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Histological, transcriptomic and *in vitro* analysis reveal an intrinsic activated state of myogenic precursors in hyperplasic muscle of trout

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1 **Abstract**

2 **Background**

3 The dramatic increase in myotomal muscle mass in post-hatching fish is related to their ability to
4 lastingly produce new muscle fibres, a process termed hyperplasia. The molecular and cellular
5 mechanisms underlying fish muscle hyperplasia largely remain unknown. In this study, we aimed to
6 characterize intrinsic properties of myogenic cells originating from fish hyperplastic muscle. For this
7 purpose, we compared *in situ* proliferation, *in vitro* cell behavior and transcriptomic profile of
8 myogenic precursors originating from hyperplastic muscle of juvenile trout (JT) and from non-
9 hyperplastic muscle of fasted juvenile trout (FJT) and adult trout (AT).

10 **Results**

11 For the first time, we showed that myogenic precursors proliferate in hyperplastic muscle from JT as
12 shown by *in vivo* BrdU labeling. This proliferative rate was very low in AT and FJT muscle.
13 Transcriptomic analysis revealed that myogenic cells from FJT and AT displayed close expression
14 profiles with only 64 differentially expressed genes (BH corrected p-val < 0.001). In contrast, 2623
15 differentially expressed genes were found between myogenic cells from JT and from both FJT and
16 AT. Functional categories related to translation, mitochondrial activity, cell cycle, and myogenic
17 differentiation were inferred from genes up regulated in JT compared to AT and FJT myogenic cells.
18 Conversely, Notch signaling pathway, that signs cell quiescence, was inferred from genes down
19 regulated in JT compared to FJT and AT. In line with our transcriptomic data, *in vitro* JT myogenic
20 precursors displayed higher proliferation and differentiation capacities than FJT and AT myogenic
21 precursors.

22 **Conclusions**

23 The transcriptomic analysis and examination of cell behavior converge to support the view that
24 myogenic cells extracted from hyperplastic muscle of juvenile trout are intrinsically more potent to

25 form myofibres than myogenic cells extracted from non-hyperplasic muscle. The generation of gene
26 expression profiles in myogenic cell extracted from muscle of juvenile trout may yield insights into
27 the molecular and cellular mechanisms controlling hyperplasia and provides a useful list of
28 potential molecular markers of hyperplasia.

29 **Background**

30 Post-hatching muscle growth in most teleost fish occurs in two processes. The first process which is
31 common with amniotes refers to increase of fibre size and is termed hypertroph. The second process
32 refers to the formation of new muscle fibers throughout the entire myotome and is termed
33 hyperplasia [1–3]. A persistence of hyperplastic growth after juvenile stage was reported in large
34 final size fish as gilthead bream [4], carp [5], european sea bass [6] and rainbow trout [7, 8].
35 Nevertheless, this production of new muscle fibers decreases with age [7], and hyperplasia was no
36 longer observed in 18-months old trout [8]. Furthermore, it is well known that fasting stops growth
37 [9] and an inhibition of *in vitro* proliferation of myogenic precursors in fasted rainbow trout has
38 been observed [10].

39 Muscle hyperplasia requires muscle stem cells, also called satellite cells [11] which are localized
40 between myofibre and basal lamina. Once activated during development, growth or after muscle
41 injury, myogenic precursors proliferate and differentiate to eventually form nascent myofibres [12,
42 13]. Satellite cells have been clearly identified *in situ* in muscle of carp [14] and zebrafish [15]. *In*
43 *vitro*, myogenic precursors extracted from trout and carp muscle proliferate and fuse into myotube
44 [10, 16, 17]. Whether myogenic progenitors of fish hyperplastic muscle exhibit specific
45 physiological state is largely unknown. To test this hypothesis, we extracted these cells from
46 hyperplastic muscle of juveniles growing trout (JT), and non-hyperplastic muscle of fasted juvenile
47 trout (FJT) and adult trout (AT), and compared their ability to proliferate *in situ*, their transcriptome
48 and their proliferation and differentiation capacities in culture.

49 Our results converge to support the view that myogenic cells extracted from hyperplastic muscle of
50 juvenile trout are intrinsically more potent than myogenic cells extracted from non-hyperplastic
51 muscle.

52 **Results**

53 **Myogenic precursors proliferate in hyperplasic muscle during post-larval growth**

54 In order to quantify the number of proliferative satellite cells in trout of 5g, 500g and fasted trout of
55 5g, we developed immunofluorescence analysis to spot proliferative nuclei in satellite cell position,
56 i.e. located under the basal lamina. For this purpose, we injected fish with BrdU and performed
57 immunofluorescence analysis with an antibody against BrdU and laminin a major component of
58 basal lamina. As shown in figure 1, the percentage of BrdU positive nuclei in juvenile trout was
59 7,2%, whereas this proportion dropped to 1.3% in larger trout (500g) and 0,1% in 3-week fasted
60 juvenile trout.

61 **Myogenic precursors extracted from hyperplasic and non-hyperplasic trout muscles exhibit** 62 **distinct transcriptome**

63 To better known the intrinsic molecular properties of myogenic precursors from hyperplasic muscle,
64 we compared the transcriptome of myogenic precursors extracted from juvenile trout (JT)
65 displaying hyperplasic muscle growth with that of myogenic precursors extracted from non-
66 hyperplasic muscle resulting from fasted juvenile trout (FJT) and adult trout (AT). For this purpose,
67 we first compared gene expression profiles between myogenic precursors from FJT and AT in order
68 to identify genes whose differential expression would be specifically related to age or fasting.
69 LIMMA statistical test [18] (BH corrected p-val < 0.001) showed that only 64 genes were
70 differentially expressed between FJT and AT samples. These differentially expressed genes (DEGs)
71 were subsequently discarded for further analysis. Using two LIMMA statistical tests (BH corrected
72 p-val < 0.001) a total of 3992 DEGs were identified between JT and FJT and 4253 DEGs between
73 JT and AT. Then, we retained common genes found in this two differential analysis and found a
74 total of 2623 differentially expressed genes between hyperplasic (JT) and non-hyperplasic muscle
75 (FJT and AT). These differentially expressed genes were then hierarchically clustered. The
76 unsupervised clustering, which is shown in figure 2 (available as supplemental data file 1), resulted

77 in the formation of two major gene clusters. The cluster 1 comprised 1865 genes up regulated in JT
78 myogenic precursors and cluster 2 comprised 758 genes down regulated in JT myogenic precursors.

79 **JT myogenic precursors exhibit transcriptomic signature of activated state cell**

80 DAVID analysis of the 1206 eligible genes from cluster 1 revealed significant enrichment (table 1)
81 in genes involved in translation ($p=2.8E^{-26}$), mitochondrial activity ($p=3,85E^{-11}$) and oxidative
82 phosphorylation ($p=7,31E^{-12}$). Among other significant functional categories inferred from up
83 regulated genes in JT myogenic precursors, we found the GO term mitotic cell cycle ($p=2.26E^{-20}$).
84 Genes belonging to this functional category included genes encoding cell division cycle (cdc)
85 proteins (8), cyclin dependent kinases (6), cyclins (6), genes involved in chromosomes segregation
86 (20) as shown in figure 3. Enrichment in gene involved in DNA metabolic process and replication
87 such as minichromosome maintenance complex components, non-homologous end-joining factor1,
88 DNA polymerases, DNA primases, DNA topoisomerases, replication proteins were also found.
89 Cluster 1 also included many genes encoding epigenetic transcriptional regulators. Among them
90 were Swi/Snf chromatin enzymes and several DNA (cytosine-5-)-methyltransferases. We found also
91 many genes encoding extracellular components including collagens (14 genes), laminin subunits (3
92 genes) and entactin as well as genes contributing to the formation of the myofibrils (i.e, 8 genes
93 encoding myosins, 5 genes encoding troponins and 3 genes encoding tropomyosins). At last,
94 besides the large number of myofibrillary proteins, we found many genes involved in myoblast
95 differentiation and fusion such as *six1b*, *six4b*, *mef2d*, *myogenin*, *tmem8c* (*myomaker*), *muscle*
96 *creatine kinase* (figure 4). Overall, cluster 1 showed enrichment in genes involved in protein
97 synthesis, cell division and myogenic differentiation.

98

99 **Genes associated with the quiescent state are down regulated in JT myogenic precursors**

100 Cluster 2 comprised genes that were down regulated in JT myogenic precursors compared to both
101 FJT and AT myogenic precursors. In this cluster, we identified genes of the Notch pathway,

102 suggesting a repression of quiescent state. Associated with this quiescence state pathway we found
103 *jagged1b*, *jagged2b*, *dll4*, *dlc*, *notch1a*, *notch1b*, *notch1*, *her6* and *hey1* among genes contained in
104 cluster 2. We detected some genes which play repression roles in proliferation as *hexim1b* [19],
105 *stat3* [20], and *Dach1* also known to inhibit Six protein activity [21]. Among the down regulated
106 genes in JT myogenic precursors, we distinguished genes which plays repression roles in myogenic
107 differentiation as *ddit3* [22], *trim33* [23], *bhlhe40* [24], *tall* [25]. Moreover, a marker of quiescent
108 satellite cells [26], *nestin* was down regulation in JT myogenic precursors. We also observed a
109 global repression of the TGF β pathway in JT myogenic precursors. Indeed, 7 genes involved in
110 TGF β pathway, were down regulated in JT myogenic precursors (*tgfb2*, *tgfbr1*, *bmpr2b*, *bmpr1bb*,
111 *smad3b*, *smad6a* and *acvrrl1*) whereas 5 inhibitors of TGF β pathway were up regulated in JT
112 myogenic precursors (*Bmp7a*, *gremlin2*, *dcn*, *fstl1b* and *fsta*). Overall, cluster 2 showed enrichment
113 in genes involved in inhibition of proliferation, repression of myogenic differentiation and
114 maintenance of cellular quiescent state.

115 **JT myogenic precursors have an enhanced intrinsic capacity of for *in vitro* proliferation**

116 To know more about the intrinsic molecular properties of myogenic precursors of hyperplastic
117 muscle compared to myogenic precursors of non-hyperplastic muscle, we carried out a primary cell
118 culture of myogenic progenitors extracted from JT, FJT and AT conditions. Cell proliferation assays
119 using BrdU showed a higher proliferation rate of JT myogenic precursors (40.1%) after two days of
120 culture compared to FJT (0.8%) and AT (10.3%) myogenic precursors (figure 5). Then, to determine
121 whether the transcriptomic activation signatures were related to a differential cell behavior
122 regarding proliferating capacity, we also measured the proliferation rate of JT, FJT and AT
123 myogenic cells at 5, 8 and 11 days after plating. In JT myogenic precursors the proliferation rate
124 increased from D2 to reach a maximum rate at D5 with 61.4% of BrdU positive nuclei, then the
125 proliferation rate decreased from D8 to 42.4% to D11 to 31.4%. In sharp contrast, proliferation rate
126 of FJT myogenic precursors remained low and tended to increase up to 12,9% at D8. For AT
127 myogenic precursors, the proliferation rate increased at D5 to 48.3% to almost reach the

128 proliferation rate in JT myogenic precursors and decreased from D8 to 31.6% and at D11 to 19.1%.
129 Thus, the kinetic of proliferation of the AT precursors was close to that one of JT but with a lower
130 rate from D5 to D11. Overall, myogenic precursors of JT exhibit a global enhanced proliferation
131 capacity under *in vitro* conditions compared to FJT and AT.

132 **JT myogenic precursors have an enhanced capacity for *in vitro* myogenic differentiation**

133 To go further on the characterization of the intrinsic molecular properties of myogenic precursors of
134 hyperplastic muscle, we quantified the *in vitro* differentiation capacities of JT, FJT and AT
135 myogenic precursors. At D2, we observed an extremely low differentiation rate in JT (1.4%), FJT
136 (1%) and AT (1.6%) myogenic precursors (figure 6). This result indicates that very few myocytes
137 were extracted at the beginning of the cell culture. Then, we also measured the differentiation rate at
138 5, 8 and 11 days after plating of JT, FJT and AT myogenic progenitors. In JT myogenic precursors
139 the differentiation rate increased at D5 to 11.6%, D8 to 24.4% and reach a maximum rate at D11
140 with 28% of nuclei contained in myosin positive cells. In sharp contrast, the differentiation of FJT
141 precursors remained very low during the first 8 days (<0.5%) then a differentiation resumption was
142 observed at D11 (6.4%). For AT myogenic precursors, no significant increase of the differentiation
143 rate was observed even after 11 days of culture. Overall, JT myogenic precursors exhibited a global
144 enhanced differentiation capacity under *in vitro* conditions.

145 Evaluation of the expression level by qPCR of *myogenin* and *myomaker* after 2 days in cell culture
146 validated the transcriptomic results as shown in figure 7. Indeed, the expression of *myogenin* and
147 *myomaker* were higher in JT myogenic precursors compared to AT and FJT myogenic precursors. In
148 addition, the expression level of *myogenin* and *myomaker* after 8 days in culture increase in FJT
149 myogenic precursors. These were contrasting with expression level in AT myogenic precursor that
150 did not exhibit such an increase between D2 and D8. Overall, qPCR data validated our previous
151 results with JT myogenic precursors as more engaged in differentiation program than AT and FJT
152 myogenic precursors.

153 **Discussion**

154 Post-hatching muscle growth in most teleost such as trout, lastingly occurs by fiber hypertrophy and
155 formation of new muscle fibers. This latter process, termed hyperplasia, requires proliferation,
156 differentiation and fusion of muscle stem cells (satellite cells) to form new multinucleated
157 myofibers. We examined in this study the hypothesis that post-hatching muscle hyperplasia in fish
158 is associated with a peculiar physiological status of myogenic precursors predetermining them to
159 self-renew and differentiate. For this purpose, we examined proliferation of trout satellite cells *in*
160 *vivo* and compared gene expression profiling and *in vitro* myogenic potential of satellite cells
161 extracted from juvenile trout muscle displaying intense hyperplastic growth (JT), with satellite cells
162 extracted from trout muscle that no longer exhibited muscle hyperplasia, namely fasted juvenile
163 trout (FJT) and adult trout (AT).

164 Many studies on mammalian isolated satellite cells were carried out on cells directly isolated from
165 muscle and purified by FACS using fluorescent reporters or cell surface marker [27]. As these
166 technologies cannot yet be used in trout fish, we took advantage of the specific adhesion of satellite
167 cells on laminin substrate to enrich them in culture [17, 28]. Although it has been reported that
168 isolation procedures alter gene expression of myogenic precursors [29, 30], we assumed in this
169 study that the differential *ex vivo* properties of trout satellite cells originating either from
170 hyperplastic or non-hyperplastic muscle, somehow reflect intrinsic differences preexisting before
171 their extraction from muscle.

172 First, we sought to identify and quantify proliferative satellite cells in muscle of growing *versus*
173 non-growing trout using *in vivo* BrdU injection followed by double immuno-labeling of laminin and
174 BrdU. In agreement with Alfei et al (1989)[31], our results clearly evidenced a higher rate of BrdU
175 positive cells in muscle of JT compared to FJT and AT, notably at sites corresponding to the satellite
176 cell niche. This shows that fish hyperplastic muscle contains proliferative satellite cells well after
177 hatching, what sharply contrasts with the mitotic quiescence of satellite cells located in mature
178 mouse muscle [32].

179 Relative to satellite cells from non-hyperplastic muscle, satellite cells from juvenile trout
180 were found to exhibit up-regulated gene set related to high metabolic activity as shown by
181 enrichment in genes involved in translational efficiency and genes encoding structural and
182 functional components of mitochondria, notably those involved in energy production for execution
183 of biosynthesis events. Mitochondrial biogenesis has been associated with the shift from quiescence
184 to proliferation of satellite cells [33, 34]. In keeping with this, our result that matches meta-analyses
185 of multiple transcriptomes revealing low expression of genes associated with oxidative
186 phosphorylation in mouse quiescent satellite cells [35], supports the view that JT cells are
187 intrinsically activated compared to satellite cells from non-hyperplastic muscle. Other major
188 functional categories inferred from genes up-regulated in myogenic precursors derived from
189 hyperplastic muscle were related to DNA replication and cell cycle. This finding, which is quite in
190 agreement with the proliferation rate of these cells measured *in vivo* and *ex vivo*, strongly reinforces
191 the view that satellite cells isolated from trout hyperplastic muscle are in an activated state. Also,
192 several major genes signing myogenic differentiation were found to be overexpressed. Among them
193 were *myogenin* which invalidation prevents myogenic differentiation in mouse [36] and *myomaker*
194 which is necessary for myoblast fusion into myotube as shown by gene invalidation [37]. In
195 keeping with this, it is interesting to note that mitochondrial activity, which is higher in JT satellite
196 cells relative to FJT and AT cells, has been reported to positively regulate myogenesis [38].
197 Conversely, transcriptome of FJT and AT myogenic precursors, compared to that of JT myogenic
198 precursors, revealed up regulation of genes involved in maintenance of stem cell quiescence,
199 notably genes involved in Notch signaling [39] or known as marker of quiescent muscle stem cell.
200 These results are in agreement with data obtained in mouse showing an up regulation of *notch* and
201 *Hey* genes in quiescent satellite cells [40]. In addition, the up regulation of several genes involved in
202 TGFbeta pathway was in line with a repression of differentiation of myogenic precursors [41].
203 Indeed, we notably observed an up-regulation of BMP receptor type 1 which knock-down in mouse
204 satellite cells caused premature myogenic differentiation [42]. All these data support the view that

205 satellite cells extracted from muscle of fasted trout or adult trout are close to a quiescent state
206 compared to satellite cells from juvenile trout.

207 Another major result of our study was that behavior of satellite cells from hyperplastic
208 muscle quite differs from that of satellite cells extracted from non-hyperplastic muscle. Specifically,
209 we found that cultured JT myogenic precursors exhibited higher proliferation rate and
210 differentiation capacities than FJT and AT myogenic precursors. These observations, that match
211 transcriptome data, further support the view that myogenic cells from hyperplastic muscle of
212 juvenile trout are intrinsically more potent to form myofibres than satellite cells from non-
213 hyperplastic muscle.

214 What could determine intrinsic myogenic capacity of JT cells ? One possible cause, inferred
215 from transcriptome analysis, could relate to epigenetic regulations of transcription. Indeed, up-
216 regulation of genes involved in DNA methylation was found in JT myogenic precursors, notably
217 several DNA methyl transferase (dnmt1, 3ab and 3b) known to be involved in muscle stem cell
218 activation [43]. Furthermore, as previously reported in hyperplastic growth zone of trout larvae [44]
219 and in activated satellite cells of mouse and trout regenerating muscle [45], we observed in JT cells
220 the overexpression of many SWI/SNF chromatin remodeling enzymes, which dynamic recruitment
221 regulate many stages of myogenesis [46].

222 **Conclusion**

223 The satellite cells from muscle of trout juveniles exhibit *in vivo* and *ex vivo* features of
224 activation that are not found in satellite cells isolated from non-hyperplastic muscle. Thus, muscle
225 hyperplastic growth in fish likely relates to the fact that satellite cells in these animals are
226 intrinsically potent to form myofibres well after hatching.

227

228 **Methods**

229 **Animals**

230 Rainbow trout (*Oncorhynchus mykiss*) weighting from 2g to 2kg were raised to a 12 h light:12 h
231 dark photoperiod and 12 ± 1 °C in a recirculating rearing system located in the Laboratory of
232 Physiology and Genomics of Fish. Fish were fed daily *ad libitum* on a commercial diet or starved
233 during 3 or 4 weeks.

234 **Measurement of satellite cells proliferation in situ**

235 Intra-peritoneal injections (150µg/g of body weight) of BrdU (Roche, no. 280 879), dissolved in a
236 solution composed with NaOH (0.02N) diluted with NaCl 0.9%, were performed on juvenile
237 rainbow trout (*Oncorhynchus mykiss*) (2g, n = 5), 4 weeks fasted juvenile rainbow trout (5g, n = 5)
238 and 400-500g rainbow trout (n = 6) which exhibited a diminution of hyperplasia.

239 Muscle tissues were fixed in Carnoy fixative solution for 48 h at 4°C, progressively dehydrated and
240 embedded in paraffin. Transverse paraffin sections (10 µm thick) were stained with laminin
241 antibody (DSHB, D18-c) and BrdU labeling and detection kit (Roche Diagnostics, no. 11 296 736
242 001) was used following the recommendations of manufacturer to measure the proliferation of the
243 cells. Briefly, tissues were incubated for 30 min at 37 °C with mouse IgG1 anti-BrdU (kit:
244 11296736001, Sigma) and, after 1h incubation at room temperature in saturation buffer (BSA 1%,
245 04-100-811C in PBST 0.1%), tissues were incubated overnight at 4°C with mouse IgG2a anti-
246 laminin (DSHB, D18-c). The secondary antibody were diluted (1/1000, Alexa 488 anti-IgG1 mouse
247 A21121 to detect BrdU and Alexa 594 anti-IgG2a mouse A21135 to detect laminin) in PBST and
248 applied for 1 h at room temperature. Tissues were then mounted in Mowiol containing 0.5 µg/ml of
249 DAPI. Tissues cross sections were photographed using a Nikon digital camera coupled to a Nikon
250 Eclipse 90i microscope. At least five images were taken per tissues and the number of nuclei BrdU
251 positive localized between basal lamina and myofiber on the total number of nuclei under basal
252 lamina (myo-nuclei) were calculated using cell counter plugin in Fiji software.

253 **Isolation of trout precursor myogenic cells**

254 For all studies, myogenic precursors were isolated from juvenile trout (5g, JT), from 3-4 weeks
255 fasted juvenile rainbow trout (5g, FJT) and from adult rainbow trout (1.5-2kg, AT) as previously
256 described [17]. Isolated myogenic precursors were plated on poly-L-lysine and laminin-coated
257 plates at 80,000 cells per cm² for every analysis except to proliferation measurement which were
258 60,000 cells per cm².

259 **Gene expression analysis**

260 Using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA were extracted from cells
261 according to the manufacturer's recommendations. The total RNA (200ng) were reverse transcribed
262 into cDNA using the High Capacity cDNA Reverse Transcription kit, (Applied Biosystems) and
263 random primers, according to the manufacturer's instructions. Target gene expression levels were
264 determined by qPCR using specific primers (forward primer sequences; *myogenin* :
265 AGCAGGAGAACGACCAGGGAAC, *myomaker* : AATCACTGTCAAATGGTTACAGA, and
266 reverse primer sequences ; *myogenin* : GTGTTGCTCCACTCTGGGCTG, *myomaker* :
267 GTAGTCCCCTCCTCGAAGT). Primers were design on two exons to avoid genomic
268 amplification. Quantitative PCR was performed on a StepOnePlus thermocycler (Applied
269 Biosystems) using SYBR FAST qPCR Master Mix (PowerUp SYBR Green Master Mix kit,
270 A25742, Applied Biosystems). Relative quantification of the target gene transcripts was made using
271 18S gene expression as reference. Quantitative PCR was performed using 10 µl of the diluted
272 cDNA mixed with 300nM of each primer in a final volume of 20 µl. The PCR protocol was initiated
273 at 95°C for 3 min for initial denaturation followed by the amplification steps (20 sec at 95°C
274 followed by 30 sec at 60°C) repeated 40 times. Melting curves were systematically monitored at the
275 end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR
276 run included replicate samples (duplicate of PCR amplification) and negative controls (RNA-free
277 samples, NTC).

278 **Microarray slides**

279 An Agilent-based microarray platform with $8 \times 60K$ probes per slide was used (GEO platform
280 record: GPL24910). Microarray data sets have been submitted to the GEO-NCBI with the accession
281 number: GSE113758.

282 **RNA labeling and hybridization**

283 RNA from (i) five distinct pools of 24H-cultured myogenic precursors from juvenile trout (JT), (ii)
284 five distinct pools of 24H-cultured myogenic precursors from 3-4 weeks fasted juvenile trout (FJT)
285 and (iii) six distinct pools of 24H-cultured myogenic precursors from adult trout (AT) were used for
286 labelling and hybridization. For each sample, 150ng of RNA was Cy3-labelled according to the
287 manufacturer's instructions (One-Color Microarray-Based Gene Expression Analysis (Low Input
288 Quick Amp Labeling) Agilent protocol). Briefly, RNA was first reverse transcribed, using a polydT-
289 T7 primer, Cy3 was incorporated by a T7 polymerase-mediated transcription and excess dye was
290 washed using an RNeasy kit (Quiagen). The level of dye incorporation was evaluated using a
291 spectrophotometer (Nanodrop ND1000, LabTech). 600 ng of labelled cRNA was then fragmented in
292 the appropriate buffer (Agilent) for 30 minutes at 60°C before dilution (v/v) in hybridization buffer.
293 Hybridizations were performed in a microarray hybridization oven (Agilent) for 17h at 65°C, using
294 two Agilent $8 \times 60K$ high-density oligonucleotide microarray slides. Following hybridization, the
295 slides were rinsed in gene expression wash buffers 1 and 2 (Agilent).

296 **Data acquisition and analysis**

297 Hybridized slides were scanned at a 3- μ m resolution using the Agilent DNA microarray Scanner.
298 Data were extracted using the standard procedures contained in the Agilent Feature Extraction (FE)
299 software version 10.7.3.1. One AT sample that did not give good quality signal on microarray was
300 discarded from the gene expression analysis. Arrays were normalized using GeneSpring software
301 version 14.5. Using R software (3.2.2) a LIMMA (3.26.9) statistical test [18] (BH corrected p-val <
302 0.001) was used to find differentially expressed genes between FJT and AT. Secondly, two LIMMA

303 statistical tests (BH corrected p-val < 0.001) were used to find differentially expressed genes
304 between JT and FJT, and between JT and AT. We kept significant differentially expressed genes
305 with an expression mean in at least one condition above or equal to 6, corresponding at 3 times
306 background (normalized values). Thirdly, we kept commons genes found in this two differential
307 analysis in the same regulation way with JT as referential condition. For clustering analysis, log
308 transformed values were median-centred and an average linkage clustering was carried out using
309 CLUSTER 3.0 software and the results were visualized with TreeView software. GO enrichment
310 analysis was performed using Database for Annotation, Visualization and Integrated Discovery
311 (DAVID 6.7) software tools.

312 **Analysis of cell proliferation**

313 Cells were cultured in presence of 10 μ M BrdU during 24H and cells were collected at days 2, 5, 8
314 and 11. The cells were fixed with ethanol/glycine buffer (100% ethanol, 50 mM glycine, pH 2). A
315 BrdU labeling and detection kit (11296736001, Sigma) was used following the recommendations of
316 manufacturer to measure the proliferation of the cells. Briefly, the cells were incubated for 30 min at
317 37 °C with mouse anti-BrdU, washed, and then incubated with the secondary antibody anti-mouse
318 FITC for 30 min. Cells were then mounted in Mowiol containing 0.5 μ g/ml DAPI. Cells were
319 photographed using a Nikon digital camera coupled to a Nikon Eclipse 90i microscope. Seven
320 images were taken per well and the number of BrdU positive nuclei on the total number of nuclei
321 was automatically calculated using a macro command on Visilog (6.7) software.

322 **Analysis of cell differentiation**

323 On days 2, 5, 8 and 11 of culture, cells on glass coverslips were briefly washed twice with
324 phosphate-buffered saline (PBS) and fixed for 30 min with 4% paraformaldehyde in PBS. After
325 three washes, cells were saturated for 1 h with 3% BSA, 0.1% Tween-20 in PBS (PBST). Cells were
326 incubated at room temperature for 3 h with the primary antibody anti-myosin heavy chain (MyHC,
327 DSHB, MF20-c) in blocking buffer [17]. The secondary antibody were diluted (1/1000, Alexa 488

328 A11001) in PBST and applied for 1 h at room temperature. Cells were mounted with Mowiol
329 containing DAPI (0.5 µg/ml). Cells were photographed using a Nikon digital camera coupled to a
330 Nikon Eclipse 90i microscope. Five images were taken per well and the number of nuclei contained
331 in MyHC positive cells on the total number of nuclei was automatically calculated using a macro
332 command on Visilog (6.7) software.

333 **Statistical analysis**

334 A two-way ANOVA analysis with a Tukey's *post hoc* multiple comparisons test was performed on
335 qPCR data, proliferation ratio and differentiation ratio. A Kruskal-Wallis test with a Dunn's *post hoc*
336 multiple comparisons test was performed on *in situ* satellite cells proliferation data. A p-value below
337 0,05 was considered significant.

338

339 **Declarations**

340 **Ethics approval**

341 Fish used in this study were reared and handled in strict accordance with French and European
342 policies and guidelines of the Institutional Animal Care and Use Committee (no. 3312-20 15121511
343 022362 and 3313-20 15121511 094929), which approved this study.

344 **Availability of data and material**

345 Gene expression data supporting the results of this article are available in the Gene Expression
346 Omnibus (GEO) repository under the accession number: GSE113758.

347 **Competing interests**

348 The authors declare that they have no competing interests.

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351 **Authors' contributions**

352 JCG conceived and supervised the study. SJ, AL and NS performed the experiments. SJ, PYR and
353 JCG analysed the data. JB helped for cell proliferation and differentiation quantification. SJ, PYR
354 and JCG wrote the paper. All authors read and approved the final manuscript.

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358

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Figures

Table 1: Functional categories inferred from up regulated genes in JT myogenic precursors.

Table of the most significant Gene Ontology terms in Biological Process and Cellular Component that were found following functional enrichment analysis (DAVID Software 6.7) among genes up regulated in JT myogenic precursors.

GO terms Biological Process	Number of genes	p-value
GO:0006412 translation	84	2,82E-26
GO:0006119 oxidative phosphorylation	30	7,31E-12
GO:0042775 mitochondrial ATP synthesis coupled electron transport	22	3,85E-11
GO:0000278 mitotic cell cycle	80	2,26E-20
GO:0000280 nuclear division	51	2,38E-14
GO:0048285 organelle fission	52	3,10E-14
GO:0007059 chromosome segregation	20	1,65E-06
GO:0006260 DNA replication	30	3,83E-05
GO:0006259 DNA metabolic process	61	2,01E-05
GO terms Cellular Component	Number of genes	p-value
GO:0005840 ribosome	72	1,61E-29
GO:0030529 ribonucleoprotein complex	102	1,59E-21
GO:0005739 mitochondrion	211	1,77E-44
GO:0070469 respiratory chain	29	6,83E-14
GO:0005839 proteasome core complex	10	3,84E-06
GO:0000776 kinetochore	16	3,10E-04
GO:0030017 sarcomere	13	4,64E-02

Figure 1: Quantification of satellite cells proliferation in hyperplastic and non-hyperplastic muscle of trout (A) Muscle cross sections stained with anti-laminin (red) and anti-BrdU (green) in trout of 2g, 500g and of 3-weeks fasted trout (5g). Nuclei were counter-stained with DAPI (blue) (scale bar = 20µm). (B) Quantification of BrdU positive nuclei (% ±SD) in satellite cells position, (under the basal lamina), in white muscle of trout weighing 2g, 500g and of 3-weeks fasted trout

weighing 5g. Different letters indicates a significant difference between means (Kruskal-Wallis and Dunn's multiple comparisons test; p -value ≤ 0.05 ; $n = 5$).

Figure 2: Hierarchical clustering of differentially expressed genes between JT myogenic precursors and FJT and AT myogenic precursors. Each row represents the expression pattern of a single gene and each column corresponds to a single sample: columns 1 to 5: JT myogenic precursors sampled; columns 6 to 10: FJT myogenic precursors sampled; and columns 11 to 15: AT myogenic precursors sampled. The expression levels are represented by colored tags, with red representing the highest levels of expression and blue representing the lowest levels of expression.

Figure 3: Hierarchical clustering of differentially expressed cell cycle genes between JT myogenic precursors and FJT and AT myogenic precursors. Each row represents the expression pattern of a single gene and each column corresponds to a single sample: columns 1 to 5: JT myogenic precursors sampled; columns 6 to 10: FJT myogenic precursors sampled; and columns 11 to 15: AT myogenic precursors sampled. The expression levels are represented by colored tags, with red representing the highest levels of expression and blue representing the lowest levels of expression.

Figure 4: Hierarchical clustering of differentially expressed myogenic genes between JT myogenic precursors and FJT and AT myogenic precursors. Each row represents the expression pattern of a single gene and each column corresponds to a single sample: columns 1 to 5: JT myogenic precursors sampled; columns 6 to 10: FJT myogenic precursors sampled; and columns 11 to 15: AT myogenic precursors sampled. The expression levels are represented by colored tags, with red representing the highest levels of expression and blue representing the lowest levels of expression.

Figure 5: Proliferation rate of JT, FJT and AT myogenic precursors after 2, 5, 8, 11 days of plating (D2, D5, D8 and D11). Each point represents the mean (% \pm SD) of BrdU positive nuclei ratio for each condition at D2, D5, D8 and D11. Different letters indicates a significant difference between means (two-way ANOVA and Tukey's multiple comparisons test; p-value \leq 0.05; n \geq 5).

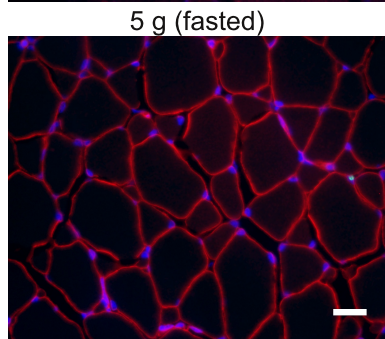
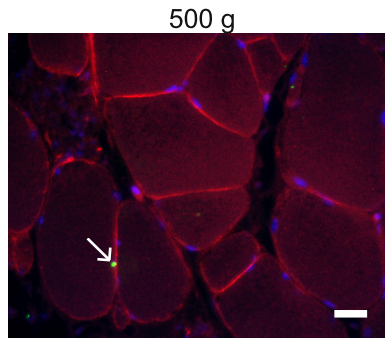
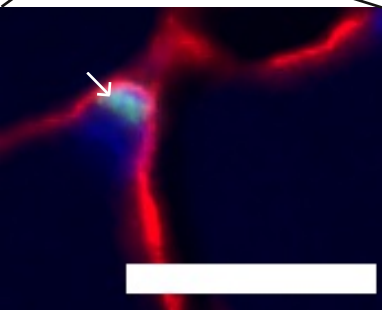
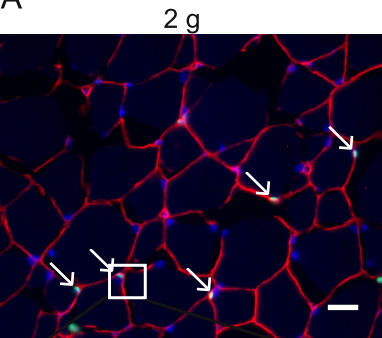
Figure 6: Differentiation rate of JT, FJT and AT myogenic precursors after 2, 5, 8, 11 days in culture (D2, D5, D8 and D11). Each point represents the mean (% \pm SD) of the percentage of nuclei contained in MyHC positive cells for each condition at D2, D5, D8 and D11. Different letters indicates a significant difference between means (two-way ANOVA and Tukey's multiple comparisons test; p-value \leq 0.05; n \geq 6).

Figure 7: Quantification of the expression of *myogenin* and *myomaker* in JT, FJT and AT myogenic precursors. Each bar represents the mean (AU \pm SD) of the expression of *myogenin* (A) and *myomaker* (B) normalized by the expression mean of 18S as referential gene for each condition at D2 and D8. Different letters indicates a significant difference between means (two-way ANOVA and Tukey's multiple comparisons test; p-value \leq 0.05; n \geq 4).

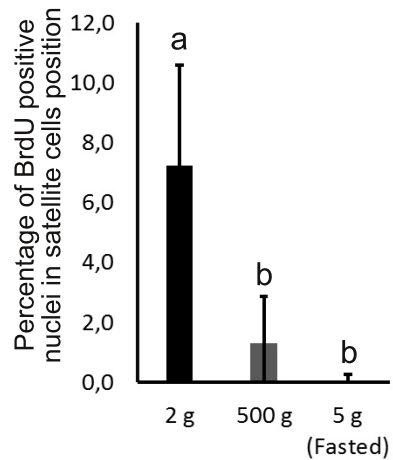
Supplemental data file 1: Differentially expressed genes in myogenic precursors from hyperplastic muscle vs non hyperplastic muscle.

Heat map file for Java treeview visualisation of hierarchical clustering of differentially expressed genes in JT myogenic precursors from hyperplastic muscle vs non hyperplastic muscle (FJT and AT). (CDT 496 ko).

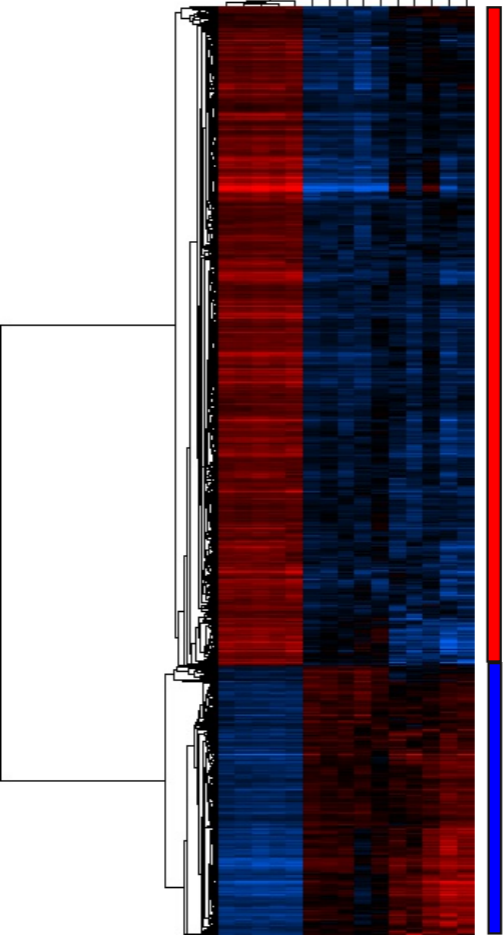
A



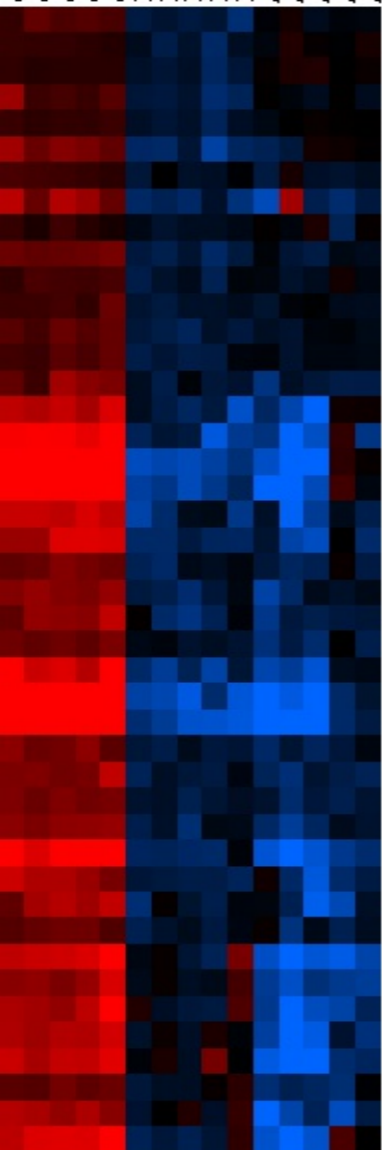
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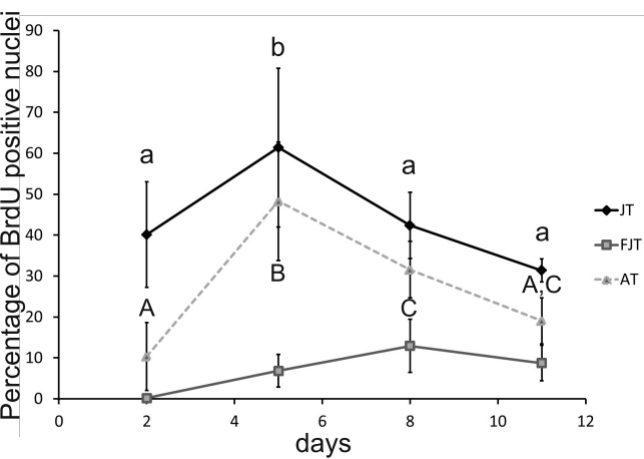
JT_2
JT_1
JT_4
JT_3
JT_5
FJT_1
FJT_2
FJT_4
FJT_3
FJT_5
AT_2
AT_1
AT_4
AT_3
AT_5

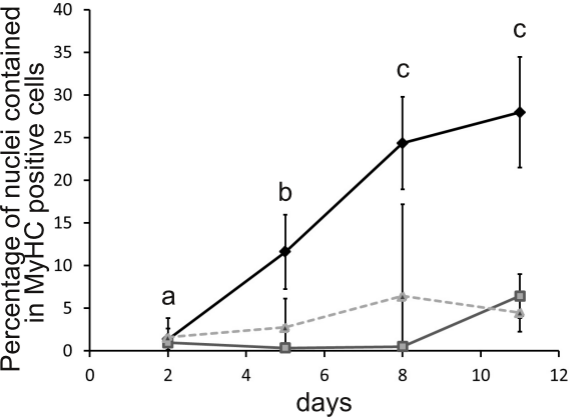


JT_2
JT_1
JT_3
JT_4
JT_5
FJT_1
FJT_2
FJT_4
FJT_3
FJT_5
AT_1
AT_3
AT_5
AT_2
AT_4



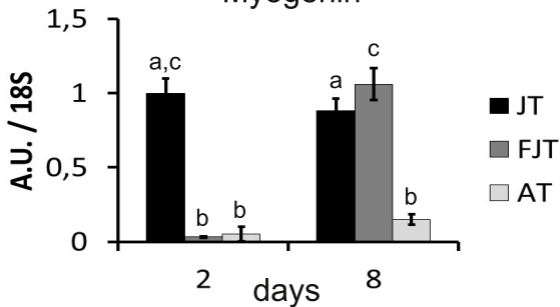
pgm5
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mef2d
sri
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atp2a1
SPTBN1 (1 of 2)
myo10l3
ANK1 (2 of 2)
capzb
cav3
ttnb
ttna
mmp2
myog
myhc4
tpm3
ckmt2a
smydlb
klhl41a
tmem8c
tnni2a.4
MYO9B (1 of 3)
klhl41b
LMOD3
myh10
tnni2a.1
tpm2
mybpc2b
tpma
tnnc2
tnnt3b
tnni2a.2
six1b
six4b
mybphb
tnnc1a
ckma





A

Myogenin



B

Myomaker

