MicroRNA *let-7b* targets Akt-1: possible implication for skeletal muscle atrophy in diabetic rats

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INTRODUCTION and OBJECTIVE

In diabetic animals occurs a marked muscle atrophy, but the molecular mechanisms underlying muscle wasting are still unclear. It is known that IGF-1/PI3K/Akt signaling is an important pathway regulating muscle mass.

During recent years, miRNAs have emerged as important regulators of a variety of biological processes as well as human diseases. MiRNAs are endogenous non-coding RNAs (21-25 nucleotides) that regulate gene expression via specific sites at the 3'-untranslated region (3'-UTR) of target mRNAs, causing translational repression or mRNA degradation. It has been estimated that miRNAs regulate approximately 30% of human genes.

Here, we hypothesize that Akt could be negatively regulated by miRNAs in skeletal muscles of steptozotocin(STZ)-induced diabetic rats. Our in silico analysis indicated that the isoform Akt1 is a potential target of *let-7b*. Thus, we investigate the expression of Akt1 and *let-7b* in the soleus muscle of normal and diabetic rats, and the luciferase assay and the endogenous inhibition of Akt1 expression by mimic *let-7b* were also performed to validate Akt1 as a target of *let-7b*.

METHODS

Soleus muscles were obtained from normal and diabetic rats (n=6) at 1, 3, 5 and 10 days after intravenous injection of STZ (45mg/kg b.w.). Expression of mature *let-7b* was evaluated by real-time RT-PCR using TaqMan MicroRNA Assasy. Rat myoblast cells (L6) were used for transfection experiments. The inhibition of Akt by *let-7b* was determinated by luciferase assay using a Psichek-2 vector with the Akt1 3'UTR (or Akt1 3'UTR mutated in the region complementary to seed region of *let-7b*) cloned downstream *Renilla luciferase* gene before poly A site. The endogenous expression of Akt was evaluated by Western blot and real time PCR of L6 cells transfected with mimic *let-7b*. The Foxo3a activity under *let-7b* overexpression was determined by luciferase assay using the vector of FRE-Luc and by citolocalization of Foxo3a fusioned to GFP. Atrogin-1/MAFbx expression was evaluated by real time PCR.

RESULTS and DISCUSSION

We found that *let-7b* is overexpressed in *soleus* at 3 days (> 6-fold; p< 0.05) after injection of STZ compared with control animals (Figure 1).

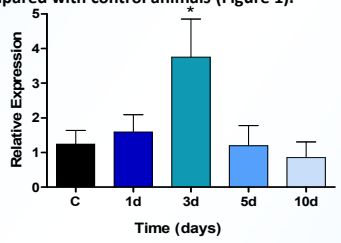

Figure 1. Expression of *let-7b* in normal and diabetic rats at 1, 3, 5 and 10 days after injection of streptozotocin. Data are presented as mean + s.e.m.*P<0.05 (ONE-WAY ANOVA).

The *let-7b* expression is consistent the proteolysis levels already reported in skeletal muscle of diabetic rats, suggesting an association between *let-7b* expression and proteolysis.

We also observed an inverse correlation of expression between *let-7b* and Akt1 protein in *soleus* of diabetic animals (data not shown) which suggests that Akt1 is a potential *let-7b* target.

The results of luciferase assay indicate that *let-7b* interacts directly with the 3'UTR of Akt1 mRNA, demonstrating that Akt1 is a direct target of *let-7b* (Figure 2). The Akt1 3'UTR mutated in the region complementary to seed region of *let-7b* abolished the interaction between Akt1 3'UTR and *let-7b*, indicating the specificity of this interaction (Fig

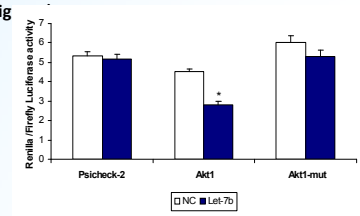

Figure 2: Analysis of the interaction of let-7b with Akt1 3'UTR by luciferase assay. L6 cells transfected with empty vector (psiCheck-2), vector with the region 3'UTR of Akt1 (Akt1) and with the region 3'UTR of Akt1 mutated at site which annealing with seed region of *let-7b* (Akt1-mut). Date represent *Renilla luciferase* activity normalized by *Firefly Luciferase* activity. Date presented as mean ± s.e.m. (*n*=3). *P<0.5 (*t Student test*).

This relevant finding was confirmed by evaluation of endogenous Akt expression in L6 cells transfected with *let-7b* mimic. *let-7b* transfection was able to reduce the Akt1 protein level (Figure 3), but not mRNA (Figure 4) indicating that the regulation occurs by translation inhibition and not by mRNA degradation.

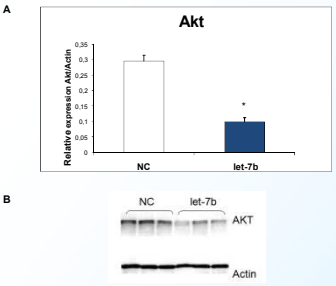

Figure 3: Inhibition of Akt endogenous protein expression by *let-7b*. Western blot of cells transfected with *let-7b* mimic or negative control. The graph shows the band intensity of Akt normalized with Actin- β (A). Date presented as mean ± s.e.m. (*n=3).* *P<0.5 (*t test of Student*). Each line is one sample and this is a representative of three independent experiments (B).

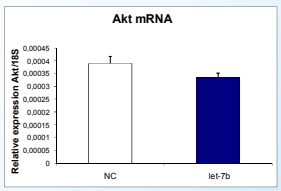

Figure 4: Expression of Akt1 mRNA of cells transfect with *let-7b* mimic. Analysis of Akt1 mRNA expression from L6 cells transfected with *let-7b* mimic or negative control by real time RT-PCR normalized with 18S expression. (*n=4*). Date presented as mean ± s.e.m. (*t Student test*).

The inhibition of Akt1 expression by *let-7b* affect downstream members of Akt signaling pathway as FoxO3a and Atrogin-1/MAFbx (Figure 5). Our results shown that *let-7b* transfection lead the FoxO3a activation (Figure 6) and nuclear localization (Figure 7) with subsequent induction of atrogin-1/MAFbx expression which is strongly associated with muscle atrophy (Figure 8).

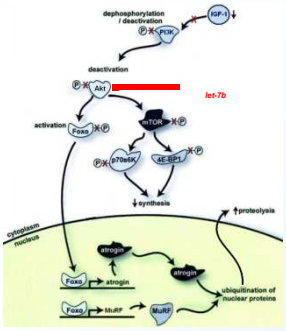

Figure 5: Proposed model of regulation of the IGF-1/PI3K/Akt signaling pathway by *let-7b* and its implications for muscle atrophy.

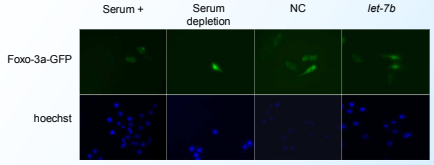

Figure 6: FoxO3a nuclear localization by *let-7b*. L6 cells transfected with GFP-FoxO3a concomitant with by *let-7b* mimic or negative control. Non-transfected cells were used as control. Under normal conditions FoxO3a is localized in cytoplasm (A). Upon serum depletion, FoxO3a accumulated in nuclei (B). *let-7b* treatment induced nuclear localization (C) which it was not observed at negative control (D). Cells also were stained with Hoechst dye to visualize the nuclei (E through H).

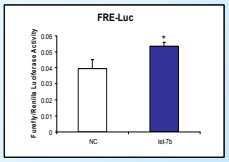

Figure 7: Activation of FoxO3a due Akt inhibition by *let-7b*. L6 cells were transfected with FRE-Luc and pRL concomitant with *let-7b mimic* or negative control. Results represent the activity of *Firefly Luciferase* normalized by *Renilla luciferase* activity at the same experiment. Date presented as mean ± s.e.m. (n=3) *P<0.5 (*t Student test*).

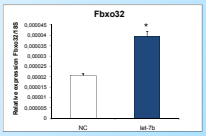

Figure 8: Induction of trasncrition of Atrogin-1/MAFbx by *let-7b*. Analysis of expression of Atrogin-1/MAFbx mRNA of cells transfect with *let-7b* mimic or negative control by real time RT-PCR normalized with 18S expression. (*n=4*). Date presented as mean ± s.e.m. *P<0.5 (*t Student test*).

CONCLUSION

The over-expression of *let-7b* in soleus muscle of diabetic rats results in down-regulation of Akt1 which could result in subsequent inhibition of protein synthesis through reduction of mTOR activation and increase in proteolysis due to nuclear translocation of the transcriptional factor FoxO3a. This work is the first demonstration of the involvement of a miRNA in skeletal muscle atrophy. Since no effective treatment has not been established for skeletal muscle atrophy, the local injection of anti-let-7b (antagomir) could be a novel therapeutic strategy for this complication of diabetes.

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