1   **A novel D$_2$O tracer method to quantify RNA turnover as a biomarker of *de***

2   ***novo* ribosomal biogenesis, in vitro, in animal models, and in human skeletal muscle**

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29 **Abstract**

30 Current methods to quantify in vivo RNA dynamics are limited. Here, we developed a

31 novel stable isotope (D_2O)-methodology to quantify RNA synthesis (i.e. ribosomal

32 biogenesis) in cells, animal models, and humans. Firstly, proliferating C2C12 cells were

33 incubated in D_2O-enriched media and myotubes (±) 50ng.ml^{-1} IGF-1. Secondly, rat

34 quadriceps [untrained n=9; 7-wks interval-"like" training n=13] were collected after ~3-

35 wks D_2O (70-Atom%) administration, with body-water enrichment (BWE) monitored

36 via blood sampling. Finally, 10 (23±1y) men consumed 150ml D_2O followed by

37 50ml/wk and undertook 6-wks resistance-exercise (RE; 6×8 repetitions, 75%-1RM

38 3/wk) with BWE monitored by saliva sampling and muscle biopsies (for determination

39 of RNA synthesis) 0-3-6-wks. Ribose mole percent excess (r-MPE) from purine

40 nucleotides was analyzed via GC-MS/MS. Proliferating C2C12 cells r-MPE exhibited a

41 rise-to-plateau while IGF-1 increased myotube RNA from 76±3ng/ul to 123±3ng/ul and

42 r-MPE by 0.39±0.1% (both P<0.01). After 3-wks, rat quadriceps r-MPE had increased

43 to 0.25±0.01% (P<0.01) and was greater with running-exercise (0.36±0.02%; P<0.01)).

44 Human muscle r-MPE increased to 0.06±0.01% and 0.13±0.02% at 3/6-wks

45 respectively equating to synthesis rates of ~0.8%/d, increasing with RE to 1.7±0.3%/d

46 (P<0.01) and 1.2±0.1%/d (P<0.05) at 3/6-wks, respectively. Therefore, we have

47 developed and physiologically validated a novel technique to explore ribosomal

48 biogenesis in a multi-modal fashion.

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Introduction

Cellular protein content is under constant renewal to maintain cellular homeostasis. Typically, the balance between protein synthesis and breakdown remains relatively stable, yet under conditions of growth, atrophy or cellular proliferation, rapid and significant changes in protein content and cell size occur (5). With protein synthesis rates determined by the number (translational capacity) and activity (translational efficiency) of ribosomes (27, 39), the ribosome provides a primary point of control in cellular homeostasis; yet our understanding of dynamic ribosome metabolism is poorly understood. Ribosomal biogenesis is the product of the coordinated synthesis of multiple ribosomal RNA's (rRNA) and proteins. In being an energy consuming process, ribosomal biogenesis is tightly regulated by multiple signaling pathways responsive to nutrition, hormones and mechanical activity (22). However, basal rates of ribosomal biogenesis and ribosome half-life across tissues are for the most part largely undescribed. With the demands to modulate and maintain protein content varying considerably across cell types, such as rapidly dividing single cells, to the coordinated maintenance repair of multicellular organs, regulation of ribosome pools is likely to be tightly linked with protein metabolism (10). Furthermore, when the coordinated control of ribosome biogenesis becomes unregulated it can be the source of many conditions such as cancer (30).

Skeletal muscle is one of the body's most plastic tissues, undergoing substantial and rapid hypertrophy or atrophy under conditions of functional overload, disuse or malnutrition (2, 9, 17). Understanding these processes is of great importance as preservation of muscle mass and function throughout life is crucial in preventing

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77   disability and maintaining quality of life- particularly in advanced ageing (16, 31). In

78   being a post-mitotic tissue, muscle mass is controlled by the balance between muscle

79   protein synthesis (MPS) and muscle protein breakdown (MPB). Many acute changes in

80   MPS (< 5h) are accompanied by the activation or suppression of proteins in the

81   mTORc1 pathway (1), modulating translational efficiency rather than translational

82   capacity (6). However, prolonged exposure to muscle loading modifies RNA content –

83   increasing with hypertrophy (3, 12, 34) and decreasing with atrophy (15). As such,

84   ribosomal biogenesis is thought to be central to muscle mass regulation.

85

86   With rRNA comprising 80% of total RNA, changes in RNA concentration are thought

87   to be indicative of changes in the balance of ribosome synthesis and breakdown.

88   However in addition to relying on long-term interventions, efficient extraction and

89   normalization to muscle weight introducing variability, measures of concentration do

90   not inform on dynamic RNA metabolism, plus changes in RNA synthesis naturally

91   precede those of content. Past measures of RNA synthesis have typically relied on the

92   incorporation of modified nucleotides such as [3H]-uridine or 5-bromouridine. However

93   the use of these techniques is limited and generally cannot be used in whole animals due

94   to their mutagenic nature. Alternatively, stable isotope tracers offer a safe method for

95   use in humans and measures of RNA synthesis using these have been made (8, 14). Yet

96   their applicability in human research has been limited due to numerous caveats,

97   including variable and complex salvage pathways, infusions and time limits (<24h)

98   resulting in a lack of methods to determine RNA synthesis rates, particularly in tissues

99   with slow renewal rates (like skeletal muscle). Heavy water (D_2O) provides alternate

100   routes in the measurement of substrate metabolism and can overcome some limitations

101 associated with other stable isotope methods. In being easily administered and with the

102 precursor pool being maintained over weeks to months, we and others have made long-

103 term cumulative measures of muscle protein synthesis (32, 40), with many other

104 substrates measured in a range of different tissues including DNA (23, 29). Deuterium

105 is incorporated via nucleotide *de novo* synthesis, overcoming previous limitations of

106 nucleotide analogues and thus similarly creates a viable route in the measurement of

107 RNA synthesis. Here, we developed sensitive GC-MS/MS universally applicable

108 methods for the measurement of RNA synthesis including in slower turning over tissues

109 requiring only minimal D_2O consumption; we validate these methods in cell cultures,

110 pre-clinical models and humans and in a cell type of contemporary interest i.e. skeletal

111 muscle.

112

113 **Materials and Methods**

114

115 **Cell culture**

116 Murine C2C12 myoblasts passage nos. 5-7; ECACC, Salisbury, UK were seeded and

117 maintained in Dulbecco's modified Eagle's medium as previously described (7)

118 containing 10% fetal bovine serum, amphoteracin B (1%), penstreptomycin (1%) and

119 4mM L-glutamine (Sigma-Aldrich, UK). Sterile 70% deuterium and U-^{13}C-Glucose

120 were added to DMEM at required enrichments and distributed amongst wells for

121 labeling consistency. In proliferating cells, media was changed every 48h and cells

122 scraped at required time points. At 90% confluency, cells were differentiated by

123 reducing serum concentrations to 2% with RNA synthesis stimulated 6 days after

124 differentiation with IGF1 50 ng.ml^{-1}.

125

Animals

Mixed population of females and males n=22 of high responder to training (HRT) rats

for aerobic training were used for the study. Rats originated from the generations 17 and

18 of selection for their training response, and were 9.2 ± 3.0 months old at the start of

the experiment (21). All experimental procedures described in this study protocol were

approved by the Animal Care and Use Committee of the Southern Finland, license

number ESAVI-2010-07989/Ym-23, STH 534A (21.9.2010) and complements

ESAVI/1968/04.10.03/2011, PH308A (30.3.2011) & ESAVI/722/04.10.07/2013,

PH275A (1.3.2013). All experiments were conducted in accordance with the Guidelines

of the European Community Council directive 86/609/EEC. Rats were kept in air-

conditioned rooms single-housed, at an ambient temperature of 21 ± 2 °C and the

relative humidity at 50 ± 10 %. Artificial lighting provided light cycles of 12 h light/12

h total darkness. Commercially available pelleted rodent diet (R36, Labfor/Lantmännen,

Malmö, Sweden) and the tap water (from the municipal water system of Jyväskylä) was

available *ad libitum* for rats throughout the study. The energy content of the feed was

1260 kJ/100 g (300.93 kcal/100g). The feed contained raw protein 18.5%, raw fat 4.0%,

NFE (nitrogen free extracts) 55.7%, fiber 3.5%, ash 6.3%, and water <12%. Rats

received a gavage of 7.2 ml/kg of 70% D_2O for the remaining 3 wks of the 7 wk

training period, with drinking water enriched to 2% to maintain body water enrichment.

Body water enrichment was determined from blood samples collected at necropsy and

used to represent the average enrichment throughout; although variability may occur

over time, enriched drinking water minimizes these effects. Interval Training consisted

of Warm-up for 5 min, at 50-60% of maximum speed (individually speed for each rat) +

149   running for 15 min: 3 min at 85 - 90%, 2 min pauses at 50%, repeated for 3 times;

150   inclination 15° uphill. Training was done 3 times per week, with one-day rest between

151   (if possible). 48 hrs after the last training bout animals were anaesthetized with carbon

152   dioxide and killed by cardiac puncture and thereafter immediately necropsied. Left

153   quadriceps were rapidly exposed, removed and immediately frozen by complete

154   immersion in liquid nitrogen.

155

156   **Subject characteristics and ethics**

157   Ten healthy younger (23±1y, BMI: 24±1) men were recruited as previously described

158   (3). All subjects provided their written, informed consent to participate after all

159   procedures and risks (in relation to muscle biopsies, blood sampling etc.) were

160   explained. Following inclusion to the study, subjects were studied over a 6-week period.

161   After baseline bilateral biopsies, subjects provided a saliva and blood sample then

162   consumed 150 ml D_2O (70 atom%; Sigma-Aldrich, Poole, UK) to label the body water

163   pool to ~0.2% APE which was maintained with weekly top-up boluses (50 ml.wk^{-1}).

164   Thereafter subjects performed progressive unilateral RET 3/wk at 75% 1RM, with

165   additional bilateral biopsies taken at 3 and 6 wks to monitor RNA incorporation. Blood

166   was collected at 0, 3 and 6 weeks to follow deuterium incorporation into peripheral

167   blood mononuclear cells, isolated using Histopaque (Sigma). For the temporal

168   monitoring of body water enrichment, each participant provided a saliva sample on RET

169   visits >30 min after their last meal or drink, with extra samples taken ~3 h after weekly

170   50 ml boluses to ensure that body water enrichment was accurately represented.

171   Samples were collected in sterile plastic tubes and immediately cold centrifuged at

172   16,000g to remove any debris that might be present; they were then aliquoted into 2ml

173   glass vials and frozen at -20°C until analysis. This study was approved by The

174   University of Nottingham Ethics Committee and complied with studies conducted in

175   accordance with the declaration of Helsinki and registered as clinical trials

176   (clinicaltrials.gov registration no. NCT02152839).

177

178   **Media and body water enrichment**

179   The deuterium enrichment was measured in media collected from cell culture plates and

180   plasma from rats by incubating 100μl of each sample with 2μl of 10 M NaOH and 1μl

181   of acetone for 24 h at room temperature. Following incubation the acetone was

182   extracted into 200μl of n-heptane, and 0.5μl of the heptane phase was injected into the

183   GC-MS/MS for analysis. A standard curve of known D_2O enrichment was run along

184   side the samples for calculation of enrichment. Human body water enrichment was

185   extracted by heating 100 μl saliva in an inverted 2 ml autosampler vial for 4 h at 100°C.

186   Vials were then placed upright on ice to condense extracted body water and transferred

187   to a clean autosampler vial ready for injection. A total of 0.1μl body water was injected

188   into a high-temperature conversion elemental analyzer (Thermo Finnigan; Thermo

189   Scientific, Hemel Hemp- stead, United Kingdom) connected to an isotope ratio mass

190   spectrometer (Delta V Advantage; Thermo Scientific)

191

192   **Protein-bound alanine muscle fraction enrichment and calculation of FSR**

193   Myofibrillar protein was isolated from human *VL* muscle biopsies and rat quadriceps by

194   homogenizing 30–50 mg muscle in ice-cold homogenization buffer, rotated for 10min,

195   and the supernatant collected after centrifugation at 13,000 g for 5 min at 4°C. The

196   myofibrillar pellet was solubilized in 0.3 M NaOH and separated from the insoluble

197 collagen by centrifugation, and the myofibrillar protein was precipitated using 1 M

198 perchloric acid (PCA). Protein-bound amino acids were released using acid hydrolysis

199 by incubating in 0.1 M HCl in Dowex H+ resin slurry overnight before being eluted

200 from the resin with 2 M NH4OH and evaporated to dryness; amino acids were then

201 derivatised as their N-methoxycarbonyl methyl esters. Dried samples were suspended in

202 60 µl distilled water and 32 µl methanol, and following vortex, 10 µl pyridine and 8 µl

203 methylchloroformate were added. Samples were vortexed for 30 s and left to react at

204 room temperature for 5 min. The newly formed N- methoxycarbonylmethyl ester amino

205 acids were then extracted into 100 µl chloroform. A molecular sieve was added to each

206 sample for ~20 s before being transferred to a clean glass gas chromatography insert,

207 removing any remaining water by size exclusion absorption. Human protein-bound

208 alanine enrichment was determined by gas chromatography: pyrolysis:isotope ratio

209 mass spectrometry (Delta V Advantage Thermo Finnigan, Thermo Scientific, Hemel

210 Hempstead, UK), with rat protein-bound alanine enrichment determined by gas

211 chromatography tandem mass spectrometry (TSQ 8000 Thermo Finnigan, Thermo

212 Scientific, Hemel Hempstead, UK) alongside a standard curve of known DL-Alanine-

213 2,3,3,3-d4 enrichment to validate measurement accuracy of the machine. Myofibrillar

214 MPS was calculated from the incorporation of deuterium-labeled alanine into protein,

215 using the enrichment of body water [corrected for the mean number of deuterium

216 moieties incorporated per alanine (3.7) and the dilution from the total number of

217 hydrogens in the derivative (i.e., 11)] as the surrogate precursor labeling between

218 subsequent biopsies. The equation used was

$$FSR = -Ln\left(\frac{1 - \left[\frac{(APEala)}{(APEp)}\right]}{t}\right)$$

219   where APEala equals deuterium enrichment of protein-bound alanine, APEp indicates

220   mean precursor enrichment over the time period, and t is the time between biopsies.

221

222   **RNA extraction, digestion and derivatisation**

223   To extract RNA, ~20-30mg of muscle was homogenized in extraction buffer (5µl/mg)

224   containing 0.1 M Tris-HCL pH 8, 0.01 M EDTA pH 8 and 1M NaCL. Proteinase K was

225   added to a final concentration of 50 µg/µl and placed at 55°C for ~2 hrs with occasional

226   mixing until complete digestion had occurred. For cell culture, each well was scraped in

227   200 µl of extraction buffer and PBMC's homogenized in 200 µl of extraction buffer. To

228   the extractions an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was

229   added, inverted several times to mix and the upper aqueous layer removed to a clean

230   eppendorf after centrifugation at 13000 rpm for 10 min. To remove additional protein

231   an equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous layer

232   and repeated as above. To precipitate RNA, an equal volume of isopropanol was added

233   to the aqueous layer, inverted several times and centrifuged at 13000 rpm for 20 min.

234   The pellet was washed 3 times in 70% ethanol; air-dried, re suspended in 22 µl of

235   molecular biology and digested with 5 µl of 375 mM sodium acetate (pH 4.8), 750 µM

236     $ZnSO_4$ containing 0.5 units of nuclease S1 and 0.25 units potato acid phosphatase and

237   placed at 37°C overnight. Hydrolysates were then reacted with 10 µl of O-

238   benyzylhyrdoxylamine (2% w/v) and 7.5 µl of acetic acid at 100°C for 30 min. Samples

239   were allowed to cool at room temperature before the addition of 10µl of 1-

240   methyimidazole and 100 µl of acetic anhydride. The reactions are transferred to a

241   boiling tubed and quenched by the addition of 2 ml ddH2O. The newly formed

242   derivatives were extracted by the addition of 750 µl dichloromethane (DCM) vortex

243   mixed and phases allowed to separate. By pre wetting the tip with DCM, the lower layer

244   is removed to a clean boiling tube and the procedure repeated. DCM extracts were then

245   dried and re suspended in 40 µl of ethyl acetate for GC-MS/MS analysis.

246

247   **GC-MS/MS instrument conditions and fractional synthesis rate calculation**

248   To measure RNA enrichment 2 µl of sample was injected into a trace 1310 gas

249   chromatograph connected to TSQ 8000 triple quadrupole GC-MS/MS (Thermo

250   Finnigan, Thermo Scientific, Hemel Hempstead, UK). Samples were injected on

251   splitless mode with inlet temperature at $280^{o}C$. GC ramp conditions were $120^{o}C$ for 1

252   min, ramp to $280\,^{o}C$ at $10\,^{o}C$ /min and hold for 3 min. Selected reaction monitoring

253   (SRM) was performed for the mass to charge ratios m/z of 273.1-111.1 and 274.1-112.1

254   representing the M and M^{+1} ions with a collision induced dissociation energy of 6.

255   Enrichment was calculated as $(M^{+1}/(M+M^{+1}))$ with the mole percent excess (MPE)

256   expressed as difference from unlabeled D_2O free samples. Fractional synthesis rates

257   were calculated as $FSR(\%.d^{-1}) = (r\text{-}MPE)/[(p\text{-}MPE) \times t] \times 100$ where r-MPE is the

258   excess enrichment of bound ribose, p-MPE is the mean precursor enrichment over the

259   time period and t is the time between samples. In cell culture and rat studies, p-MPE

260   was calculated as the water enrichment multiplied by the amplification factor of 2.098

261   determined in cells. In human studies, p-MPE was taken as the ribose PBMC

262   enrichment measured over the labeling period. Samples were run in triplicate alongside

263   standard curves of known ribose standards, with the average of both peaks were used in

264   the results. Additionally, unlabeled samples were injected in different quantities to

265   determine abundance effects.

266

267 **Statistical Analysis**

268 Descriptive statistics were produced for all data sets to check for normal distribution

269 (accepted if P>0.05) using a Kolmogorov-Smirnov test. All data are presented as means

270 ± SEM. Differences between the effects of interval training and control on RNA

271 synthesis in rates were analyzed by t-test. All other data sets were analyzed by repeated

272 measures one-way or two-way ANOVA with a Bonferroni correction using GraphPad

273 Prism (La Jolla, CA) Software Version 5. Correlations were assessed using Pearsons

274 product moment correlation coefficient. The α level of significance was set at $P<0.05$.

275

Results

GC-MS/MS chromatography and SRM transitions

Addition of O-benyzylhyrdoxylamine and 4 acetyl groups to ribose produces a derivative with a molecular weight of 423.1 (Fig 1A). Upon gas chromatography the derivative produces two peaks representing the cis-trans isomers formed due to the anomeric carbon of ribose (Fig 1B). Full scan MS analysis of the derivative produces a most abundant fragment with best chromatography of 273.1, with second fragmentation producing a most abundant fragment of 111.1. Analysis of this transition is highly selective and produces GC-MS/MS spectra with very low background, detecting standard enrichments as little as 0.02 APE (Fig 1C). Further this SRM encompasses all backbone carbons of ribose confirmed by +5 enrichment from U-^{13}C-Glucose incorporation (Fig 1D).

Deuterium incorporation into RNA bound ribose

The MPE of ribose (Fig 2A) extracted from purine nucleotides of RNA from C2C12 cells increased linearly with increasing concentrations of media D_2O enrichment being 0%, 1.6±0.08%, 4.1±0.1%, 9.5±0.15%, 18.8±0.18% at media concentrations of 0% 0.67%, 1.9%, 4.6% and 8.9% respectively. Linear regression revealed that on average 2.1 ^{2}H are incorporated into purine ribose during synthesis of new RNA. PBMC'S from human subjects showed an increase in MPE to 0.37±0.04% and 0.42±0.04% (both P<0.01), revealing the average accessible hydrogen's to be 2.6±0.2 (Fig 2B).

Validation of deuterium incorporation in RNA *in vitro*

300 C2C12 cell number increased from 0.083±0.001 million per well to 1.2 ±0.03 million

301 after 117 h of proliferation (Fig 3A), whilst RNA MPE followed a rise to plateau

302 relationship from 0.28±0.03% after 15 h and progressed to 0.52±0.02% by 117 h (Fig

303 3B). In response to IGF1 treatment, RNA content increased by 27.5 h from 76.1±3

304 ng.ul^{-1} to 123.4±3 ng.ul^{-1} (Fig 3C). Similarly, RNA MPE significantly increased in

305 control to 0.15 ±0.01% at 27.5 h, with the increase in IGF1 treatment being significantly

306 greater (Fig 3D).

307

308 **RNA Synthesis in rat muscle *in vivo***

309 After 3 weeks of continuous D_2O administration, RNA MPE significantly increased to

310 0.25±0.01% in control and was significantly greater with interval training to

311 0.36±0.01% P<0.001 (Fig 4A). The calculated RNA FSR was 0.97±0.05 %.d^{-1} with a

312 significant increase in response to interval training to 1.3±0.05 %.d^{-1} P<0.001 (Fig 4B).

313 Rat quadriceps MPS showed a correlation with quadriceps RNA synthesis % of r^2=0.17

314 and P=0.05

315

316 **RNA synthesis in human muscle**

317 The MPE from RNA bound ribose increased in rest legs to 0.064 ±0.01% and 0.137

318 ±0.02% at 3 and 6 weeks respectively (P<0.001). In RET legs, the MPE increased to

319 0.125 ±0.02% and 0.211 ±0.01% at 3 and 6 week respectively (P<0.001), being greater

320 than rest at both time points (P<0.005). Corrected for PBMC RNA enrichment an FSR

321 of 0.86 ±0.1%.d^{-1} and 0.78 ±0.1%.d^{-1} was determined in rest legs at 3 and 6 weeks

322 respectively. In RET legs the RNA FSR was significantly greater than rest being 1.69

14

323   ± 0.2 %.d^{-1} (P<0.01) and 1.24 ± 0.1%.d^{-1} (P<0.05) at 3 and 6 weeks respectively. Human

324   VL MPS was highly correlated with VL RNA synthesis P=0.009 and an r^2=0.32.

325

326

Discussion

We have developed and validated D_2O-based methods for the measurement of *in vitro* and *in vivo* RNA synthesis that can be used safely and effectively in humans and with the potential for application to any tissue- this is a step forward from current practices in providing methods for long term measures of RNA synthesis in humans, particularly those of slow turnover pools such as skeletal muscle. RNA content is closely linked to cellular metabolism, with ribosomal biogenesis being required for cellular proliferation (10) and growth (34). Currently, changes in RNA content are primarily determined by crude measures of RNA concentrations, with limited methods in place to determine dynamic rates of RNA synthesis *in vivo*. Our approach will provide insight into the workings of dynamic ribosomal biogenesis in vitro, in animal models and in humans – across cell types.

Method development and validation of *in vitro* RNA synthesis in skeletal muscle cells

Nucleotide synthesis involves complex precursor pools with variable nucleotide salvage (23) making the incorporation of stable isotope labeled compounds difficult to interpret (13). The advantage of using D_2O is that deuterium becomes incorporated into the backbone hydrogen of ribose during nucleotide synthesis, with deoxyribose from purine deoxyribonucleosides primarily synthesized via *de novo* synthesis; as such, providing a reliable input of isotope (23, 29). As deoxyribonucleotides are reduced from ribonucleotides, this further creates a viable method for measures of RNA synthesis and

16

350 for the first time we have shown a constant incorporation of deuterium across a range of

351 media concentrations into purine ribose.

352

353 Total RNA encompasses rRNA, tRNA and mRNA that will have variable turnover rates

354 (33). The quickest of these will be tRNA and mRNA that will contribute to early

355 increases in detectable enrichment. However in making up <20% of total cellular RNA,

356 these pools become quickly saturated and deuterium incorporation follows a rise to

357 plateau in proliferating cells reflecting the required expansion of rRNA for cell division

358 (Fig 3B) (8, 10). Further, as initial validation using established stimulators of *in vitro*

359 myotube hypertrophy and ribosomal biogenesis (i.e. IGF-1)(7), we were able to detect

360 simultaneous increases in both RNA content and deuterium incorporation into RNA.

361 Therefore, deuterium incorporation into RNA was reflective of newly synthesized

362 RNA.

363

364 To use precursor product calculations, a measure of the precursor, or a proxy thereof, is

365 required (38). Alternatively, when using D_2O, an amplification factor can be used to

366 represent the amount of accessible hydrogen in the precursor that can incorporate

367 deuterium and be multiplied by the body water enrichment (4). Nucleotide precursor

368 pools are difficult to measure, with continuous input of unlabeled substrates such that

369 the maximal theoretical plateau is never achieved (24). To investigate the number of

370 accessible hydrogen in ribose *in vitro*, murine C2C12 skeletal muscle cells were

371 repeatedly passaged in a range of D_2O media enrichments, revealing a constant

372 incorporation of ~2.1 deuterium's out of a total 6. Previously, values of ~3.1 have been

373 reported for deoxyribose out of a total 7 (26, 29), expectedly higher due to the

374 additional hydrogen that exchanges with ribonucleotide reductase.

375

376 **Validation of *in vivo* RNA synthesis in an animal model**

377 Compared to many tissues, skeletal muscle has a relatively slow habitual protein

378 renewal rates, with little to no active DNA synthesis (11); in contrast actual RNA

379 synthesis rates are practically unknown and will vary considerably across tissues. As

380 D_2O can be simply administered by oral consumption and easily maintained, D_2O can

381 be used to capture a vast range of synthesis rates. Recently, D_2O has been used to

382 measure ribosome renewal in mouse liver, although in using GC-MS this requires high

383 levels of enrichment (5% APE) and fast rates of turnover (~10%) that can be

384 burdensome and limit applications(25). Applying our validated *in vitro* methods to

385 rodents, to our knowledge, we made the first long term measures of RNA synthesis in

386 skeletal muscle. In doing so, we demonstrated there is active renewal of RNA pools of

387 ~1%.d^{-1}. Furthermore, using an exercise stimulus to activate ribosomal biogenesis (39)

388 we validated the existence of a significant increase in deuterium incorporation into

389 RNA, demonstrating increased RNA synthesis. Intriguingly, RNA synthesis rates were

390 correlated with MPS, which we speculate is due to a co-ordinate regulation in response

391 to exercise.

392

393 **RNA Synthesis in Human Muscle and the Effect of Resistance Exercise**

394 Presumably most tissues will have a constant level of rRNA synthesis to maintain

395 functional ribosomes for cellular protein synthesis. That said, since ribosome biogenesis

396 consumes considerable energy and will therefore likely be maintained at minimal

397   requirements. Using the methods described here, to our knowledge we report the first

398   measures of RNA synthesis in human muscle, showing a constant synthesis rate of ~0.8

399   %.d^{-1} during "habitual activity". Further, to asses precursor enrichment, we measured

400   the plateau enrichment of a population of cells 100% replenished (PBMCS) over the

401   labeling period (29). This accounts for individual variability in the number of accessible

402   hydrogens and further we showed on average 2.6 deuterium's were incorporated,

403   similar to our *in vitro* measures.

404

405   Skeletal muscle RNA content is highly responsive to functional overload (28, 37) and

406   here we have shown that in response to RET, RNA synthesis was significantly

407   increased after 3 and 6 weeks of exercise training in humans. Once again, RNA

408   synthesis was correlated with MPS which further validates that in muscle, ribosome and

409   protein metabolism are likely to be inextricably related (likely via mTORc1 (18)).

410   Similarly, although there is little other data for us to compare our results to, whole body

411   rRNA turnover determined by breakdown products in urine have been estimated ~2.5%

412   (36). Further this showed a strong relationship between whole body protein degradation

413   rates of r^2=0.7 supporting that these are closely linked process in muscle homeostasis.

414

415   **Further Application of Methods for RNA synthesis**

416   Previously, measures of RNA synthesis have been made in humans using 6,6^{2}H$_2$-

417   Glucose, however this requires large amounts of tracer to be consumed (1g/Kg) and is

418   generally limited to fast turnover cells (8). Further, achieving high levels of enrichment

419   to perform GC-MS analysis in humans is costly, requiring high levels of D$_2$O

420   consumption that is burdensome on the individual and may potentially cause adverse

421   effects such as nausea and vertigo (19). Further, rates of RNA synthesis will vary

422   considerably across tissues, making the detection of slow turnover pools such as muscle

423   using GC-MS techniques difficult. Here, by combining sensitive GC-MS/MS

424   techniques (detection limits of $\geq$0.02% MPE) and the ability to administer D_2O from

425   days to weeks, this method creates opportunities to measure RNA synthesis over a

426   range of rates and tissues. Such measures can be employed through simple D_2O

427   administration and access to tissue samples or blood- with some prior expectation of

428   synthesis helpful. For instance, human body water enrichment can be simply maintained

429   ~ 0.15-0.2% APE using an initial bolus of 150 ml D_2O, followed by weekly doses of 50

430   ml (3). In this situation, sampling from a tissue after 5 days with an RNA turnover rate

431   of ~10 %.d^{-1} would result in an easily detectable product enrichment using GC-MS/MS

432   of ~ 0.075-0.1%, whereas an RNA turnover rate of ~1 %.d^{-1} would result in an

433   undetectable enrichment of ~ 0.0075%-0.01%. This is not to say these measure can't be

434   made by other means. Raising body water enrichment will increase end point

435   enrichment and body water enrichments as high as 2% would make GC-MS techniques

436   an option. However D_2O consumption of such high levels is costly and burdensome on

437   subjects. As such the methods used here can be readily applied to many situations.

438

439   **Conclusion**

440   In summary we have developed and validated the use of D_2O in measurement of RNA

441   synthesis both *in vitro* and *in vivo*. With many RNA synthesis rates unknown, these new

442   methods will have a significant impact in being able to measure a wide range of RNA

443   turnover rates in varied tissues. Further, ribosomal biogenesis has been the interest of

444   recent publications in muscle adaptive mechanisms (12, 20, 34, 35) and will likely play

445   a significant role elucidating muscle metabolism at rest and in response to

446   hypertrophic/atrophic conditions.

447

467

468 **Disclosures**

469 No conflicting interests

470

471 **Author contributions**

Figure Legends

Figure 1. A) Structure and mass of the ribose BHTA derivative. B) Typical GC-MS/MS chromatogram of the ribose derivative on a DB-17 column for the SRM transitions of (273.1-111.1). Blue line represents the M and red the M+1 C) Standard curve of $1\text{-}^{13}C$-Ribose 0.02, 0.05, 0.1, 0.5 D) measurement of the +5 isotopomer of RNA bound ribose from C2C12 myotubes incubated in $U\text{-}^{13}C$ glucose enriched media

Figure 2. A) MPE of RNA from maximally labeled C2C12's vs. D_2O media MPE and B) the amplification of deuterium into PBMC'S from human subjects. *** Significantly different from baseline P<0.001

Figure 3. Time course of A) cell number B) MPE of RNA in proliferating C2C12's. Time course in the concentration of C) RNA and D) MPE of RNA in non-treated condition (Control) and in responses to IGF1. Dotted line represents plateau enrichment.* Significantly different from baseline P<0.05, *** P<0.001, **** P<0.0001. § significantly different from control at that time point P<0.01

Figure 4. A) MPE of RNA bound ribose from control and exercised rat quadriceps B) FSR of RNA from control and exercised rat quadriceps C) correlation between quadriceps $MPS\%.d^{-1}$ and RNA $FSR\%.d^{-1}$. Dotted line represents plateau enrichment *** Significantly different than control P<0.001

Figure 5. A) Time course of body water enrichment measured through saliva samples over the 6 weeks of labeling B) MPE of RNA bound ribose from human *VL* in rest and

512   RET legs. Dotted line represents the average plateau PBMC enrichment **C)** FSR of

513   RNA bound ribose from human *VL* in rest and RET legs **D)** correlation between *VL*

514   MPS%.d^{-1} and RNA FSR %.d^{-1}. *** Significantly different from baseline P<0.001 §

515   Significantly different from control at that time point P<0.05

516

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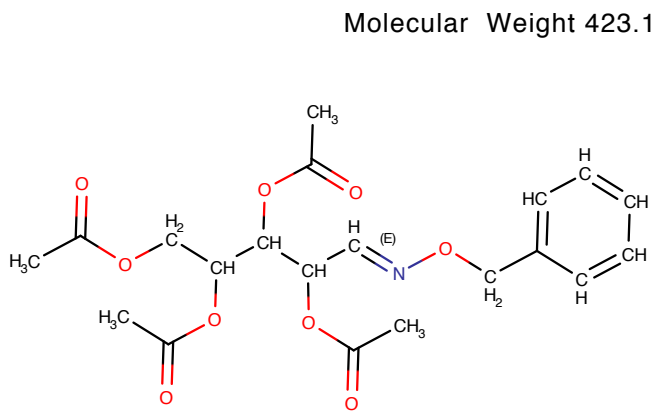
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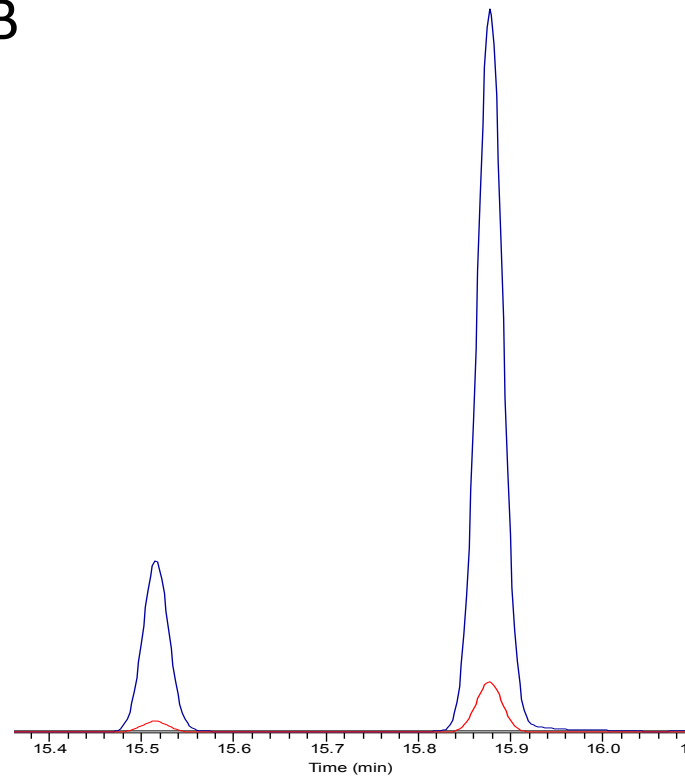
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Figure 1

A

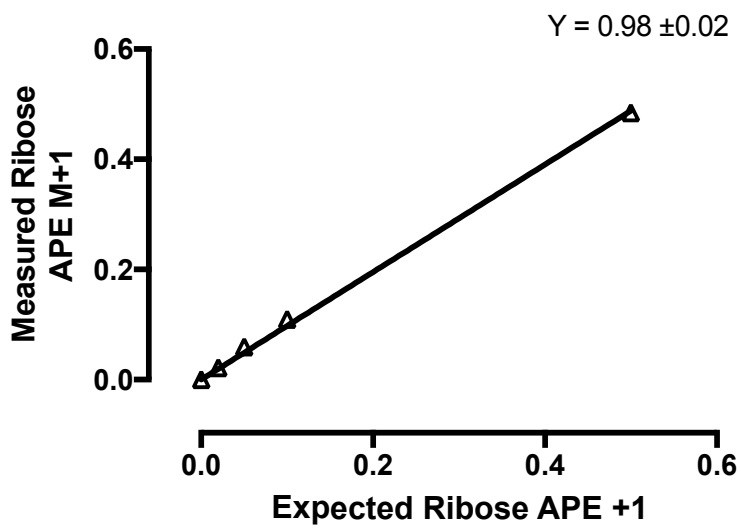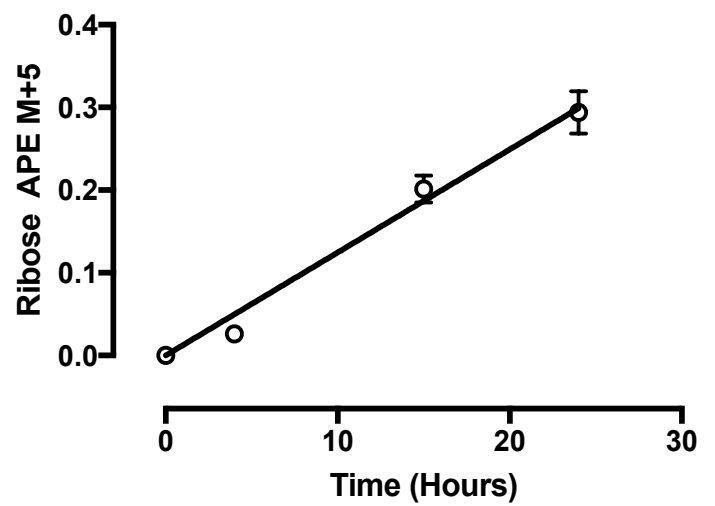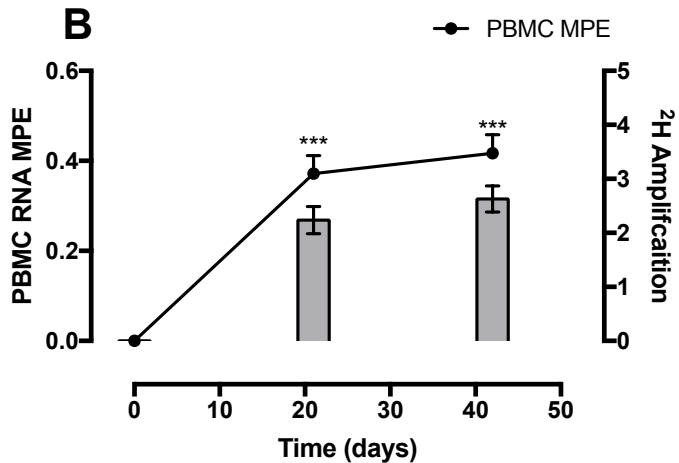Molecular Weight 423.1

B

Time (min)

C

Y = 0.98 ±0.02

D

Figure 2

Figure 3

Figure 4

Figure 5