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Report

Plasticity of Mammalian Myotubes Upon Stimulation with a Thrombin-Activated Serum Factor

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KEY WORDS

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ABSTRACT

Salamanders display unique regeneration abilities among adult vertebrates. An intriguing feature of salamander regeneration is the dedifferentiation of cells, such as myofibers and myotubes at the injury site, a process that involves cell cycle reentry from the differentiated state. A thrombin-activated serum factor that is distinct from conventional growth factors is known to cause S-phase reentry in salamander myotubes. While mammalian myotubes do not reenter S-phase upon serum stimulation, an upregulation of some immediate early genes such as jun and fos has been observed. Until now, it was unknown whether this transcriptional response was stimulated by conventional growth factors or by the thrombin-activated serum factor. By measuring transcriptional activity in individually purified C2C12 mouse myotubes using quantitative reverse transcription polymerase chain reactions, we show that a set of immediate early genes are activated in response to the thrombin-activated serum factor in a distinct manner from the growth factors PDGF, FGF and EGF. A partially purified fraction of the thrombin activated serum factor elicited stronger upregulation of a broader set of genes compared to individual growth factors and additionally caused downregulation of E2F6. Despite this robust transcriptional response in mammalian myotubes, we did not detect a large-scale change in histone H3K9 di-methylation or S-phase, a feature that characterizes salamander serum-stimulated myotubes. Our results indicate that mammalian myotubes have retained responsiveness to the thrombin-activated serum factor, but full re-entry into S-phase is prevented by factors downstream of the immediate early genes.

INTRODUCTION

Most cells in the body are differentiated and quiescent, such as skin, nerve or muscle cells. Skeletal muscle is a classical example of a differentiated cell, which is formed by the fusion of mononucleate progenitors and by their withdrawal from the cell cycle.[1] Work on multinucleated myotubes showed however that the postmitotic arrest and the differentiated state are maintained by reversible mechanisms rendering cells plasticity.[2-4] The ultimate proof of such plasticity was demonstrated by transferring nuclei from adult somatic cells into enucleated oocytes. These experiments demonstrated that entire organisms could be cloned through nuclear transfer and reprogramming.[5-7] Nuclear reprogramming is a key concept in regenerative medicine since in principle it could lead to the regeneration of any cell type in the adult organism through transplantation or appropriate stimulation of local progenitors.

Adult salamanders, such as the newt and axolotl, respond to major injuries by rebuilding complex structures.[8] Salamanders can regenerate entire limbs, tails, cardiac tissues, lens and retina of the eye, and parts of the central nervous system.[9] Injury to salamanders can cause local reversal of the differentiated state, reprogramming and the generation of a regenerative progenitor cell population, which proliferates and builds up the exact replica of the lost body part.[10]

Lens regeneration for example proceeds through cell cycle reentry and phenotypic dedifferentiation of pigmented epithelial cells of dorsal iris, and their subsequent differentiation into lens cells.[11,12] New limbs and tails form through a mesenchymal growth zone, called the blastema, from which cells of the regenerated appendage originate.[13] During limb and tail regeneration, resident myofibers or implanted multinucleated myotubes can undergo a cellularization process giving rise to mononucleate cells, which in turn incorporate into the regeneration blastema.[14,15]

An important question is how injury is linked to dedifferentiation, cell cycle reactivation and reprogramming. Through a series of studies, the clot component thrombin

has emerged as a critical determinant of salamander regeneration. Selective activation of thrombin is required for proper lens regeneration in newts, and blocking the proteolytic activity of thrombin can stop S-phase reentry by pigmented epithelial cells both in vivo and in vitro.[16-18] Similarly, thrombin activity is apparent in the limb regeneration blastema, and S-phase reentry by cultured newt myotubes is induced by a serum component, that is activated by thrombin proteolysis and which is enriched in crude thrombin preparations.[19] The thrombin activated factor was previously partially purified and characterized.[20] A feature that distinguishes the thrombin-activated serum factor from polypeptide growth factors such as PDGF, FGF and EGF is its selective ability to induce S-phase reentry of differentiated myotubes but not quiescent mononucleate myogenic cells. Since the mononucleate cells readily reenter S-phase upon stimulation by peptide growth factors, the thrombin-derived factor appears to be a different type of molecule.[19]

While sera from all mammals tested can elicit S-phase reentry in newt myotubes, mammalian myotubes themselves do not initiate S-phase in response to serum or the thrombin activated factor. Northern and Western blot analyses indicated, however that partially purified C2C12 myotubes do progress through the G_1-phase of the cell cycle following serum stimulation.[21-23] These experiments suggested the typical immediate early gene response as well as the upregulation of cyclin D1 in mammalian myotubes that characterize the initial phases of the cell cycle.

We endeavored to test whether the mitotic response by mammalian myotubes after serum exposure is due to conventional peptide growth factors, to thrombin or to a combination of these two alternatives. We also wanted to create an assay system in which contamination by the transcriptionally more active mononucleate myogenic precursors could be avoided. By analyzing individually purified myotubes using quantitative reverse transcription PCR (qRT-PCR) we show that thrombin and peptide growth factors elicit partially overlapping transcriptional response in C2C12 myotubes.

MATERIALS AND METHODS

Cell culturing, immunocytochemistry, microscopy, image processing. C2C12 cells (ATCC) were grown in DMEM supplemented with 12% fetal bovine serum (FBS). Differentiation was induced in high density cultures grown on gelatin-coated dishes in DMEM containing 1% horse serum (DM). Salamander A1 myogenic cells were cultured as described earlier.[24] Differentiation was induced on fibronectin coated dishes in the presence of 0.5% FBS. The following antibodies were used according to the manufacturers recommendations: anti-BrdU (Trichem Aps), anti-phosphorylated histone 3 (Upstate, USA), anti-c-jun (Santa Cruz Biotechnology, Inc), anti-myosin heavy chain (Hydridoma Bank, USA), anti di-methylated H3K9 (Upstate, USA). Immunofluorescent staining was analyzed by a Zeiss inverted microscope. A LSM 510 Meta laser microscope with LSM 5 Image browser software (both Carl Zeiss MicroImaging, Inc) was used for confocal analyses for visualization of c-jun expression in myotubes. Linear image adjustments were done using Photoshop 6.0. Experimental and control images were always taken under identical conditions and manipulated the same way.

Stimulation of myotubes. Following differentiation, myotube cultures were stimulated with either of the following: 10% FBS (Invitrogen), crude bovine thrombin (Calbiochem) at 0.1 mg/ml added to DM, EGF (Invitrogen) at 20 ng/ml added to DM, FGF2 (Invitrogen) at 20 ng/ml added to DM, PDGF-BB (Gift from Dr. U. Eriksson, Ludwig Institute of Cancer Research, Stockholm) at 10 ng/ml added to DM. The HEP3 fractionation is described elsewhere.[20] The HEP3 fraction was desalted on a NAP-5 column (Amersham Biosciences) and eluted in DMEM containing 0.1% protease free BSA (Serva). Before addition to the cells, HEP3 was diluted 20x in the elution buffer, which was also used for the control cells. Control myotubes were always harvested at the same time as samples for every type of stimulation and time point. To test the effect of the HEP3 fraction on A1 myotubes, purified myotubes were stimulated with the HEP3 fraction as described earlier.[20] To test the effect of HEP3 on A1 mononucleate cells, cells were serum starved for one day followed by addition of HEP3 fraction in the presence of 10 μM BrdU. Cells were fixed with ice cold methanol after 24 hours and stained for BrdU as described earlier.[18] Alternatively, serum starved A1 mononucleate cells were stimulated by HEP 3 for 4 hours, fixed with 2% PFA and stained for phosphorylated Histone 3 as described earlier.[25]

Isolation of myotubes. To avoid any response from contaminating myoblasts, myotubes were picked individually using a glass capillary. At the end of the stimulation, cells were trypsinized briefly and transferred to DM in a 3 cm dish coated with horse serum to avoid attachment of the cells. Myotubes were then picked under light microscope, using a horse serum-coated glass capillary attached to a syringe through plastic tubing. To ensure absolute absence of myoblasts, the myotubes were expelled in a new 3 cm dish containing PBS and picked once more making sure only myotubes with no mononuclear cells attached were picked. In this step 10 myotubes per sample were expelled in a 1.5 ml tube and frozen on dry ice.

RNA extraction and cDNA synthesis. RNA was extracted using the RNeasy microkit (Qiagen) according to the instructions of the manufacturer. cDNA synthesis was performed in a 20 μl reaction, using 4 μl of RNA, 0.5 μM dNTPs, 750 ng anchored oligo$(dT)_{20}$, 10 μM random nonamers, 1x first strand buffer, 40 units Rnase out, 1mM DTT and 200 units Superscript III (Invitrogen). RNA, primers and dNTPs were first incubated at 65°C for 5 min and then chilled on ice. After adding the remaining reagents samples were first incubated at 25°C for 5 minutes, followed by incubating for 60 minutes at 50°C. The reactions were stopped by heat inactivating at 70°C for 15 minutes.

Quantitative reverse transcription PCR. The following primer pairs were used for template amplification; forward (f), reverse (r):

β-actin(f)	TGT TAC CAA CTG GGA CGA CA
β -actin(r)	GGG GTG TTG AAG GTC TCA
GAPDH(f)	CAT GGC CTT CCG TGT TCC
GAPDH(r)	GCG GCA CGT CAG ATC CA
c-fos(f)	GAA TGG TGA AGA CCG TGT CA
c-fos(r)	TGC AAC GCA GAC TTC TCA TC
c-jun(f)	TCC CCT ATC GAC ATG GAG TC
c-jun(r)	TTT TGC GCT TTC AAG GTT TT
c-myc(f)	GAC CAG ATC CCT GAA TTG GA
c-myc(r)	CTC GTC TGC TTG AAT GGA CA
Id1(f)	GGT ACT TGG TCT GTC GGA GC
Id1(r)	TCA TGT CGT AGA GCA GGA CG
Cyclin D1(f)	AGT GCG TGC AGA AGG AGA TT
Cyclin D1(r)	CAC AAC TTC TCG GCA GTC AA
E2F6(f)	CTG GGG GCA TTC TTG ACT TA
E2F6(r)	CTT TTC CAC CAG TTC GAT GC

qRT-PCRs were performed in an ABI prism 7000 sequence detection system (Applied Biosystems). 2 μl of cDNA, 0.8 μM of each primer and 1x SYBR green PCR master mix (Applied Biosystems)

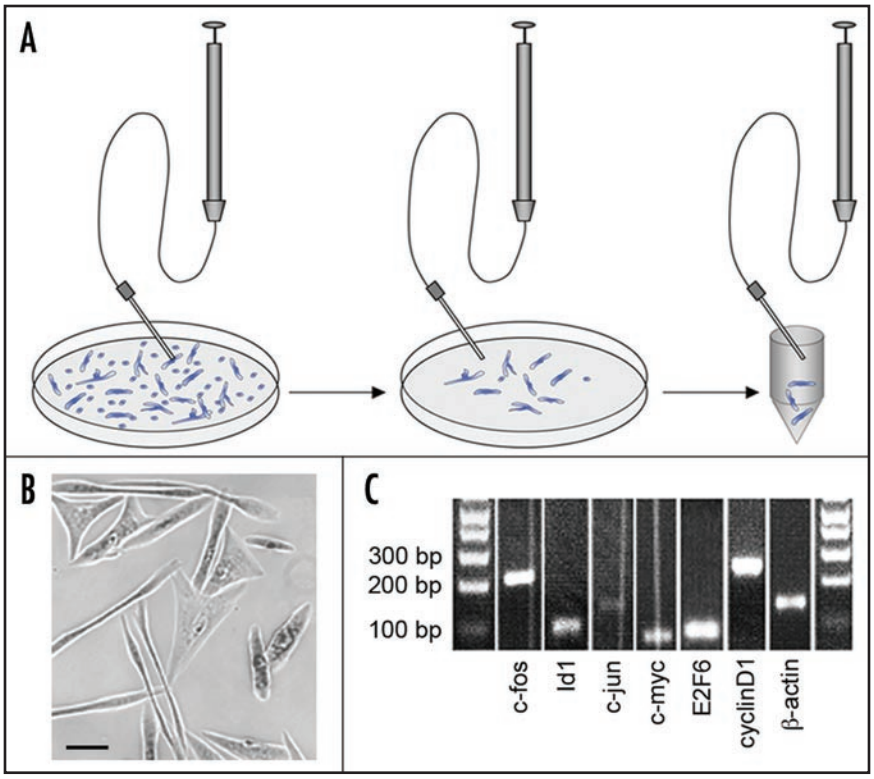

Figure 1. Purification of myotubes using glass capillary. (A) Cartoon illustrating the purification steps before mRNA preparation and PCRs. (B) Representative sample of purified myotubes. Scale bar: 100 μm (C) Specificity of the primers used to amplify various mRNAs.

in a 25 μl reaction, was amplified as suggested by the manufacturer. A dissociation curve was run at the end of the cycling to ensure the absence of primer-dimer and alternative products and relative mRNA levels were calculated as recommended by the manufacturer. (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). B-actin and GAPDH was used to normalize expression levels. Student's t-test was used for the statistical analyses.

RESULTS AND DISCUSSION

To examine C2C12 myotube responsiveness to serum and other growth factors, we chose to analyze the transcriptional activation or repression of c-fos, c-myc, c-jun, Id-1, cyclinD1, and E2F6. C-fos, c-myc and c-jun can promote cell cycle progression and activation of cyclins, such as cyclin D1, in most cells.[26,27] In addition to promoting cell cycle progression, Id-1 also counteracts myogenic differentiation by inhibiting bHLH proteins, such as MyoD.[28,29] E2F6 has been shown to be involved in maintaining cells in the quiescent G_0 state by gene silencing as part of a protein complex, which contains polycomb group-related proteins.[30,31] Hence downregulation of E2F6 could be associated with the traversing of the G_0/G_1 boundary.

In order to analyze the transcriptional response of mammalian myotubes following various external stimuli and to avoid contamination by mononucleate myogenic progenitor cells, we double purified individual C2C12 myotubes using a glass capillary immediately before the mRNA preparations. Figure 1A shows a schematic cartoon of the purification process and Figure 1B illustrates a representative sample of purified myotubes with no contamination by mononucleate cells. cDNAs were synthesized from mRNA samples obtained from ten myotubes, which in turn served as templates in each qRT-PCR. The specificity of the oligonucleotide primers was determined by control PCRs using mRNA prepared from cycling mononucleate myogenic cells as template. Figure 1c shows that each primer pair amplifies only one DNA species of the expected size. The specificity was further confirmed by one single melting point in the qRT-PCRs indicating no heterogeneity in the amplified DNA fragments (data not shown).

We first stimulated myotubes with serum. In agreement with previous observations[23] we saw a significant upregulation of c-fos after 1 hour, but the c-fos response was no longer evident after 4 hours (Fig. 2A). Serum also caused significant upregulation of Id-1 both at 1 hour and 4 hours (Fig. 2B). In contrast to earlier observations,[21,23] we could not detect any significant upregulation of c-myc (Fig. 2D) and we saw only a moderate upregulation of c-jun after 4 hours (Fig. 2C). Increased amounts of c-jun proteins could however be visualized in cultured myotubes by indirect immunocytochemical analyses (Fig. 3), indicating that transcriptional activation was followed by the translation of c-jun mRNA. No upregulation of cyclin D1 could be detected (data not shown). In fact, in most of the cases no cyclin D1 mRNA could be detected. In contrast cycling mononucleate cells contained detectable levels of cyclinD1 mRNA (data not shown). It is likely that the discrepancy between the current and previous results stems from the differences in purity of the myotubes. In previous studies mononucleate contamination of C2C12 myotube culture was reduced using AraC, a protocol which does not eliminate all cycling progenitors.

We next set out to define which extracellular factors are able to elicit these responses. In particular we were interested in comparing the effects of conventional peptide growth factors and the thrombin-activated serum factor. We treated cells either with PDGF, FGF, EGF, crude preparation of thrombin, or with a partially purified fraction from serum that is a substrate of thrombin and elicits S-phase reentry in newt myotubes.[20] This serum fraction was characterized earlier and was designated the working name HEP3 based on its purification through a heparin column.[20] The HEP3 fraction caused S-phase reentry by quiescent salamander myotubes (data not shown). Quiescent mononucleate myogenic cells did not replicate their DNA above the background level (data not shown), indicating that the HEP3 fraction is largely free from peptide growth factors.

We saw that PDGF, FGF, EGF, crude thrombin and HEP3 evoked different transcriptional responses. Similarly to serum c-fos mRNA was significantly upregulated after stimulation by crude thrombin, HEP3, FGF, and EGF (Fig. 2A). However, Id-1 mRNA did not increase as a response either to PDGF, FGF or EGF (Fig. 2B). In contrast both crude thrombin and HEP3 treatment caused significant upregulation of Id1. c-jun upregulation was only caused by the purified HEP3 fraction, but not by PGDF, FGF or EGF (Fig. 2C). Interestingly none of the tested factors could evoke upregulation of c-myc (Fig. 2D). None of the serum components caused upregulation of cyclin D1 (data not shown). These findings indicate that except for c-fos, immediate early gene activation in mouse myotubes is not due to conventional peptide growth factor. Instead the thrombin-activated serum factor appears to be responsible for a broader reactivation of immediate early genes. The observation that neither c-myc nor cyclinD1 became activated after

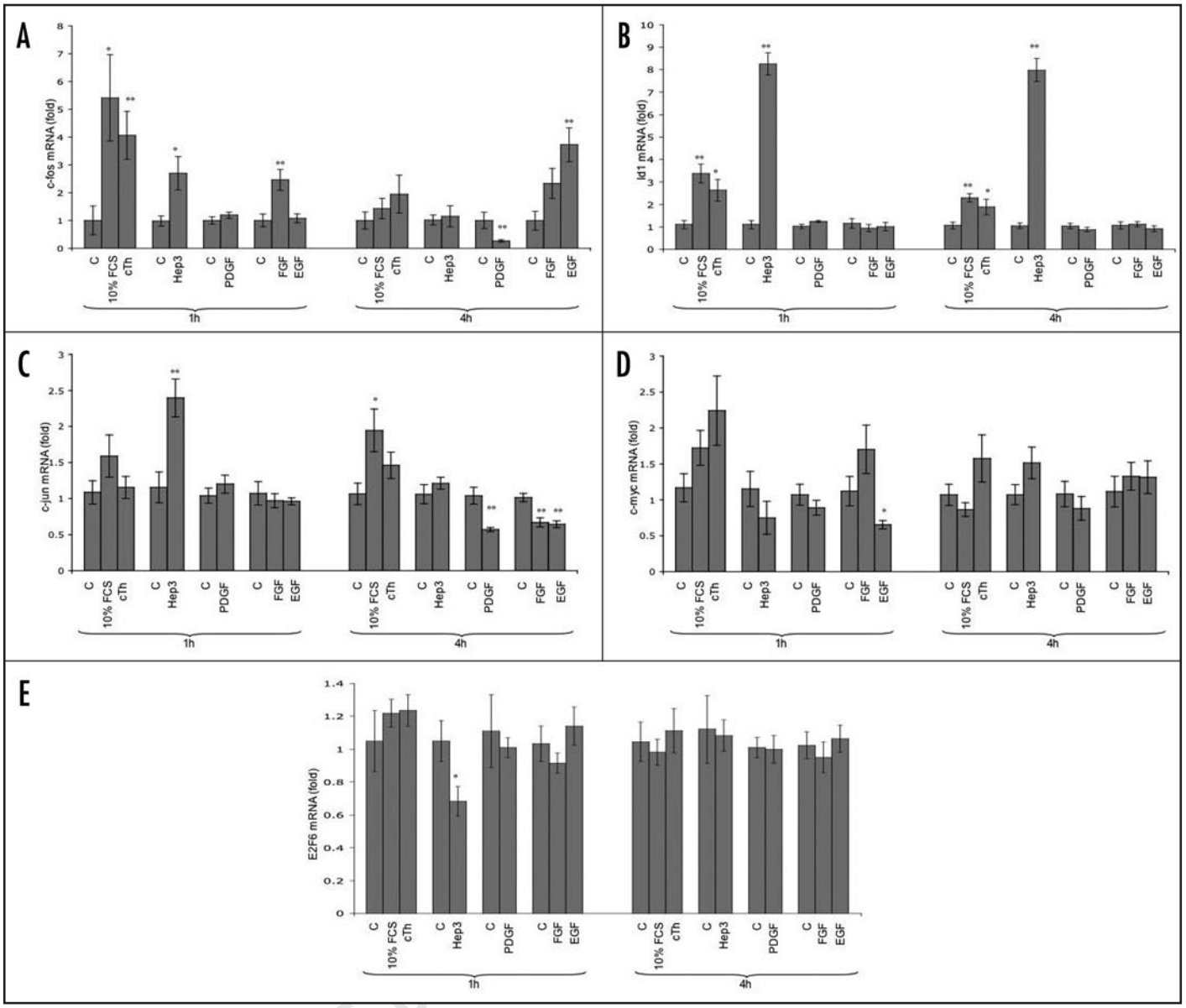

Figure 2. Quantification of various mRNAs after myotube stimulation. Relative levels of mRNAs are indicated compared to controls in differentiation medium (C). cTh, crude preparation of thrombin; HEP3, partially purified thrombin activated factor. $p \leq 0.05$ is indicated by *, $p \leq 0.01$ is indicated by **.

any of the stimuli is consistent with the view that c-myc upregulation precedes cyclinD1 upregulation and subsequent S-phase transition by differentiated myotubes.[22]

We also examined the regulation of E2F6. E2F6 has been implicated in maintaining cells in the quiescent G_0 phase by silencing c-myc responsive genes, such as cyclinD1, and its downregulation is associated with cell cycle reentry. We saw that the HEP3 fraction was the sole stimulus that caused a small but significant downregulation of E2F6 (Fig. 2E), further indicating that HEP3 fraction is more potent in undermining the postmitotic arrest than PDGF, FGF or EGF. The HEP3 fraction evokes a broader response than serum from which it was purified. This may be due to the presence of additional inhibitory or anti-mitotic signals present in serum.

E2F6 is an unusual member of the E2F family of proteins since it cannot bind pocket proteins, such as the retinoblastoma protein (pRb), and does not possess a transactivation domain.[32] Rather, E2F6 forms complex with chromatin-modifiers. The complex contains among others histone methyl transferases and polycomb proteins that are associated with silent promoters containing a classical repressor mark, histone 3 methylated on lysine 9 (H3K9m).[33] G_1 transition and cell cycle progression is concomitant with the release of silencing components and E2F6 is not present on promoters where H3K9 is unmethylated.[31] Since the role of E2F6 is intimately coupled to H3K9 methylation we tested by immunocytochemical analyses whether serum stimulation causes large-scale changes in H3K9 methylation in myotubes. In accordance with the lack of E2F6 downregulation upon serum stimulation we could not detect any global change in the methylation status using antibodies raised against methylated H3K9 in mouse C2C12 myotubes. In contrast salamander myotubes responded to serum stimulation by large-scale reversal of di-methylation on H3K9 (Fig. 4). Immunoreactivity in undifferentiated mononucleate cells is less as compared to myotubes also in differentiation medium, indicating that H3K9 dimethylation occurs upon terminal differentiation (Fig. 4B and H).

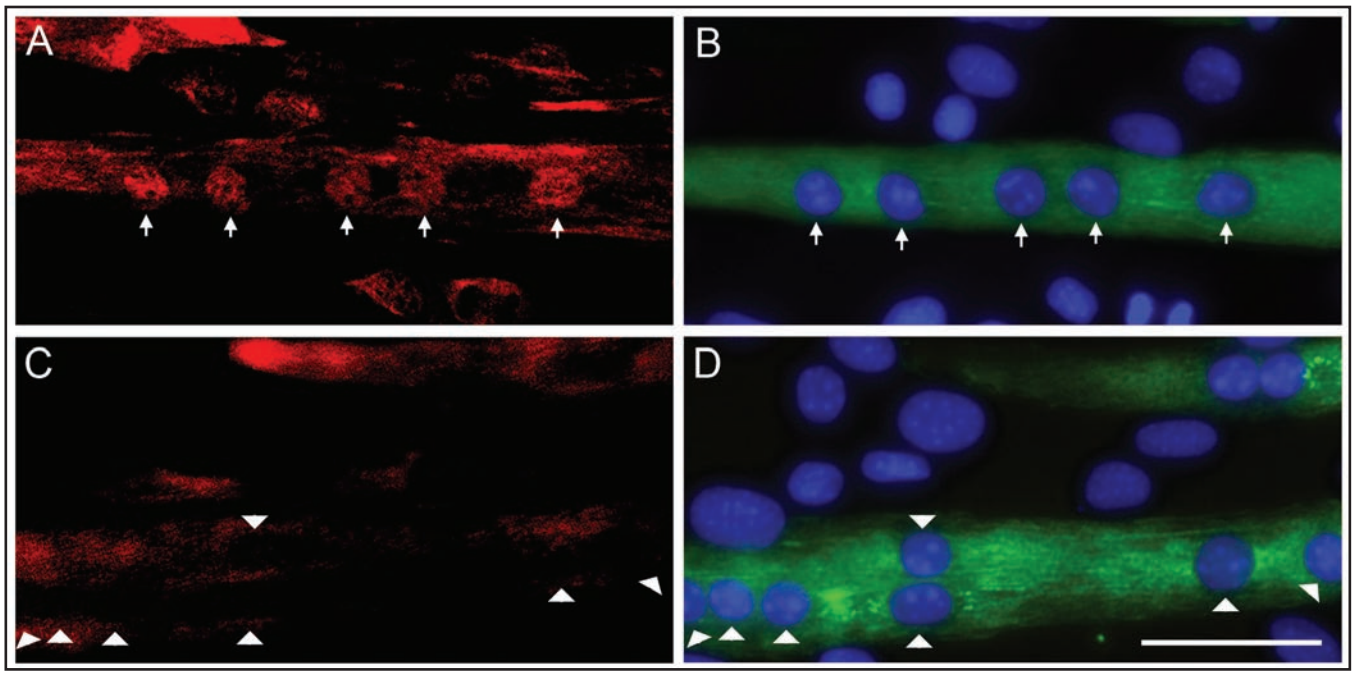

Figure 3. Increased amount of c-jun protein in myotubes stimulated by serum. Example of C2C12 myotube showing increased c-jun immunoreactivity after serum stimulation (A and B), compared to myotubes in differentiation medium (C and D). Red: c-jun, Blue: DAPI, Green: MHC. Arrows point to myonuclei showing c-jun immunostaining. Arrowheads point to myonuclei negative for c-jun immunostaining. Scale bar: 50 μm.

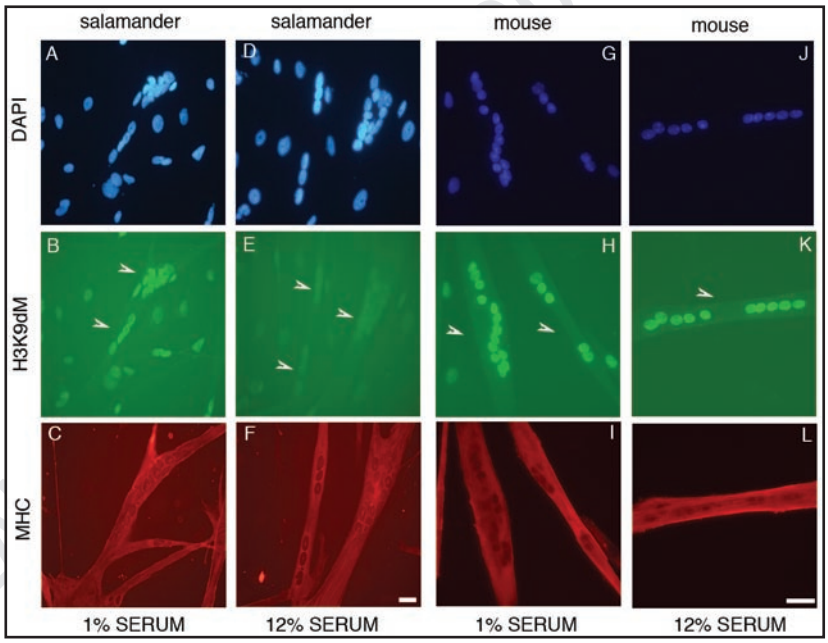

Figure 4. Reversal of H3K9 di-methylation in salamander but not in mouse myotubes after serum stimulation. Intensive immunoreactivity for dimethylated H3K9 after 4 days in differentiation medium both in salamander (A–C) and mouse myotubes (G–I). Reversal of H3K9 dimethylation in salamander (D–F) but not in mouse myotubes (J–L) 1 day after serum stimulation. Arrows point to myotubes. Note that nuclei in myotubes show more intense immunoreactivity than in mononucleate cells. Scale bars: 50 μm.

These data suggest that, although some components of the molecular machinery of cell cycle reentry can be activated also in quiescent mammalian myotubes, the lack of H3K9-demethylation is one aspect of mammalian myotubes' inability to advance further in the mitotic cycle. One way forward could be to address which factors are responsible for demethylation in salamanders and whether demethylation could activate the pathways in mammalian myotubes, which lead to cell cycle progression. The role of chromatin modifiers, such as recently identified demethylases,[34] and their associated partners may be crucial in these processes.

Further comparison of the cell cycle regulators in mammalian and salamander myotubes is needed to understand why mammalian

myotubes do not replicate the DNA after stimulation with the thrombin activated factor. S-phase reentry in salamander myotubes requires the inactivation of the retinoblastoma protein (pRb)[35] but, interestingly, inactivation of pRb is not sufficient for inducing S-phase in terminally differentiated mammalian skeletal muscle.[36,37] Whether inactivation of pRb is sufficient for S-phase reentry in salamanders is still unclear. The increasing amount of genetic information in salamanders will allow large-scale cross species comparisons to identify the crucial components of S-phase progression and to reveal the signaling pathways through which the thrombin activated serum factor causes DNA replication.

Taken together our results support the conclusions from earlier experiments that mammalian myotubes are able to exit quiescence. Importantly, in contrast to previous assumptions we show that conventional peptide growth factors such as PDGF, FGF and EGF contribute relatively little to this process. Instead our data suggests that signaling through the thrombin-activated serum factor in myotubes is an evolutionarily conserved mechanism. However full reentry into S-phase is prevented by yet unidentified factors downstream of the immediate early genes.

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