced to differentiate for three days and stained with anti-My-HC antibody and DAPI. *Scale bars, 20 μm.* The images shown are representative fields for each cell treatment from three independent experiments. **(d)** Fusion index of cells in (c). Error bars represent S.E.M. of three independent experiments. ***P<0.0001 (student's t test). **(e)** Total extracts from cells treated as described above were blotted and probed with antibodies against HuR, HMGB1, My-HC, myoglobin, myogenin and α-tubulin. A representative blot of two independent experiments is shown. **(f)** Schematic model illustrating how HuR promotes the myogenesis in undifferentiated muscle cells. HuR recognizes an U_8 element in the *HMGB1*-3′UTR adjacent to the seed element of miR-1192, and promotes HMGB1 translation and myogenesis. HuR mediates this effect even if miR-1192 is bound to its seed element (upper panel). In the absence of HuR however,

miR-1192 recruits Ago2 thus inhibiting HMGB1 translation and preventing myogenesis (lower panel).