Myostatin stimulates myosatellite cell differentiation in a novel model system: evidence for gene subfunctionalization

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Garikipati DK, Rodgers BD. Myostatin stimulates myosatellite cell differentiation in a novel model system: evidence for gene subfunctionalization. Am J Physiol Regul Integr Comp Physiol 302: R1059–R1066, 2012. First published January 18, 2012; doi:10.1152/ajpregu.00523.2011.—Myosatellite cells play an important role in mammalian muscle regeneration as they differentiate and fuse with mature fibers. In fish, they also contribute to postnatal growth and the formation of new fibers. The relative conservation of fish systems, however, is not well known nor are the underlying mechanisms that control myosatellite cell differentiation. We therefore characterized this process in primary cells from rainbow trout and determined the effects of two known regulators in mammalian systems: IGF-I and myostatin. Unlike mammalian cell lines, subconfluent and proliferating trout myosatellite cells differentiated spontaneously and at a rate proportional to serum concentration. The expression of key myogenic markers (Myf5, MyoD1, myogenin, and MLC) and of the different myostatin paralogs (MSTN-1a/1b/2a) increased with serum-stimulated differentiation, although MSTN-1a expression was consistently higher than that of the other paralogs. In addition, MSTN-2a was only expressed as an unprocessed transcript. In low serum, where differentiation is normally suppressed, recombinant myostatin stimulated myogenic marker expression over time. The opposite was true for IGF-I as it stimulated proliferation, not differentiation, and additionally antagonized myostatin. This includes myostatin’s effects on marker expression and on the autoregulation of MSTN-1a and -1b expression. These results conflict with studies using mammalian cell lines and suggest, alternatively, that myostatin is a positive, not negative, regulator of myosatellite cell differentiation. Mammalian myoblasts differentiate when confluent and with serum withdrawal, which differs considerably from how myosatellite cells differentiate in vivo. Thus the primary rainbow trout myosatellite cell culture system appears to be more physiologically relevant.

growth/differentiation factor-8; rainbow trout; muscle stem cells; myogenesis

MYOGENESIS results from two processes, the determination of somatic cells to myosatellite cells and their subsequent differentiation and fusion to mature myotubes (4, 11). The second process requires cell cycle withdrawal and the sequential expression of key myogenic regulatory factors and muscle-specific genes (46, 47). Mammals maintain a small number of quiescent myosatellite cells, which help repair or regenerate damaged muscle. These cells are present in the basal lamina and are activated in response to exercise or muscle damage (10, 27). They are also being used to develop cell-based therapeutics for treating muscle growth disorders (57, 59). Because primary mammalian myosatellite cells are difficult to isolate, especially in large quantities, several rodent myoblast cell lines have been developed (e.g., mouse C2C12 and rat L6) and are commercially available. Fish, however, are a rich source of primary myosatellite cells as both hyperplastic and hypertrophic muscle growth occurs even in adults (41).

Several studies have implicated insulin-like growth factor (IGF)-1 and myostatin as potent stimulators and inhibitors, respectively, of muscle cell differentiation (17, 39, 51). Those involving myostatin, however, were mostly performed with cell lines (28, 32, 49, 51, 63) that, unlike primary myosatellite cells, must be induced to differentiate. In fact, immortalized myoblasts differentiate only when confluent and after removing mitogenic signals with serum withdrawal. By contrast, primary myosatellite cells from a variety of species differentiate in the presence of serum even when subconfluent (5, 22, 35, 62, 70). This suggests that the processes that regulate differentiation in myoblasts and myosatellites differ in subtle, yet significant ways.

Growth arrest is a necessary prerequisite for initiating cellular differentiation and is stimulated by myostatin in both myoblasts (28, 50) and myosatellites (25, 37). This involves the downregulation and inactivation of Cdk2, the upregulation of p21, a Cdk inhibitor, and the hypophosphorylation of Rb. All of this is consistent with initiating, not attenuating, differentiation (28, 32, 49). However, myostatin has been demonstrated to inhibit myoblast differentiation in vitro (28, 32, 49, 61), which conflicts with its well-established actions on cellular proliferation. Limited studies with myosatellite cells report conflicting results as myostatin has been demonstrated to both inhibit differentiation and to stimulate cellular quiescence (37, 68). The use of alternative models for investigating myostatin’s effects on myogenesis could therefore resolve the controversy and help explain how the myokine regulates both muscle development as well as muscle repair.

One such model, the rainbow trout primary myosatellite cell, is gaining popularity and has been recently used to characterize basic myogenic processes in vitro as well as the comparative actions of insulin and IGF-1 (6, 8, 14, 42). The system is somewhat more complicated than those of mammals, however, as several gene duplication events have resulted in the retention of four myostatin genes in salmonids including rainbow trout: MSTN-1a, -1b, -2a and -2b, a pseudogene (51). Nevertheless, preliminary results indicate that myostatin inhibits the proliferation of these cells and may actually initiate differentiation (25). The goal herein, therefore, is to further examine myostatin’s role in regulating muscle cell differentiation, using this novel system, and to determine the relative contribution of each paralog to controlling the process. Our results suggest that myostatin indeed stimulates terminal differentiation, likely via MSTN-1a and -1b, although MSTN-2a may mediate the stimulatory effects of IGF-1. Thus portraying myostatin’s effects on
myogenesis as purely inhibitory may misrepresent the myo-
kine’s actions as they appear to contribute to the normal
differentiation process.

MATERIALS AND METHODS

Cell culture and differentiation assays. Rainbow trout were ob-
tained from the Washington State University hatchery, a Center of
Reproductive Biology core facility. Fish were reared and used accord-
ing to protocols preapproved by the Institutional Animal Care and Use
Committee. Primary myosatellite cells were isolated as described (30)
and seeded in 12-well tissue culture plates pretreated with laminin (5
μg/ml, Sigma) and poly-l-lysine (100 μg/ml, Sigma) at a density of
100,000 cells/plate. Cells were cultured in Dulbecco’s modified
Eagle’s medium (DMEM, Sigma) supplemented with different con-
centrations of fetal bovine serum (FBS, 1, 2, 5, and 10%; Atlas
Biological) to determine the effects of serum on the differentiation of
confluent subcultures. Culture periods lasted up to 2 wk as cells were
terminated after 1, 5, 10, and 14 days. Cells were also cultured in 1%
serum supplemented with either 50 nM recombinant mouse myostatin
(R&D Systems), 50 nM IGF-I (Diagnostic Systems Laboratories), or
both. These cells rarely differentiate at such low serum concentrations
(see below). Thus this determined the ability of these hormones to
initiate and maintain differentiation at a serum concentration that
otherwise does not support it. Culture medium was changed after 5
and 10 days, and cells were retreated with hormones at these times.

Actin and nuclear staining. Myosatellite cells were grown on glass
coverslips treated with poly-l-lysine and laminin as described above.
Cells were washed twice with PBS, fixed in 2% paraformaldehyde for
15 min on ice, washed three times with PBS, permeabilized with 0.2%
 Triton X-100, and washed three more times before being stained.
Rhodamine-conjugated phalloidin was then added at a concentration
of 70 nM. Nuclei were stained with 5 μM Hoescht stain, and cells
were imaged using a Zeiss fluorescent microscope. The fusion index
of 9 μl primer mixture containing 450 nM each primer. The reactions
were performed using the iCycler iQ Real-Time PCR detection system
(Bio-Rad) and specific primer sets (Table 1). For each sample, 1 μl
cDNA was combined with 7.5 μl of 2× SYBR Green PCR master
mix (Bio-Rad). For each reaction, 6 μl of this mixture was added to
9 μl RT-PCR mixture was used to calculate the relative
abundance of each transcript. Sample values were then normalized to
those of 18s to control for differences in RNA and cDNA loading.
Each sample was run in duplicate and each plate was run in duplicate.
Assays were repeated with different samples, and all data are pre-
presented as normalized gene expression.

Expression of the MSTN-2b paralog was not quantified because this
pseudogene is expressed at very low and physiologically irrele-
vant levels (23). For qualitative assessment of MSTN-2a expression,
skeletal muscle and brain samples along with the differentiated cells
from days 1, 5, 10, and 14 were collected and snap frozen. Previously
validated primers and conditions (23) were used for RT-PCR and the
primers annealed to sites that spanned the first intron of the MSTN-2a
gene, enabling the detection of both spliced and unspliced transcripts
in a single reaction. PCR was then performed for 35 cycles using 18S
as a loading control.

Statistical design. For gene expression analysis, three independent
experiments were performed, each with two plate-replicates/experi-
ment. Target gene values were normalized to those of 18s, again as
described (25). Cells were terminated at distinct time points rather
than continuously so differences between means were determined by
a one-way analysis of variance coupled to Tukey’s post hoc test for
multiple mean comparisons (P ≤ 0.05).

RESULTS

Serum regulation of the myosatellite cell phenotype. Serum
withdrawal is a common means for stimulating the differenti-
ation of confluent myogenic cells, whereas by contrast, serum
itself stimulates the proliferation of subconfluent cells (10, 19,
54). Myosatellite cells from rainbow trout were very respon-

Table 1. Real-time qRT-PCR primer sequences

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’–3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myf5 F</td>
<td>CCG ACA GCA TGG TGG ACT GT</td>
<td>*AY751283.1</td>
</tr>
<tr>
<td>Myf5 R</td>
<td>TGC CAT AGG TGG TGT TCA TCT GA</td>
<td>*AY751283.1</td>
</tr>
<tr>
<td>MyoD1 F</td>
<td>GTC CTT GGA TAA AAA CAG CCG</td>
<td>(33)</td>
</tr>
<tr>
<td>MyoD1 R</td>
<td>CAA AGA AAT GCA TGT CCG</td>
<td>(33)</td>
</tr>
<tr>
<td>Myogenin F</td>
<td>GTC GAG ATC TGG AGG AGT GC</td>
<td>(33)</td>
</tr>
<tr>
<td>Myogenin R</td>
<td>GGT CCT CTT TGG AGG TCG</td>
<td>(33)</td>
</tr>
<tr>
<td>MLC F</td>
<td>GCC CCC AGT AAC TTC AC</td>
<td>(66)</td>
</tr>
<tr>
<td>MLC R</td>
<td>CTC CTC CTT TCC TCC CGG TG</td>
<td>(66)</td>
</tr>
<tr>
<td>18 s F</td>
<td>TGC GCC TTA ATT TCA CTC AAG A</td>
<td>(23)</td>
</tr>
<tr>
<td>18 s R</td>
<td>CAA CTA AGA AGG GCG ATG GA</td>
<td>(23)</td>
</tr>
<tr>
<td>MSTN-1a F</td>
<td>CCT CCA TCA TCA TTA TAC ATA TTA</td>
<td>(24)</td>
</tr>
<tr>
<td>MSTN-1a R</td>
<td>GCA ACC ATG AAA CTA TCA TAA A</td>
<td>(24)</td>
</tr>
<tr>
<td>MSTN-1b F</td>
<td>TTC AGC CAA ATA CTA ATT CAG</td>
<td>(24)</td>
</tr>
<tr>
<td>MSTN-1b R</td>
<td>GAT AAA TTA GAA CCT GCA TCA GAT TC</td>
<td>(24)</td>
</tr>
<tr>
<td>MSTN-2a F</td>
<td>AAT CTC CCC GCA TAA AAG CAA CGG C</td>
<td>(23)</td>
</tr>
<tr>
<td>MSTN-2a R</td>
<td>CAC CAG AAG CCA CAT CGA TCT T</td>
<td>(23)</td>
</tr>
</tbody>
</table>

All primers were used at 60°C. MLC, myosin light chain; MSTN, myostatin. *NCBI Accession number.
sive to medium FBS as concentrations were positively correlated with cell number and with a differentiated phenotype (i.e., elongated/fused cells; Fig. 1). Previous studies have even determined that these cells grow better in FBS than in rainbow trout serum (18, 30). This was best illustrated by comparing cellular confluency and the relative number of myotubes at day 7. Indeed, cells cultured in 5% FBS were 100% confluent and appeared fully differentiated by day 10, whereas cells cultured in 2% FBS did not reach confluency until day 14 and these cells did not appear fully differentiated. Cellular phenotypes and relative confluency were similar in cells cultured in 5% and 10% FBS, suggesting no additional benefit to stimulating cells with concentrations above 5%. Cells grown in 1% serum survived, although there was no evidence of significant proliferation, cellular fusion, or myotube formation. Together, these results indicate that serum stimulates the proliferation and differentiation of subconfluent rainbow trout myosatellite cells. This is in contrast to the effects of serum on myoblast cell lines, but is consistent with studies using primary myosatellite cells (2, 5, 22, 35, 48, 62, 70).

Serum regulation of differentiation marker expression. The serum effect on differentiation was quantified by measuring the gene expression of markers for early (Myf5 and MyoD1), mid (myogenin), and late (MLC) stages of differentiation in cells cultured with 1% and 5% FBS. Expression patterns for each marker were similar in that cells cultured in 5% FBS had higher levels of marker expression at most time points (Fig. 2, A–D). A notable exception, however, was the Myf5 expression on day 14, which began to fall in the 5% group while expression in the 1% group began to peak (Fig. 2A). Net differences in MRF expression were also reflected in the MLC expression patterns as the day 14 levels in the 1% group were comparable to those in the day 5 group (Fig. 2D). This suggests that cells differentiated nearly three times faster in 5% FBS. Terminal differentiation was also verified by phalloidin staining of actin and in calculating the fusion index for each treatment (Fig. 2, E–L). These results mirrored the time-dependent changes in MLC expression in the formation of elongated myotubes (Fig. 1) and in response to serum as actin staining and cellular fusion occurred earlier in cells grown with 5% serum. Furthermore, actin staining was also more intense in these cells. A maximal fusion index of 91% occurred at day 14 in cells grown with 5% serum. The remaining 9% of undifferentiated cells are composed of either nonmyogenic cells (i.e., fibroblasts) or myogenic reserve cells, which is consistent with previous reports of cellular purity following our isolation procedure (18, 22, 30).

Serum regulation of MSTN paralog expression. Changes in the temporal expression pattern for each paralog were similar with cells in 1% and 5% FBS (Fig. 3) as expression increased progressively over time. However, absolute levels were dependent on serum concentrations and, apparently, differentiation status. In fact, expression levels for each paralog was higher in the 5% group at all time points (Fig. 3A–C). MSTN-1a expression was higher than that of MSTN-1b and -2a at all time points, although expression of both MSTN-1 genes plateaued at day 10, whereas MSTN-2a expression continued to rise (Fig. 3D). The similar expression patterns indicate that the basic mechanisms governing gene expression, at least during differentiation, have been retained with each paralog. Subtle differences nevertheless exist as the patterns are not identical. This indicates that promoter function is actively diverging and that the genes are subfunctionalizing.

Myostatin and IGF-I regulate differentiation marker and MSTN paralog expression. To determine whether myostatin and/or IGF-I influenced the rate of spontaneous differentiation, subconfluent myosatellite cells were cultured in 1% FBS and with different combinations of 50 nM myostatin and/or 50 nM IGF-I. Low serum concentration was used as it prevents cells from rapidly differentiating (Figs. 1 and 2). As expected, expression of all markers increased over time, regardless of treatment, indicating that differentiation progressed at a normal rate (Fig. 4) yet delayed rate (compare controls with 1% FBS group in Fig. 3). On day 3, however, Myf5 expression was almost fivefold higher in myostatin-treated cells than in control cells, and this pattern was similar at days 10 and 14, clearly indicating that myostatin initiates differentiation (Fig. 4A). MyoD1, myogenin, and MLC expression was similarly stimulated by myostatin at all time points (Fig. 4B–D). Thus, myostatin not only initiates differentiation, but also maintains the process in vitro without stimulating quiescence.

The expression of Myf5, MyoD1, and myogenin in cells treated with IGF-I were similar or less than those in control cells (Fig. 4A–C). By contrast, IGF-I increased MLC expression (Fig. 4D). These data appear somewhat contradictory, although IGF-I also stimulated proliferation and, as discussed below, MSTN-2a expression and transcript processing. The reduced MRF expression is therefore consistent with increased proliferation while increased MLC expression is consistent with the differentiation of a minority of cells (25). In fact, MLC
expression was lower in IGF-treated than in myostatin-treated cells at all time points. In addition, marker expression levels in cells cocultured with both hormones were either similar to that of control cells or between control and myostatin-treated cells. This indicates that IGF-I attenuated myostatin’s effects on differentiation as it does on proliferation.

In the current study, cells were cultured in low serum for 2 wk. Although paralog expression changed over time and as cells differentiated, myostatin induced both MSTN-1a and -1b expression at all time points while IGF-I inhibited expression (Fig. 5, A and B). The two hormones also antagonized one another as exposing cells to both myostatin and IGF-I compromised their individual effects on MSTN-1 paralog expression. By contrast, MSTN-2a expression was slightly elevated by both hormones (Fig. 5C) and costimulating cells had similarly effects. Myostatin and IGF-I differentially regulate myosatellite cell proliferation and differentiation. Thus their effects on MSTN-2a expression likely result from different cellular processes and responses.

**MSTN-2a transcript processing.** Analysis of MSTN-2a expression over time, using intron-spanning primers and RNA isolated from cells differentiating under 5% serum indicated that MSTN-2a was expressed as an unspliced transcript at all times (Fig. 6). RNA isolated from the brain was used as a positive control as MSTN-2a is correctly spliced only in this tissue (23). Thus, the differentiation-induced increase in MSTN-2a expression likely has no effect on myosatellite cells because the transcript cannot be translated.

**DISCUSSION**

This study is the first to demonstrate direct actions of myostatin in any fish species. It also suggests that the current understanding of myostatin action, based mostly on immortalized cell lines, should be reconsidered. We further explore myostatin’s role during differentiation by suppressing the process with, to our surprise, low serum supplementation. Our results suggest that myostatin stimulates differentiation marker expression in cells with suppressed rates of growth and differentiation and that the dominant paralog expressed throughout the process is MSTN-1a. Our data additionally indicate that myostatin attenuation of IGF-I may help the latter to stimulate cell proliferation.
muscle cell differentiation and that myostatin’s control of differentiation is mediated by different MSTN paralogs (see below). Thus, the characterization of myostatin as a global inhibitor of muscle growth is inaccurate because these and other recent studies suggest that it stimulates, rather than inhibits, myosatellite cell differentiation.

Rainbow trout primary myosatellite cells spontaneously differentiate at low cell densities and high serum levels (5, 6, 8, 14, 36, 42). In fact, primary myosatellite cells isolated from other vertebrates, including mammals, also differentiate in the presence of high serum concentrations (2, 5, 22, 35, 48, 62, 70). The use of immortalized muscle cell lines in differentiation studies is therefore problematic as these cells only differentiate at high densities and after serum withdrawal, which hardly resembles the low cell density and cytokine-rich environment of the myosatellite niche in vivo. Our studies not only suggest that serum stimulates differentiation, as is evident by a conserved MRF expression pattern (15, 31, 40, 65), but that manipulating serum concentrations in vitro can control the process; 5% is optimal and 1% retards growth and differentiation, but is sufficient for survival. In fact, exploiting the serum-sensitive nature of these cells will prove useful in determining whether myostatin can initiate differentiation in proliferating cells (25) as the current study indicates that myostatin maintains the process even when proliferation and differentiation are suppressed.

Myostatin induced expression of key myogenic regulatory factors (Fig. 4) that are necessary for the initiation and maintenance of muscle cell differentiation (9, 17, 54). It also increased the expression of MLC, a marker of terminal differentiation and maturation. In fact, myostatin’s effects were similar to those of 5% serum (Fig. 3). Myostatin therefore stimulates, not inhibits, the differentiation of myosatellite cells. These results are in contrast to previous studies with myoblast cell lines where differentiation of confluent cells was first induced by serum withdrawal (28, 32, 49). Proliferating myo-

![Fig. 3](image-url)  
Fig. 3. Expression of MSTN-1a, -1b, and -2a at different stages of myosatellite cell differentiation. A–C: cells were cultured in DMEM supplemented with 1% or 5% FBS and terminated on days 1, 5, 10, and 14 for analysis. Levels of mRNA for each transcript were then measured by quantitative RT-PCR. Time points with different superscripts are statistically different (P < 0.05). Same letters denote no statistical difference. D: values from the 5% FBS groups are plotted together to facilitate the relative comparison between each paralog. Values are means ± SE; n = 9/group.

![Fig. 4](image-url)  
Fig. 4. Myostatin and insulin-like growth factor (IGF-I) differentially regulate the expression of myogenic differentiation markers. Primary myosatellite cells were cultured in DMEM supplemented with 1% FBS and treated with the indicated combinations of 50 nM myostatin and/or 50 nM IGF-I. Cells were terminated on days 1, 5, 10, and 14, and mRNA levels for Myf5, MyoD1, myogenin, and MLC were then measured by qRT-PCR. Treatments with different superscripts are statistically different (P < 0.05). Same letters denote no statistical difference. Values are means ± SE, n = 9/group.
blast cell lines do not normally express myostatin (53), although it does increase during differentiation (7, 15, 50) due to MyoD transactivation (16, 55, 60). McFarlane et al. (38) recently demonstrated myostatin inhibition of human myoblasts, not primary cells, although differentiation was induced as when using cell lines. The discrepancies between these studies and those reported herein could therefore be due to the use of immortalized cell lines and/or to the "artificial" induction of differentiation. In fact, Manceau et al. (35) suggested that myostatin regulation of muscle progenitors is context dependent because it not only inhibits muscle progenitor cell proliferation in vivo but stimulates terminal differentiation by promoting MyoD expression and p21 activation. Sato et al. (56) further demonstrated that myostatin knock-down with RNA interference (RNAi) delays differentiation in primary chick cells, and Ge et al. (26) demonstrated impaired differentiation of myosatellite cells with impaired myostatin signaling (i.e., Smad3 deficient). These results are consistent with those presented herein and with the normal differentiation process of primary myosatellite cells. They also suggest that nonmyostatin factors likely facilitate the process in myostatin null animals.

Myostatin and IGF-I are arguably the two most important regulators of skeletal muscle growth and development and are similarly important to cardiac muscle (13, 17, 51, 52). Their opposing actions do more than just help control or even optimize muscle growth as IGF-I stimulates myostatin expression in skeletal and cardiac muscle (1, 21, 44, 64). Myostatin is therefore a proposed chalone for both tissues since it appears to suppress IGF actions (43, 51, 52, 58), and as a result, facilitate IGF-stimulated muscle cell differentiation as well as limit tissue hypertrophic responses.

All three functionally active MSTN paralogs likely contribute to the differentiation process, albeit their specific roles have functionally diverged. The combined expression changes in MSTN-1a, and to a lesser extent MSTN-1b, throughout differentiation, as well as their autoregulation, suggests that these paralogs are particularly important to the differentiation process. The IGF-induced increased expression (Fig. 5) and transcript processing of MSTN-2a (25), combined with the down-regulation of both MSTN-1 paralogs, is extremely novel and is indicative of selective pressures that favored gene subfunctionalization. Indeed, this further indicates that the mechanisms responsible for serum- and IGF-stimulated muscle cell differentiation are fundamentally different as myostatin’s role during differentiation has been partitioned among the paralogs. The specific mechanisms responsible for subfunctionalization, however, are uniquely diverse because they involve differences in gene promoter structure/function and also transcript splicing.

Autoregulation of myostatin expression also occurs in mammalian cells (20). The fact that three novel aspects of the IGF-myostatin relationship (IGF-induced myostatin expression, myostatin autoregulation, and myostatin/IGF-I attenuation of action) are well conserved across vertebrate class boundaries highlights the importance of the relationship. It also suggests that the rainbow trout primary myosatellite cell culture system is a potentially valuable model for investigating this relationship, despite the apparent complexities of the salmonid myostatin gene family.

Perspectives and Significance

A comprehensive knowledge of myostatin’s effects is required prior to the use of myostatin attenuating technologies that are currently being developed (3, 12, 29, 34, 67). The data presented herein provide a platform for future studies that
utilize a novel comparative system with biomedical potential. In fact, large quantities of myosatellite cells from rainbow trout are easily obtained and cultured, much more than from mammals, and their phenotypic responses to myostatin and IGF-I are consistent with responses in primary myosatellites from other vertebrates, including mammals, and with muscle cell progenitors in vivo. Myostatin also inhibits protein synthesis in mature muscle and in mammalian cell lines (1, 64, 69). Rainbow trout myosatellite cells could therefore prove useful in better understanding muscle cell hypertrophy and, in general, how subfunctionalization of gene duplicates impact complex physiological processes and thus, adaptation.

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