

Stau1 negatively regulates myogenic differentiation in C2C12 cells

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Sequential expression of myogenic regulatory factors (MRFs) such as MyoD and myogenin drives myogenic differentiation. Besides transcriptional activation of MRFs, this process is also coordinated by post-transcriptional regulation; MyoD and myogenin mRNAs are stabilized by RNA-binding protein HuR. Stau1 is known to regulate mRNA stability in a complex with Upf1, which is termed Stau1-mediated mRNA decay (SMD). We describe here that Stau1 is involved in the regulation of myogenesis. We found that knockdown of Stau1 promotes myogenesis including the expression of a muscle-specific marker protein, myoglobin, in C2C12 myoblasts. MyoD induces myogenin expression in response to induction of myogenesis, which is a key step to start myogenesis. The level of MyoD protein was not affected when Stau1 was depleted by siRNA, whereas the levels of myogenin mRNA and protein were increased in Stau1-knockdown cells. Interestingly, myogenin promoter activity was also increased in Stau1-knockdown cells in the absence of induction of myogenesis. Furthermore, Stau1-knockdown cells spontaneously progressed myogenesis including the expression of muscle-specific protein. Although Stau1 is involved in mRNA decay together with Upf1, Upf1-knockdown did not affect progression of myogenesis. Our results suggest that Stau1 negatively regulates myogenesis in C2C12 myoblasts through a mechanism that is different from SMD.

Introduction

Over the past two decades, lots of investigations have revealed that skeletal muscle development is coordinated by a set of transcription factors termed myogenic regulatory factors (MRFs) (Berkes & Tapscott 2005). MyoD is a first identified MRF that directs myogenic specification in several cultured non-muscle cells (Lassar *et al.* 1986; Davis *et al.* 1987; Weintraub *et al.* 1989). Following discovery of MyoD, Myf5, MRF4 and myogenin, genes encoding MRFs closely related to MyoD, have been found to regulate myogenic differentiation *in vitro* (Braun *et al.* 1989; Edmondson & Olson 1990; Miner & Wold 1990). Indeed, genetic studies have revealed roles of MRFs in muscle development *in vivo* (Hasty *et al.* 1993; Nabeshima *et al.* 1993; Rudnicki *et al.* 1993; Zhu & Miller 1997; Sumariwalla & Klein 2001; Kassam-Duchossoy *et al.* 2004). MyoD and Myf5 are expressed in proliferating myoblasts during muscle development *in vivo*. Mice deleted for

either MyoD or Myf5 does not exhibit apparent defects in muscle development, whereas disruption of both MyoD and Myf5 results in the loss of myoblasts and failure of muscle development, indicating that MyoD and Myf5 function redundantly in specification of muscle lineage (Rudnicki *et al.* 1993). On the other hand, in myogenin knockout mice, severe defects in myogenic differentiation are observed although myoblasts are present, suggesting that myogenin has a role in terminal differentiation (Hasty *et al.* 1993; Nabeshima *et al.* 1993). MRF4 has been reported to have roles in both early myogenic determination and terminal differentiation (Zhu & Miller 1997; Sumariwalla & Klein 2001; Kassam-Duchossoy *et al.* 2004). Thus MRFs are sequentially expressed at specific stage during myogenesis and promote the program of myogenic differentiation by transactivating target genes.

Regulation of mRNA stability is one of post-transcriptional mechanisms that control protein levels. It has been shown that the half-lives of MyoD and myogenin mRNAs are extended upon myogenic differentiation (Figueroa *et al.* 2003). HuR is an RNA-binding protein

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that associates with AU-rich element (ARE) in 3'-untranslated region (3'-UTR) of its target mRNAs and protects the target mRNAs from rapid mRNA degradation (Myer *et al.* 1997; Fan & Steitz 1998; Peng *et al.* 1998). Based on the observations that the half-lives of MyoD and myogenin mRNAs are extended by exogenous expression of HuR and that C2C12 myoblasts expressing exogenous HuR efficiently differentiate to myofiber, it has been suggested that HuR protects MyoD and myogenin mRNAs from mRNA decay during myogenesis (Figueroa *et al.* 2003). Indeed, HuR associates with ARE in 3'-UTR of MyoD and myogenin mRNAs and a depletion of HuR in C2C12 myoblasts results in a failure of myogenic differentiation (van der Giessen *et al.* 2003). Taken together, HuR contributes a progression of myogenesis by stabilizing the mRNAs encoding MRFs. MRFs induce not only muscle-specific genes but also a cell cycle inhibitor p21 during myogenesis, and this p21 induction by MRFs is required for exit from mitosis and normal muscle differentiation (Guo *et al.* 1995; Zhang *et al.* 1999). As well as MyoD and myogenin mRNAs, half-life of p21 mRNA is also regulated by HuR (Figueroa *et al.* 2003; van der Giessen *et al.* 2003). Another RNA-binding protein, NF90, is also known to be involved in expression of MyoD, myogenin and p21 *in vivo*. NF90 has two double-stranded RNA binding domains (dsRBDs) (Kao *et al.* 1994). In NF90 knockout mice, MyoD, myogenin and p21 mRNAs are reduced and disorganized arrangement of skeletal muscle are observed (Shi *et al.* 2005). NF90 stabilizes its target mRNAs by binding to ARE in 3'-UTR of the mRNAs (Shim *et al.* 2002), and NF90 binds to MyoD and p21 mRNAs in developing muscle (Shi *et al.* 2005), proposing a model that NF90 regulates myogenesis by stabilizing the mRNAs.

Drosophila Staufen is a conserved RNA-binding protein that possesses five dsRBDs. Staufen colocalizes with oskar mRNA at the posterior pole in oocyte and then observed at anterior pole together with bicoid mRNA when egg is laid. This process is required for proper anterior-posterior axis formation (St Johnston *et al.* 1991). During neuroblast cell division, Staufen colocalizes with prospero mRNA at apical cortex of neuroblast at interphase (Li *et al.* 1997; Broadus *et al.* 1998). Staufen mutant flies display aberrant mRNA localization, suggesting that Staufen regulates development via mRNA localization. In vertebrates, *Xenopus* Staufen orthologue is colocalized with Vg1 mRNA in oocyte and dominant-negative *Xenopus* Staufen blocks proper mRNA localization (Yoon & Mowry 2004). In mammals, two Staufen orthologs, Stau1 and Stau2, have been identified (Buchner *et al.* 1999; Kiebler *et al.* 1999; Marion *et al.* 1999; Wickham *et al.* 1999). Human Stau1 associates

with polysomes and localizes in rough endoplasmic reticulum (Marion *et al.* 1999; Wickham *et al.* 1999). In neurons, Stau1 is localized in dendrites and found in kinesin-associated mRNA granules, suggesting that Stau1 is involved in mRNA transport (Kiebler *et al.* 1999; Kohrmann *et al.* 1999; Kanai *et al.* 2004). Furthermore, a novel function of Stau1 has been achieved by analysis of functional interaction with Upf1 (Kim *et al.* 2005, 2007). Upf1 is a component of mRNA decay machinery that is involved in non-sense mRNA decay (NMD) in a complex with Upf2 and Upf3 (Hentze & Kulozik 1999; Isken & Maquat 2007). Stau1 forms a complex with Upf1, but not Upf2 or Upf3, and promotes mRNA decay, which is distinct from canonical NMD and is termed Stau1-mediated mRNA decay (SMD) (Kim *et al.* 2005). It has also been shown that Stau1 is a component of the postsynaptic apparatus in muscle and that expression of Stau1 is increased during myogenic differentiation (Belanger *et al.* 2003). Upf1 is also up-regulated during myogenic differentiation as well as Stau1 and SMD activity is increased in differentiated myotubes (Kim *et al.* 2007). Thus Stau1 and SMD are implicated in myogenesis; however its physiological significance still remains unknown.

In this manuscript, we addressed a possible involvement of Stau1 in myogenesis in C2C12 myoblasts. We found that knockdown of Stau1 allowed myoblasts to spontaneously progress myogenic differentiation in the absence of induction of myogenesis. On the other hand, Upf1 knockdown did not progress the myogenesis. Our findings indicate that Stau1 negatively regulates myogenesis in C2C12 myoblasts by an Upf1-independent mechanism.

Results

Knockdown of Stau1 enhanced myogenesis in C2C12 myoblasts

It has been shown that Stau1 is increased upon myogenesis in C2C12 myoblasts (Belanger *et al.* 2003; Kim *et al.* 2007), arguing a hypothetical role of Stau1 in myogenic differentiation. To explore a possible involvement of Stau1 in myogenesis, we generated Stau1-knockdown C2C12 myoblasts. In our experimental condition, both Stau1 mRNA and protein were expressed in growing C2C12 myoblasts at detectable level (Fig. 1A,C, control). C2C12 myoblasts were transfected with control or Stau1 siRNA and then the levels of endogenous Stau1 mRNA and protein were examined. As shown in Fig. 1A, Stau1 mRNA was significantly reduced in cells transfected with Stau1 siRNA as compared with control cells, whereas GAPDH mRNA was detected at similar level in both cells, indicating that Stau1 siRNA specifically

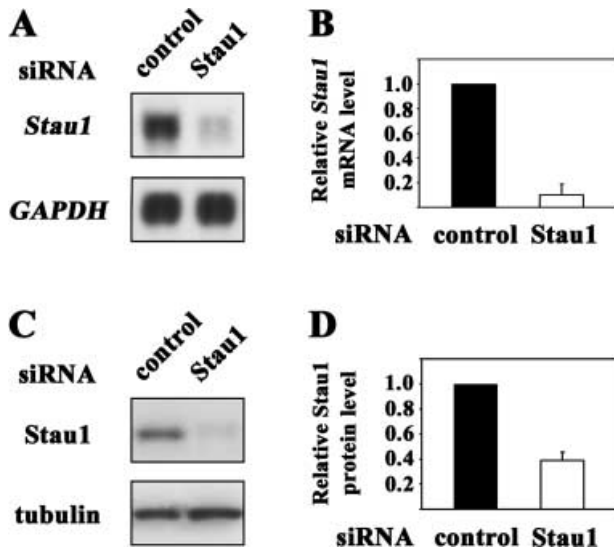


Figure 1 Knockdown of Stau1 in C2C12 myoblasts. (A), (B) Stau1 mRNA level. C2C12 myoblasts were transfected with control or Stau1 siRNA. Forty-eight hours after transfection, total RNAs were extracted from cells and subjected to Northern blot analysis with a specific probe for Stau1 or GAPDH. Three independent experiments were performed and relative mRNA level of Stau1 normalized by that of GAPDH was represented in (B). (C), (D) Stau1 protein level. Total cell lysates were extracted from cells 48 h after transfection of siRNA. The protein level of Stau1 was examined by Western blotting with anti-Stau1 antiserum. Tubulin was detected as a loading control. Three independent experiments were performed and relative protein level of Stau1 normalized by that of tubulin was represented in (D).

reduced endogenous Stau1 mRNA. Consistent with the reduction of Stau1 mRNA, the level of Stau1 protein was also reduced in cells transfected with siRNA for Stau1 as compared with control cells (Fig. 1C,D). Both Stau1 mRNA and protein were reduced < 40% in Stau1-knockdown cells (Fig. 1B,D). Stau1-knockdown cells showed no obvious change in growth rate (data not shown). To assess the effect of Stau1 knockdown on myogenesis, control and Stau1-knockdown cells were cultured until confluence and then induced to differentiate into myotubes (Fig. 2). In control cells, myotubes expressing a muscle-specific marker protein, myoglobin, were rarely detected in control cells at post-induction day 2, whereas lots of myoglobin-positive cells were observed in Stau1-knockdown cells at post-induction day 2 (Fig. 2A). Statistical analysis revealed that 20% of Stau1-knockdown cells expressed myoglobin at post-induction day 2, whereas only 3% of control cells were myoglobin-positive (Fig. 2B). To confirm the effect of Stau1 knockdown on myoglobin expression, the level of

myoglobin protein was examined by Western blotting. Consistent with the results by immunostaining, the level of myoglobin protein was significantly increased in Stau1-knockdown cells as compared with control cells (Fig. 2C,D). We further examined the effect of in Stau1 knockdown on myoglobin expression by using another Stau1 siRNA and confirmed that another Stau1 siRNA had a similar effect (data not shown). In addition, expression of the siRNA-resistant form of Stau1 complemented the effect of Stau1 knockdown (see below). These results indicate that knockdown of Stau1 promotes myogenic differentiation in C2C12 myoblasts.

Myogenin was expressed in Stau1-knockdown cells prior to induction of myogenesis

As the expression of a muscle-specific marker protein myoglobin was increased in Stau1-knockdown cells, we next analyzed the effect of Stau1-knockdown on earlier events of myogenic differentiation program. It has been shown that myogenesis is regulated by sequential expression of MRFs such as MyoD and myogenin. MyoD is expressed in proliferating myoblasts and induces the expression of myogenin upon muscle differentiation, which promotes terminal differentiation (Berkes & Tapscott 2005). To examine whether knockdown of Stau1 affects to an early step in myogenic differentiation program, we analyzed the expression of MyoD and myogenin in Stau1-knockdown cells (Fig. 3). Total cell extracts were prepared from control or Stau1-knockdown cells before and after induction of myogenesis, and then analyzed by Western blotting. In control cells, MyoD was expressed prior to induction of myogenesis and myogenin was induced in response to the induction. MyoD was detected at a similar level in control and Stau1-knockdown cells before and after induction (Fig. 3A,B). In contrast, myogenin was expressed prior to induction in Stau1-knockdown cells at similar level to that in control cells after induction (Fig. 3A,C). These results indicate that myogenic differentiation program is promoted in Stau1-knockdown cells without induction of myogenesis.

Myogenin promoter activity was increased in Stau1-knockdown cells

It has been reported that myogenin expression is regulated at both transcriptional and post-transcriptional level (Figueroa *et al.* 2003; van der Giessen *et al.* 2003). To investigate how myogenin protein level was increased in Stau1-knockdown cells, we first analyzed the level of myogenin mRNA. In control cells, myogenin mRNA was not detected prior to induction of myogenesis and

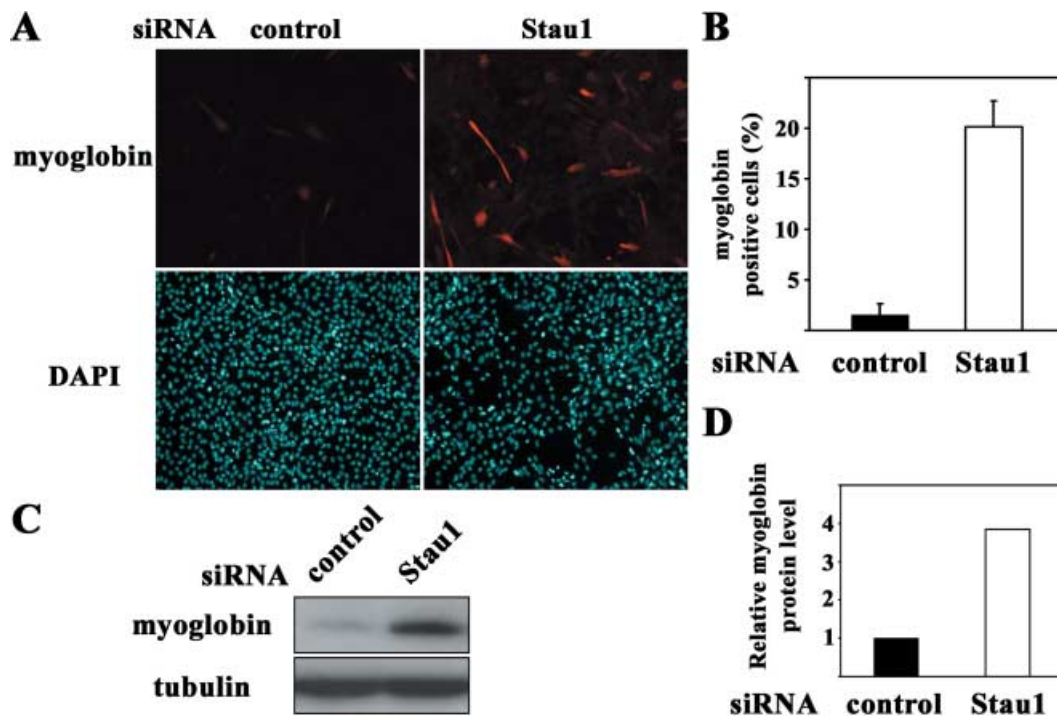


Figure 2 Effect of Stau1 knockdown on myogenesis. (A), (B) Immunostaining of myoglobin in Stau1-knockdown cells. Control or Stau1-knockdown cells were grown to confluence in growth medium and then cultured in differentiation medium to induce myogenesis. Two days after induction, myoglobin was detected by immunohistochemistry. Three fields (more than 1000 cells) were examined and percentage of myoglobin-positive cells was represented in (B). (C), (D) Western blotting of myoglobin in Stau1-knockdown cells. The level of myoglobin protein shown in (C) was normalized by tubulin and relative amount of myoglobin was represented in (D).

detected in response to the induction (Fig. 4A,B). Consistent with the result of myogenin protein, myogenin mRNA was also detected in Stau1-knockdown cells prior to induction at similar level to that in control cells after induction (Fig. 4A,B).

To determine whether the increase of myogenin mRNA in Stau1-knockdown cells result from activation of myogenin promoter, we next examined the activity of myogenin promoter using reporter construct containing myogenin proximal promoter. In control cells, the transcriptional activity of myogenin promoter was activated about fourfold upon induction of myogenesis (Fig. 4C). In Stau1-knockdown cells, myogenin promoter activity was increased about fourfold as compared with control cells before and after induction of myogenesis (Fig. 4C). These results suggest that Stau1 negatively regulates myogenesis by inhibiting the event prior to the activation of myogenin promoter.

Stau1-knockdown cells differentiated into myotubes in the absence of induction of myogenesis

Since activation of myogenin promoter, a key step for myogenesis, was observed in Stau1-knockdown cells in

the absence of induction, we next examined whether Stau1-knockdown cells differentiated into myotubes without induction. Control or Stau1-knockdown cells were maintained in growth medium for 2 days and then progression of myogenesis was evaluated by the expression of a muscle-specific marker myoglobin. As shown in Fig. 5A, myoglobin-positive cells were rarely detected in control cells without induction. In contrast, 10% of Stau1-knockdown cells expressed myoglobin in growth medium (Fig. 5A,B). We further confirmed the expression of myoglobin in Stau1-knockdown cells by Western blotting. Consistent with the results by immunostaining, myoglobin protein was detected in Stau1-knockdown cells but not in control cells in growth medium (Fig. 5C). These results indicate that Stau1-knockdown results in the expression of myogenin and allows myoblasts to differentiate in the absence of induction of myogenesis.

Stau1-knockdown cells differentiated into myotubes in an Upf1-independent mechanism

It has been shown that Stau1 associates with Upf1 and promotes mRNA decay and activity of SMD is

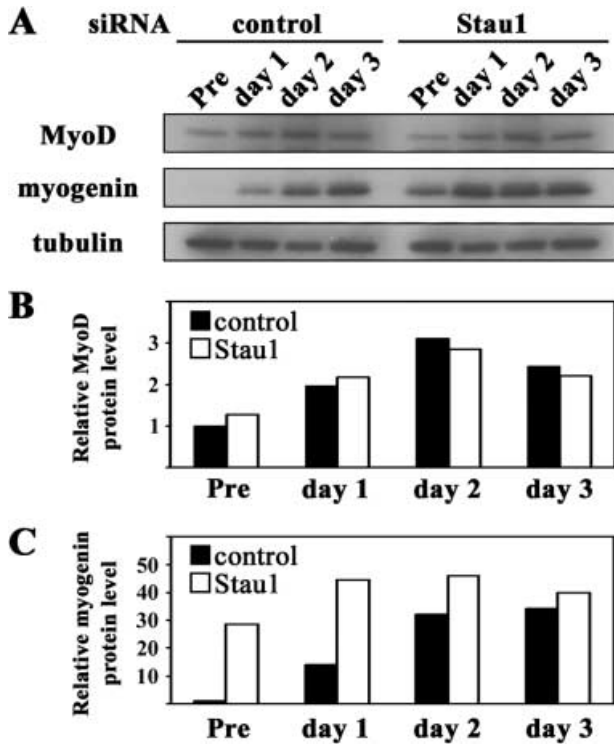


Figure 3 Expression of MyoD and myogenin proteins in Stau1-Knockdown cells during myogenesis. (A) C2C12 myoblasts transfected with control or Stau1 siRNA were grown to confluence. Total cell lysates were extracted from cells prior to induction (Pre) and after induction at indicated days (day 1–3). MyoD and myogenin proteins were detected by Western blotting. (B), (C) The level of MyoD and myogenin proteins shown in panel A were normalized by that of tubulin and values were represented in (B) and (C), respectively.

up-regulated in differentiated myotubes (Kim *et al.* 2007). To test whether Upf1 is involved in negative regulation of myogenesis together with Stau1, we generated Upf1-knockdown cells. C2C12 myoblasts were transfected with siRNA for Upf1. Endogenous Upf1 was specifically reduced in cells transfected with Upf1 siRNA (Fig. 6A). While Stau1-knockdown cells expressed myogenin without induction, Upf1-knockdown cells did not (Fig. 6A). Furthermore, Stau1-knockdown cells expressed myoglobin in growth medium, whereas Upf1-knockdown cells did not (Fig. 6B). These results suggest that Stau1 negatively regulates myogenesis in an Upf1-independent mechanism.

RNA-binding activity of Stau1 was not required for Stau1-mediated inhibition of myogenesis

To uncover the molecular mechanism of negative regulation of myogenesis by Stau1, we tested whether an

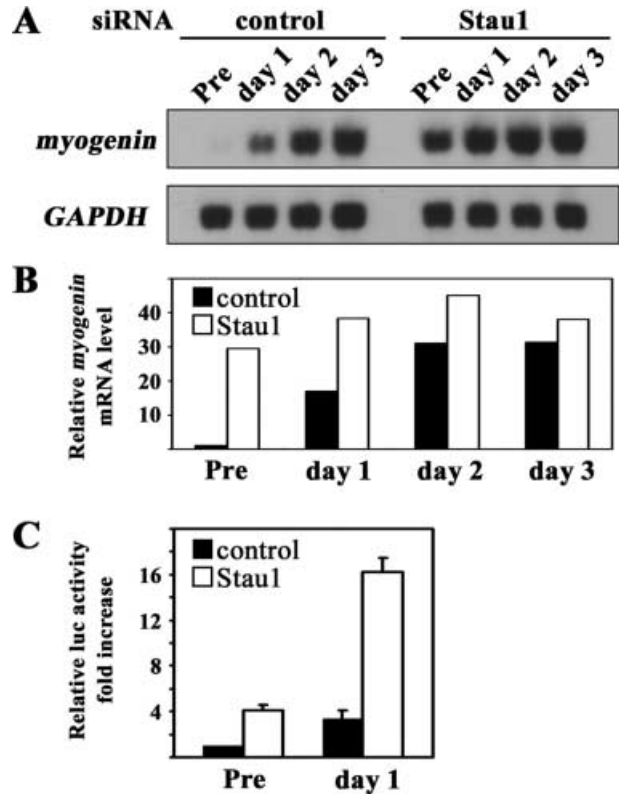


Figure 4 Effect of Stau1-knockdown on myogenin expression. (A), (B) Myogenin mRNA level. C2C12 myoblasts transfected with control or Stau1 siRNA were grown to confluence. Total RNA were extracted from cells at indicated days, and then subjected to Northern blot analysis with a specific probe for myogenin and GAPDH. The level of myogenin mRNA shown in panel A was normalized by that of GAPDH mRNA and values were represented in (B). (C) Myogenin promoter activity. Control or Stau1-knockdown cells were transfected with reporter plasmids as described in experimental procedures. Total lysates were extracted from cells prior to induction or post-induction day 1 and myogenin promoter activity was examined. The values in (C) are the average of triplicate from three independent experiments.

RNA-binding activity of Stau1 was required for the negative regulation of myogenesis. *Drosophila* Stau1 has five dsRBDs, and mammalian Stau1 has four dsRBDs but lacks one dsRBD correspond to first N-terminal dsRBD of *Drosophila* Stau1. It has been shown that dsRBD3 of *Drosophila* Stau1 directly associates with mRNAs such as bicoid and prospero *in vitro* (St Johnston *et al.* 1992; Li *et al.* 1997). As well as *Drosophila* Stau1, dsRBD3 of mammalian Stau1 (second dsRBD of mammalian Stau1 correspond to dsRBD3 of *Drosophila* Stau1) also possesses RNA-binding activity *in vitro* (Marion *et al.* 1999; Wickham *et al.* 1999). Stau1ⁱ, an isoform

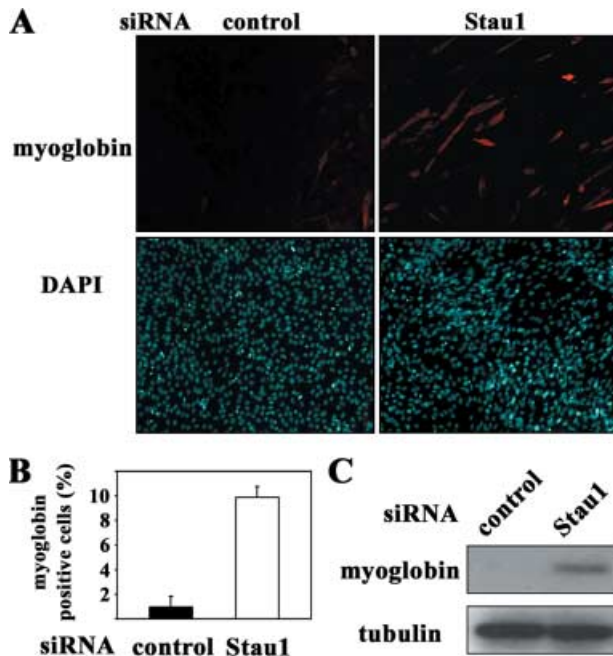


Figure 5 Stau1-Knockdown cells spontaneously differentiated without induction. (A), (B) Immunostaining of myoglobin in Stau1-knockdown cells. C2C12 myoblasts were transfected with control or Stau1 siRNA. Cells were grown to confluence and then cultured in growth medium for more 2 days. Cells were fixed and immunostained with anti-myoglobin antibody together with DAPI. Three fields (more than 1000 cells) were examined and percentage of myoglobin-positive cells was represented in (B). (C) Western blotting of myoglobin in Stau1-knockdown cells. Total cell lysates were prepared from cells cultured as described in (A) and then subjected to Western blot analysis with anti-myoglobin antibody.

of Stau1, has inserted six amino acids within dsRBD3, and shows impaired RNA-binding activity *in vitro* (Duchaine *et al.* 2000). Stauⁱ is expressed in various tissues (Duchaine *et al.* 2000) and we found that Stauⁱ was also expressed in C2C12 myoblasts as well as Stau1 (data not shown).

To examine whether Stauⁱ, which has an impaired RNA-binding activity, complements the phenotype of Stau1-knockdown cells, we generated virus expressing siRNA-resistant Stau1 and Stauⁱ by introducing silent mutation at the site of siRNA target sequence. C2C12 myoblasts were infected with virus expressing siRNA-resistant Stau1 and Stauⁱ and then transfected Stau1 siRNA to knockdown of endogenous Stau1 (Fig. 7A). Myogenin promoter activity was increased by Stau1 siRNA in cells infected control virus (Fig. 7B, black bars). Expression of siRNA-resistant Stau1 reversed this

increase of myogenin promoter activity by Stau1-knockdown (Fig. 7B, grey bars). Similarly, expression of siRNA-resistant Stauⁱ also reversed the increase of myogenin promoter activity by Stau1-knockdown (Fig. 7B, open bars). These results indicate that effect of Stau1 siRNA on myogenin promoter is indeed responsible for knockdown of endogenous Stau1, and inhibition of myogenin promoter by Stau1 is not required for its RNA-binding activity via dsRBD3.

Discussion

Myogenic differentiation program is highly coordinated by sequential expression of a set of transcriptional factors termed MRFs (Berkes & Tapscott 2005). This process is also coordinated by post-transcriptional regulation (Figuroa *et al.* 2003; van der Giessen *et al.* 2003). Expression of MRFs are regulated by a mechanism that protects mRNA from rapid degradation via 3'-UTR (Figuroa *et al.* 2003). In response to myogenic induction, an RNA-binding protein HuR has shown to translocate from nucleus to cytoplasm, bind to the 3'-UTRs of MRFs mRNAs, and ensure the expression of MRFs by stabilizing MRFs mRNAs (van der Giessen *et al.* 2003). Another mRNA stabilizing factor, NF90, is also known to contribute the expression of MRFs (Shi *et al.* 2005). Thus, stabilization of MRFs mRNAs by RNA-binding proteins is a key mechanism required to promote proper myogenic differentiation. Recently, Stau1, a conserved RNA-binding protein, has shown to mediate mRNA degradation via 3'-UTR of target mRNAs together with Upf1, a component of mRNA decay machinery (Kim *et al.* 2005). In this report, we considered a possible involvement of Stau1 in myogenesis and found that Stau1-knockdown cells exhibited the enhanced expression of muscle-specific genes, including myogenin and myoglobin following induction of myogenesis (Figs 2 and 3). Furthermore, Stau1-knockdown cells spontaneously differentiated to myotubes in the absence of induction (Fig. 5), indicating that Stau1 is required to maintain undifferentiated state in C2C12 myoblasts. In contrast to HuR which positively regulates myogenesis through the 3'-UTRs of MRFs, our results suggest Stau1 negatively regulates myogenesis through the promoter activity of myogenin.

It remains unknown how Stau1 regulates the promoter activity of myogenin. We have shown here that Stauⁱ, an isoform of Stau1, which impaired RNA-binding activity *in vitro*, could suppress the activation of myogenin promoter similar to Stau1. Thus, the negative regulation of myogenin promoter activity by Stau1 might be independent to RNA-binding. There are several possible

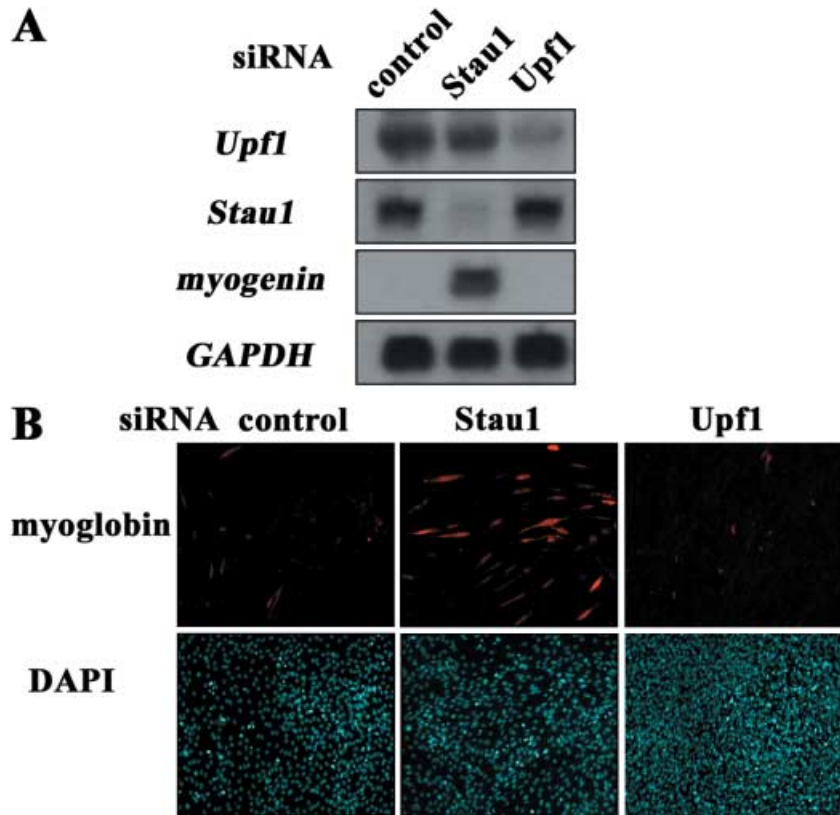


Figure 6 Expression of myogenic markers in UPF1-Knockdown cells in the absence of myogenic induction. (A) Effect of Stau1- or Upf1-knockdown on the expression of myogenin. C2C12 myoblasts were transfected with control, Stau1 or Upf1 siRNA. Forty-eight hours after transfection, total RNA was extracted from cells and subjected to Northern blot analysis with specific probes for Upf1, Stau1, myogenin and GAPDH. (B) Effect of Stau1- or Upf1-knockdown on the expression of myoglobin. Cells transfected each siRNA were grown to confluence and cultured with growth medium for more 2 days. Immunofluorescence was performed with anti-myoglobin antibody. The nuclei were stained with DAPI.

mechanisms for this regulation of Stau1. First possibility is that Stau1 directly acts to the myogenin promoter. Several RNA-binding proteins and RNA modification enzymes have been shown to regulate transcription at promoter region; RNA helicase, p68, has shown to be recruited to the promoter region of myosin heavy chain and muscle creatine kinase in differentiated myotubes (Caretti *et al.* 2006), and RNA-binding protein NF90 directly associate with IL-2 promoter in activated T-cells (Corthesy & Kao 1994). Although it has been reported that Stau1 localizes in cytoplasm (Marion *et al.* 1999; Wickham *et al.* 1999), Stau1 has been also reported to have a bipartite nuclear localization signal (Martel *et al.* 2006). In addition, we found that Stau1 localized in the nucleus of growing C2C12 myoblasts (our unpublished data). Thus, it is possible that Stau1 negatively regulates myogenin expression via directly acting to the promoter region during growing stage. Second possibility is that Stau1 affects the expression level or activity of the inhibitory factors for myogenin expression. It has been shown that, in growing myoblasts, the transcriptional activity of MyoD is restricted by various inhibitory factors such as Id, twist, MyoR and Mist-1 (Berkes & Tapscott 2005). In Stau1-knockdown cells, these inhibitory factors would

be somehow down-regulated, and then myogenesis would be stimulated in the absence of myogenic induction. Third possibility is that Stau1 affects the expression level or activity of some positive regulators for myogenin expression. During myogenesis, MyoD activates the transcription of various target genes including myogenin in cooperate with co-factors such as E-proteins, HDACs, HATs and SWI/SNF chromatin remodeling factors (Berkes & Tapscott 2005). In Stau1-knockdown cells, these cofactors would be somehow up-regulated even in the absence of myogenic induction, and then myogenesis would be stimulated. Fourth possibility is that Stau1 activates the myogenin promoter through the SMD pathway. However, it is unlikely, because knockdown of Upf1, a cofactor for the SMD pathway, did not affect myogenesis (Fig. 6). Since our knockdown of Upf1 did not completely stop Upf1 expression, it remains the possibility that a residual level of Upf1 is sufficient for normal progression of myogenesis.

It has been shown that both Stau1 and Upf1 are increased during myogenesis and the activity of SMD is up-regulated in differentiated myotubes (Kim *et al.* 2007). Although Stau1 might regulate myogenin expression in an Upf1-independent way, Stau1 together with Upf1

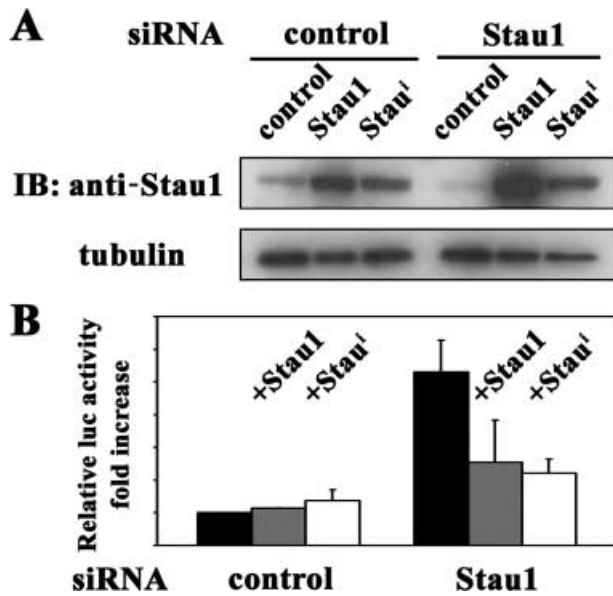


Figure 7 Effect of exogenously expressed Stau1 and Stau1 on myogenin promoter activity in Stau1-Knockdown. (A) The protein level of Stau1 and Stau1. C2C12 myoblasts were infected with control virus or virus containing Stau1 or Stau1 cDNA. Infected cells were transfected with control or Stau1 siRNA. Total lysates were extracted from cells prior to induction and then subjected to Western blot analysis with anti-Stau1 antiserum. (B) Relative activity of myogenin promoter was examined as described in Fig. 4B. The values are the average of triplicate from three independent experiments.

may have some other roles in muscle maturation and/or function in differentiated myotubes as mRNA decay machinery. Interestingly, Stau1 is expressed in skeletal muscle *in vivo* and accumulates postsynaptic sarcoplasm at neuromuscular junction (Belanger *et al.* 2003), so it may be possible that SMD has some roles there.

It has been shown that activation of Notch signaling results in a reduction of MyoD expression and inhibits myogenic differentiation in C2C12 cells (Kuroda *et al.* 1999). Furthermore, previous studies have also revealed that Notch has pivotal roles in maintenance of muscle progenitor cells (Luo *et al.* 2005) and that inhibition of Notch signaling enhances MyoD expression in muscle progenitor cells (Kuang *et al.* 2007). Because knockdown of Stau1 enhanced myogenesis without affecting MyoD expression, Stau1 may have a role in a fine tuning of myogenesis in differentiating myoblasts expressing MyoD rather than maintenance of muscle progenitor cells. MRFs have significant roles in muscle specification, maturation and regeneration *in vivo* (Hasty *et al.* 1993; Nabeshima *et al.* 1993; Rudnicki *et al.* 1993; Zhu

& Miller 1997; Sumariwalla & Klein 2001; Kassari-Duchossoy *et al.* 2004). It will be interesting to investigate the role for Stau1 in developing and regenerating muscle *in vivo*.

Experimental procedures

Cell culture, differentiation and transfection

C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum. To induce myogenic differentiation, cells were grown to confluence and then cultured in DMEM supplemented with 2% horse serum. After myogenic induction, medium was exchanged everyday. Transient transfections of C2C12 myoblasts were performed using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) as described by the manufacture.

Knockdown of Stau1 and Upf1

Double-stranded Stealth RNA duplexes (Invitrogen, Carlsbad, CA) corresponding to mouse Stau1 coding region (5'-CCCTGCCAGAAAGGTTGGAGGTAA-3') or mouse Upf1 coding region (5'-CAATGGGCCTGTACTGGTTTGTGCT-3') were transfected into C2C12 cells by Lipofectamine RNAiMAX reagent using reverse transfection protocol as described by manufacture (Invitrogen). As a control, we used Stealth RNAi negative control duplexes (Invitrogen) which sequence has no significant homology to any mammalian gene.

Plasmid construction

Mouse Stau1 is a generous gift from Michael Kiebler (Medical University of Vienna, Austria). To construct retroviral vector expressing Stau1, coding region of Stau1 is amplified by PCR with the set of primers TN103 (5'-GGATCCATGTATAAGCCCGTGGAC-3') and TN-104 (5'-GTCGACTCAGCACCTCCCGCACGC-3'). The *Bam*HI-*Sal*I-digested fragment of Stau1 was cloned into the *Bam*HI-*Xho*I site of pMX vector. Insertion sequence of Stau1 was introduced by PCR with the set of primers TN103 and TN287 (5'-CCCGGGCCACCTGTGT CAGAAGGAAAAGACTCAAAATTCACAGG-3'). A construct to examine myogenin promoter activity, pRL-myogenin, was constructed as follows. Mouse myogenin proximal promoter region (-525 to -1) was amplified by PCR using genomic DNA as a template and cloned into pRL-null vector (Promega, Madison, WI).

Retroviral experiments

PLATE cells were transfected with 4 µg of pMX vector at 50% confluence. After 2 days, the retroviral supernatant was centrifuged and resuspended in fresh medium containing 8 µg of polybrene. C2C12 cells were plated prior to infection and then incubated with viral medium for 24 h.

Northern blotting

Total RNA was prepared from cells with TRIzol Reagent (Invitrogen). Ten µg of each RNA samples was loaded on a 1% agarose gel containing 5.5% formaldehyde and resolved by electrophoresis. RNA was transferred to a nylon membrane and then hybridized with digoxigenin (DIG)-labeled antisense probes. After washing and blocking, membrane was incubated with AP-conjugated anti-DIG antibodies and the signal was detected by CDP-star (Roche).

Western blotting and antibodies

Cells were washed with cold PBS and then solubilized in lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF and 10 µg/mL leupeptine, 0.075 U/mL aprotinin, 1 mM DTT and 1% Triton-X100). Extracts were centrifuged at 24 000 × g for 10 min at 4 °C and supernatant was recovered. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad) and equal amount of protein was loaded on each lane. Extracts were boiled in 1× SDS-PAGE sample buffer and resolved by SDS-PAGE. After transferred to nylon membrane, membrane was incubated with antibodies, anti-myoglobin (Dako, Glostrup, Denmark), anti-MyoD (Santa Cruz Biotechnology, Santa Cruz, CA), anti-myogenin (Santa Cruz), or anti-α-tubulin (Sigma, St Louis, MO) antibodies and subsequently probed with HRP-conjugated secondary antibody (BD Bioscience, Rockville, MD) and detected by enhanced chemiluminescence (Millipore, Bedford, MA). C-terminal region of mouse Stau1 (327 aa to 487 aa) was used for production of a rabbit anti-Stau1 antiserum.

Immunofluorescence

Cells were cultured on gelatin-coated coverslips. After washing with PBS, cells were fixed with 3.7% formaldehyde for 10 min. After blocking with 5% BSA and permeabilization with 0.1% Triton X-100, cells were incubated with anti-myoglobin antibody (Dako) and subsequently probed with the rhodamine-conjugated anti-rabbit IgG antibody. Coverslips were mounted on a slide glass with Fluoromount-G (Southern Biotech, Birmingham, AL) containing DAPI and analyzed by fluorescence microscopy (Carl Zeiss).

Reporter assay

Myogenin promoter activity was examined as follows. Cells were transiently transfected with pRL-myogenin and pGL vectors (Promega). Twenty-four hours after transfection, cells were lysed and then luciferase activities were measured by Dual-Luciferase Reporter Assay System as described by manufacture (Promega).

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