1   **Nucleosome repositioning in cardiac reprogramming**

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Abstract

Early events in the reprogramming of fibroblasts to cardiac muscle cells are unclear. While various histone undergo modification and re-positioning, and these correlate with the activity of certain genes, it is unknown if these events are causal or happen in response to reprogramming. Histone modification and re-positioning would be expected to open up chromatin on lineage-specific genes and this can be ascertained by studying nucleosome architecture. We have recently developed a set of tools to identify significant changes in nucleosome architecture which we used to study skeletal muscle differentiation. In this report, we have applied these tools to understand nucleosome architectural changes during fibroblast to cardiac muscle reprogramming. We found that nucleosomes surrounding the transcription start sites of cardiac muscle genes induced during reprogramming were insensitive to reprogramming factors as well as to agents which enhance reprogramming efficacy. In contrast, significant changes in nucleosome architecture were observed distal to the transcription start site. These regions were associated with nucleosome build-up. In summary, investigations into nucleosome structure do not support the notion that fibroblasts to cardiac muscle cell reprogramming involves chromatin opening and suggests instead long-range effects such as breaking closed-loop inhibition.

Introduction

In a previous report, we described the development of a set of tools to investigate nucleosome architecture (1). The approach differs from others in several ways. Firstly, the approach utilizes common software that is easily and readily available. In contrast, other approaches require the use of specific programs which pose significant barriers due to requirements to understand computer languages and a reliance on repositories which are not maintained. Secondly, and perhaps more importantly, our approach starts with specific groups of genes. The standard

46 approach starts by investigating nucleosome architecture changes with the entire genome as a

47 single group. Groups of genes are then identified that fit a pattern and biological information is

48 subsequently extracted through ontological methods. A problem that we have found with gene

49 ontology is there is often a mismatch between the constituent genes of gene ontological terms

50 and the genes whose expression is relevant to the research question (1).

51 Once we developed our novel approach, we applied it to study nucleosome architectural changes

52 during skeletal muscle cell differentiation. Three groups of muscle genes were chosen for study:

53 those expressed solely in skeletal muscle; those expressed in both skeletal muscle and cardiac

54 muscle; and those expressed solely in cardiac muscle. By way of a control, a fourth group was

55 comprised of non-muscle genes. Each gene group was verified for specificity and was of sufficient

56 size to avoid any potential bias from outliers. Our approach revealed that during myogenic

57 differentiation, skeletal muscle and common muscle genes underwent specific and unique

58 nucleosome changes not observed in the other groups. Nucleosomes in three regions (-2500bp

59 to -2400bp, -650bp to -400bp and +600bp to +700bp) relative to the transcription start site shifted

60 in a 3' direction. In addition, there was notable a loss of nucleosomes surrounding the transcription

61 start site.

62 Moving forward, we wanted to apply our approach to direct cardiac reprogramming. We, along

63 with others, have reported that partial functionality can be restored to the infarcted heart by directly

64 reprogramming scar tissue fibroblasts into heart muscle cells (2-5). The initial stages of direct

65 reprogramming are characterized by several epigenetic events, notably loss of H3K27me3 and

66 gain of H3K4me3 (6, 7). These epigenetic markers are believed to promote gene repression and

67 gene activation, respectively, and are thought to work co-operatively to open chromatin, enabling

68 RNApol-II to bind and initiate transcription (7). However, chromatin remodeling has not been

69 proven. Moreover, other studies in different gene systems contend that epigenetic changes are

70 merely bystander effects (8). The tools at our disposal enable us to address these questions

71 further. As detailed in this report, direct reprogramming does induce changes to the nucleosome

72 architecture specific to cardiac muscle genes. However, these changes occur at some distance

73 from the TSS and appear to coincide with those observed in skeletal muscle genes during

74 myogenic differentiation. Notably, there were no changes in the nucleosome architecture

75 surrounding the TSS. Moreover, agents that are known to enhance reprogramming efficacy

76 through enhanced gene expression also had no effect on TSS nucleosome architecture. In

77 conclusion, our findings suggest that epigenetic events initiated by direct reprogramming do not

78 open up chromatin for RNApol-II binding.

79

80 **Materials & Methods**

81 _Databases:_ the RNA-seq(9) and MNase-seq(10) datasets are available on the NIH Single-Read

82 Archive under accession numbers PRJNA1067687 and PRJNA837987 respectively.

83 _Gene groups:_ per our previous publication(1), genes were ascribed to specific gene groups.

84 The cardiac & common muscle gene group includes Actn2, Cacna1c, Kcna4, Kcnj2, Mb, Mef2C,

85 Myh6, Myl2, Nebl, Ryr2, Scn5a, Sln, Tnni3, Tnnt2, and Ttn.

86 The skeletal muscle gene group includes Cacna1s, Myh1, Myh2, Myh3, Myh4, Myod1, Myog,

87 Neb, Ryr1, Scn4a, Tnni1, and Tnni2.

88 The non-muscle group includes Atl1, Cd34, Cdh1, Col1a1, Col1a2, Dcx, Eno2, Eng, Flt1, Flt4,

89 Map2, Mapt, Ncam1, Neurod1, Nlgn1, Pecam1, Postn, Rbfox3, Scn4a, S100a4, Syn1, Tek,

90 Tcf21, Thy1, Vcam1, Vegfa, and Vwf. This group is larger to ensure sufficient coverage of

91 alternative reprogramming trajectories (fibroblast, endothelial, neuronal).

92 _RNA-seq:_ analysis of our dataset (PRJNA1067687) was carried out as described. Briefly,

93 neonatal cardiac fibroblasts were transfected with miR combo (reprogramming agent) or a control

94   miRNA. Four days later, total RNA was extracted using the Quick-RNA MiniPrep Kit (Zymo

95   Research, 11–328) and high-throughput sequencing was performed by the Duke Genomic Core

96   on an Illumina HiSeq 4000. Gene expression was determined via bioinformatics programs within

97   the Galaxy suite(11).

98   *Investigating nucleosome architecture:* the tools used to investigate nucleosome architecture are

99   described at length in our earlier report(1). The starting point was MNase-digested chromatin

100   isolated from miR combo and control miRNA transfected neonatal cardiac fibroblasts and

101   sequenced on a NovaSeq 6000 Kit (Illumina). The dataset has an accession number of

102   PRJNA837987 in the NIH single-read archive(10). Bowtie was used to align sequences to the

103   mouse genome and filtered for quality, true pairs and mitochondrial genome sequences.

104   BamCoverage (1bp window, normalized to effective mouse genome size and in MNase mode)

105   was used to determine read count for each promoter (-3kb upstream of the transcription start site

106   to + 1kb downstream of the transcription start site) for the genes listed above. Data was exported

107   as bedgraph and the bedgraph files were merged with the bedtools Merge BedGraph files tool.

108   Once merged, read count data was normalized to ensure gene had equal weight via the equation:

109   For gene x, normalized read count$_{position-n}$ = read count$_{position-n}$/$\sum$read count$_{genex}$ where position-n is

110   the position of the 1bp window with respect to the transcription start site.

111   To determine the effects on nucleosome architecture between two conditions the following

112   formula was applied:

113   For gene x, Δnormalized read count$_{position-n}$ = (read count$_{position-n}$)myotube– (read count$_{position-n}$)control.

114

115   Significances in Δnormalized read counts were determined by paired T-tests comparing

116   normalized read counts in the two groups for each 1bp window.

117   *Statistics:* Statistical methods are described in the figure legends.

118

Results

120 Our approach for analyzing nucleosome architecture involves starting with a defined group of

121 genes. A group of genes must be greater than 10 to ensure insensitivity to outliers (**Fig.1A**).

122 Consequently, all gene groups were comprised of more than 10 genes.

123 Our analysis of nucleosome architecture in fibroblasts to cardiac muscle cells focused on three

124 groups of genes: (1) cardiac and common muscle genes; (2) genes specific to skeletal muscle;

125 and, (3) non-muscle genes. In fibroblasts reprogramming to cardiac muscle cells, cardiac and

126 common muscle genes are activated. In contrast, genes specific to skeletal muscle, as well as

127 non-muscle genes, remain silent or are repressed (**Fig.1B**).

128 Regions of significant increase or decrease in nucleosome content were determined by

129 comparing nucleosomes following transfection with the reprogramming agent miR combo and

130 control miRNA. With respect to cardiac and common muscle genes, miR combo significantly

131 altered nucleosome architecture in three regions of the promoter. These positions were -2600bp

132 to -2300bp (Region A); -1800bp to -1400bp (Region B); and +350bp to +900bp (Region E) relative

133 to the transcription start site (**Fig.2A**). In these regions there was a significant increase in

134 nucleosome density. The efficacy of miR combo is enhanced by the addition of RNA-sensing

135 receptor agonists (10, 12). The actions of these agonists on cardiac and common muscle genes

136 was focused on a region -900bp to -550bp (Region C) relative to the transcription start site

137 (**Fig.2A**). Neither miR combo alone, nor miR combo plus an RNA-sensing receptor agonist, had

138 an effect on nucleosome content close to the transcription start site (-200bp to +200bp (Region

139 D); **Fig.2A**).

140 The regions described above were studied in more detail by comparing cardiac and common

141 muscle genes with skeletal muscle and non-muscle genes. The comparisons were broken down

142   into two measurements. Firstly, comparisons were made to determine if the test condition altered

143   nucleosome position. Secondly, comparisons were made to determine if the test conduction

144   induced nucleosomes to build up or are depleted. To address changes in nucleosome position,

145   correlation matrices were determined. The R^2-value represents how nucleosome positions

146   change. For any given region, if nucleosomes in two genes occupy the same position relative to

147   the transcription start site, they are in phase and the R^2-value will be +1. Whereas, the R^2-value

148   will be -1 if the nucleosomes are fully out of phase (nucleosome in one group is completely absent

149   in the comparator group). As shown in **Fig.2B**, in miR combo transfected cells, the position of

150   nucleosomes across the whole promoter show no correlation between the three groups of genes.

151   Correlations and anti-correlations are more evident in the aforementioned five regions of the

152   promoter. Of the five regions, -2600bp to -2300bp (Region A), -900bp to -550bp (Region C) and

153   +350bp to +900bp (Region E) are notable because they appear to define cardiac and common

154   muscle genes from skeletal muscle and non-muscle genes on the basis of negative R^2-values in

155   the latter two gene groups (**Fig.2B**). Region -1800bp to -1400bp distinguishes cardiac and

156   common muscle genes from skeletal muscle but not non-muscle genes and there is no difference

157   in nucleosome positioning in cardiac and common muscle genes, skeletal muscle and non-muscle

158   genes in the -200bp to +200bp (Region D) (**Fig.2B**).

159   In terms of nucleosome content, miR combo generally only affected cardiac and common muscle

160   genes. While there was no effect over the whole promoter, miR combo induced significant

161   nucleosome build-up in the -2600bp to -2300bp (Region A), -1800bp to -1400bp (Region B) and

162   +350bp to +900bp (Region E) regions (**Fig.3**). The only significant event in the skeletal muscle

163   and non-muscle groups was a significant increase in nucleosome content in the -900bp to -550bp

164   (Region C) region of the latter group (**Fig.3**). Again, there was no significant change in

165   nucleosome content for any group of regions in the region immediately adjacent to the

166   transcription start site (Region D) (**Fig.3**).

167   As mentioned above, fibroblast to cardiomyocyte reprogramming efficacy is improved by the

168   addition of RNA-sensing receptor agonists(10, 12). RNA-sensing receptors are a broad group of

169   proteins that are believed to share a common signaling pathway. We have found evidence for the

170   role for two RNA-sensing receptors, TLR3 and Rig1. TLR3 and Rig1 appear to influence fibroblast

171   to cardiomyocyte reprogramming through different pathways. We wanted to understand if TLR3

172   and Rig1 agonists potentially enhance reprogramming efficacy by changing nucleosome

173   architecture.

174   Over the whole 4kb promoter, neither the TLR3 agonist PolyIC, nor the Rig1 agonist 3p-hpRNA

175   influenced nucleosome content in any gene group (**Fig.4**). However, differences were noted in

176   the analysis of the five aforementioned promoter regions. Again, the first comparisons were made

177   to determine the effect on nucleosome movement by determining how in phase they were. In

178   cardiac and common muscle genes, both PolyIC and 3p-hpRNA induced significant nucleosome

179   re-arrangement in all five of the aforementioned promoter regions. Moreover, the effects of the

180   two agonists differed from each other as evidenced by the low R^2-values (**Fig.4**). The same trends

181   were observed in skeletal muscle and non-muscle groups, whereby PolyIC and 3p-hpRNA

182   induced nucleosome re-arrangement when compared to the control and the re-arrangements

183   differed between the two ligands (**Fig.4**).

184   Following measurements of nucleosome movement, nucleosome content in the five promoter

185   regions was quantified. Generally, both RNA-sensing receptor ligands induced the same trends.

186   With respect to cardiac and common muscle genes, both PolyIC and 3p-hpRNA increased

187   nucleosome content in the -1800bp to -1400bp and -200bp to +200bp regions while decreasing

188   it in the -2600bp to -2300bp, -900bp to -550bp and +350bp to +900bp regions (**Fig.5**).

189   Interestingly, PolyIC and 3p-hpRNA had the same effect on the equivalent regions of skeletal

190   muscle genes (**Fig.5**). Many of the same features were also observed with non-muscle genes.

191 However, in contrast to muscle genes, PolyIC and 3p-hpRNA induced nucleosome loss in the -

192 200bp to +200bp region and nucleosome gain in the +350bp to +900bp region (**Fig.5**).

193

194 **Discussion**

195 When early events in the reprogramming of fibroblasts to cardiac muscle cells are studied' there

196 has been a focus on epigenetic changes. Indeed, early on there is a significant re-positioning of

197 the epigenetic motifs H3K27me3, H2AK119ub and H3K4me3 (6, 7). H3K27me3 and H2AK119ub

198 are typically labeled "repressive" while H3K4me3 is often labeled "activating". They are believed

199 to close and open chromatin respectively through the movement of nucleosomes. The idea is that

200 open chromatin allows transcription factors to bind and activate specific genes. Due to the

201 correlations between where these motifs are found and gene activity, researchers have assumed

202 causality (7). However, this assumption is problematic because so-called repressive marks are

203 also found on active genes and so-called activating marks are found on repressed genes (13-16).

204 A further issue is one of universality. Antibody immunoprecipitations and sequencing are used to

205 identify where the epigenetic motifs are located and biological function is subsequently inferred

206 through ontology. While informative, gene ontological terms are curated lists and as such they

207 can be incomplete, potential sources of bias, and not fully represent complex biological processes

208 (17). What this means is that genes affected by reprogramming and do not fit the epigenetic

209 paradigm have the potential to be missed. It was with these questions in mind that we developed

210 an approach to study chromatin whereby we used novel analytical tools to identify significant

211 changes in nucleosome structure. We first applied this approach to skeletal myoblast

212 differentiation as this process is well characterized. Bearing in mind that gene ontological terms

213 applied at the end of an analysis may miss important genes, we started with large panels of genes

214 that are known to be specifically expressed in skeletal muscle cells. For controls, we also

215 generated gene groups comprised of genes expressed solely in cardiac muscle and genes

216   specific to alternate lineages. MNase-seq was used to measure nucleosome density at a single

217   base-pair resolution over a 4kb region surrounding the transcription start site (-3kb to +1kb) of all

218   the a priori chosen genes. After normalization to equally weight each gene and subtraction to

219   calculate changes in nucleosome density, statistical methods were used to elucidate the effects

220   of skeletal muscle differentiation on nucleosome structure (1). The approach we developed

221   demonstrated that myogenic differentiation induced nucleosome loss immediately around the

222   transcription start site of skeletal muscle genes. There was no such loss of nucleosomes around

223   the transcription start site of cardiac muscle or non-muscle genes (1). These findings were in

224   agreement with earlier studies (18) and demonstrated the validity our approach.

225   Having validated our approach in a model system, we have now applied it to study how

226   nucleosomes respond to reprogramming. The expectation, based on the epigenetic data as well

227   as the similarities between skeletal and cardiac muscle, was that nucleosomes would deplete

228   from around the transcription start site of cardiac muscle genes. Unexpectedly this was not

229   observed. Moreover, agents which enhance reprogramming efficacy by increasing cardiac

230   muscle gene activity also had no effect on nucleosomes around the transcription start site.

231   Instead, changes in nucleosome architecture were observed at some distance from the

232   transcription start site. There were three significant regions specific to cardiac muscle genes: -

233   2600bp to -2300bp (Region A), -900bp to -550bp (Region C) and +350bp to +900bp (Region E)

234   relative to the transcription start site. Rather than showing nucleosome depletion, as might be

235   expected, all three regions showed significant nucleosome accumulation. Taken together, this

236   data is hard to square with the aforementioned model of repressive and activating epigenetic

237   motifs regulating chromatin opening to modify gene expression. Analyses of nucleosome

238   architecture in fibroblast to cardiac muscle reprogramming are hard to come by and we are aware

239   of only one other report where the authors studied nucleosome architecture via the alternative

240   ATAC-seq method (19). Like our study, they found that the majority of changes in nucleosome

10

241   occupancy were at some distance away from the transcription start site (19). Moreover, the

242   authors provided evidence that changes in nucleosome occupancy were due to transcription

243   factors binding to chromatin. Long distance regulation of gene activity, as suggested in both

244   studies, could occur through mechanisms such as breaking closed-loop inhibition, whereby

245   nucleosome architecture is such that the gene is silenced through loop formation (20). The further

246   implication from both studies is that epigenetic marker re-positioning are not causative agents in

247   reprogramming, but are more to stabilize the gene activity patterns (8).

248   We have reported that the efficacy of reprogramming fibroblasts to cardiac muscle cells is

249   increased by RNA-sensing receptor agonists. To date, we have found evidence for the

250   involvement of two RNA-sensing receptors: TLR3 (12) and Rig1 (10). Both TLR3 and Rig1

251   enhance efficacy by increasing cardiac muscle gene activity. However, the two receptors achieve

252   this effect differently. While TLR3 utilizes the transcription factor NFkB, Rig1 instead utilizes the

253   transcription factor YY1. We were interested if this difference in transcription factor utilization was

254   mirrored in differences in nucleosome structure. Overall, both TLR3 and Rig1 agonists had a

255   significant impact on nucleosome architecture. Interestingly, and mirroring their disparate

256   transcription factor utilization, TLR3 and Rig1 agonists induced the adoption of distinct

257   nucleosome architectures as there was little correlation in terms of nucleosome positions. TLR3

258   and Rig1 agonists appeared to affect muscle and non-muscle genes differently. While both

259   agonists induced nucleosome depletion on muscle genes, the opposite was found on non-muscle

260   genes. Thus, TLR3 and Rig1 agonists appear to enhance muscle gene activity through

261   nucleosome re-arrangement and depletion.

262   In summary, studies of nucleosome architecture suggest that in the context of cellular

263   reprogramming cardiac muscle genes are activated via changes in nucleosome positioning distal

264   to the transcription factor start site.

265   **Acknowledgements**

266   The work was supported by the Edna and Fred L. Mandel, Jr. Foundation as well as the National

267   Heart, Lung and Blood Institute (R01 HL131814-01A1).

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322

323   **Figures & Figure Legends**

324   **Figure 1. Expression of muscle and non-muscle genes during the direct reprogramming of**

325   **fibroblasts into cardiac muscle cells.**

326   **(A)** To determine the minimum number of genes for analysis of nucleosome architecture, an

327   experiment was conducted whereby genes were additively compared to their full-group. The

328   comparisons were made on nucleosome density at a 1bp resolution across the full promoter and

329   the R^2 value noted after the addition of each gene. Five random sequences of genes were

330   investigated.

331   **(B)** RNA-seq data derived from fibroblasts four days after transfection with the reprogramming

332   cocktail miR combo were investigated for the expression of the members of each listed gene-

333   group. The data is expressed as a fold change in expression when compared to cells transfected

334   with a control miRNA. N=3. Significances were determined by Wilcoxon Signed Rank Test (Pratt

335   method, median = 1); ns – not significant, **$P<0.01$.

336

337   **Figure 2. Direct reprogramming of fibroblasts into cardiac muscle cells is associated with**

338   **significant changes in nucleosome architecture.**

14

339   **(A)** Our PRJNA837987 MNase-seq dataset was analyzed to determine the effects of direct

340   reprogramming on nucleosome positioning. To compare nucleosome patterns within each group

341   and across the three groups, the read counts were normalized by dividing the read count at each

342   base-pair of the promoter by the sum of read counts across the promoter (a full description is

343   provided in the methods). The schematic shows those regions in cardiac and common muscle

344   gene promoters where nucleosomes were significantly enriched or depleted in response to

345   reprogramming as well as in response to the reprogramming enhancers PolyIC and 3p-hpRNA.

346   **(B)** Regions of significant enrichment or depletion (A, B, C and E) were analyzed further alongside

347   a region encompassing the TSS (D) by determining the nucleosome correlations.

348

349   **Figure 3. Cardiac and common muscle genes show significant nucleosome re-positioning**

350   **away from the TSS.**

351   For each analyzed promoter, the change in read number (Δ normalized read count) following

352   direct reprogramming (miR combo versus control miR) was calculated at a 1bp resolution and

353   summed over the genomic region analyzed. Significances were determined by Wilcoxon Signed

354   Rank Test (Pratt method, median = 0); ns – not significant, **P<0.01.

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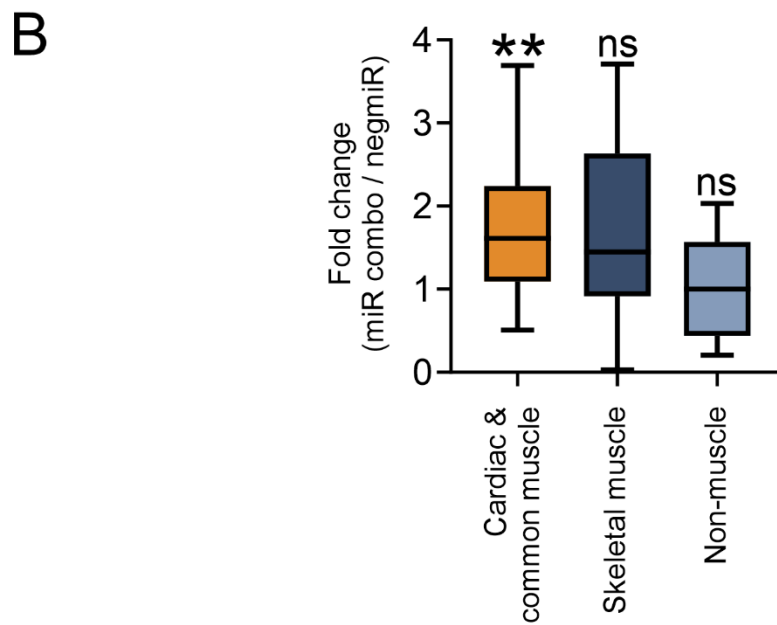356   **Figure 4. TLR3 and Rig1 agonists affect nucleosome architecture differently.**

357   For the whole promoter as well as regions A to E, the change in read number (Δ normalized read

358   count) following direct reprogramming (miR combo plus PolyIC/3p-hpRNA versus miR combo)

359   was calculated at a 1bp resolution and compared between the various gene groups. The matrices

360   report the correlations between the various groups.

361

362 **Figure 5. TLR3 and Rig1 agonists have opposing effects on nucleosome architecture in**

363 **muscle and non-muscle genes.**

364 For the whole promoter as well as regions A to E, the change in read number (Δ normalized read

365 count) following direct reprogramming (miR combo plus PolyIC/3p-hpRNA versus miR combo)

366 was calculated at a 1bp resolution and summed. Significances were determined by Wilcoxon

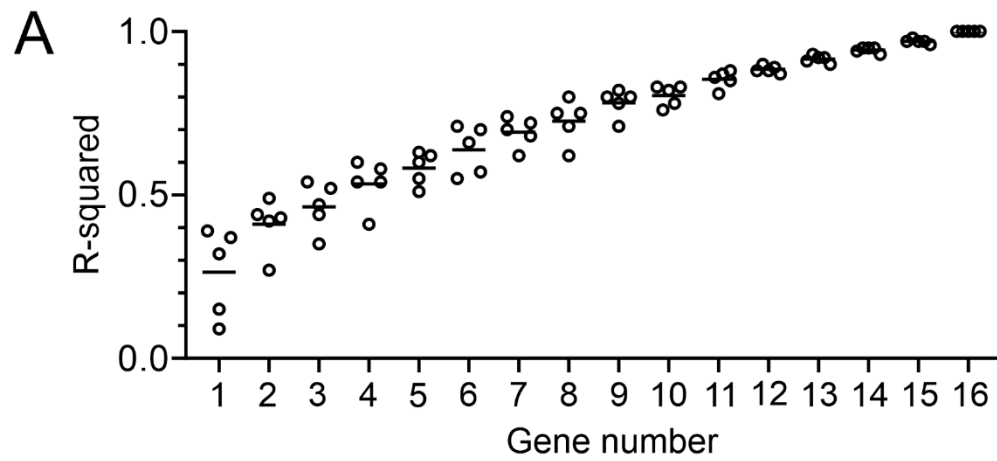367 Signed Rank Test (Pratt method, median = 0); ns – not significant, *P<0.05, **P<0.01, ***P<0.001.
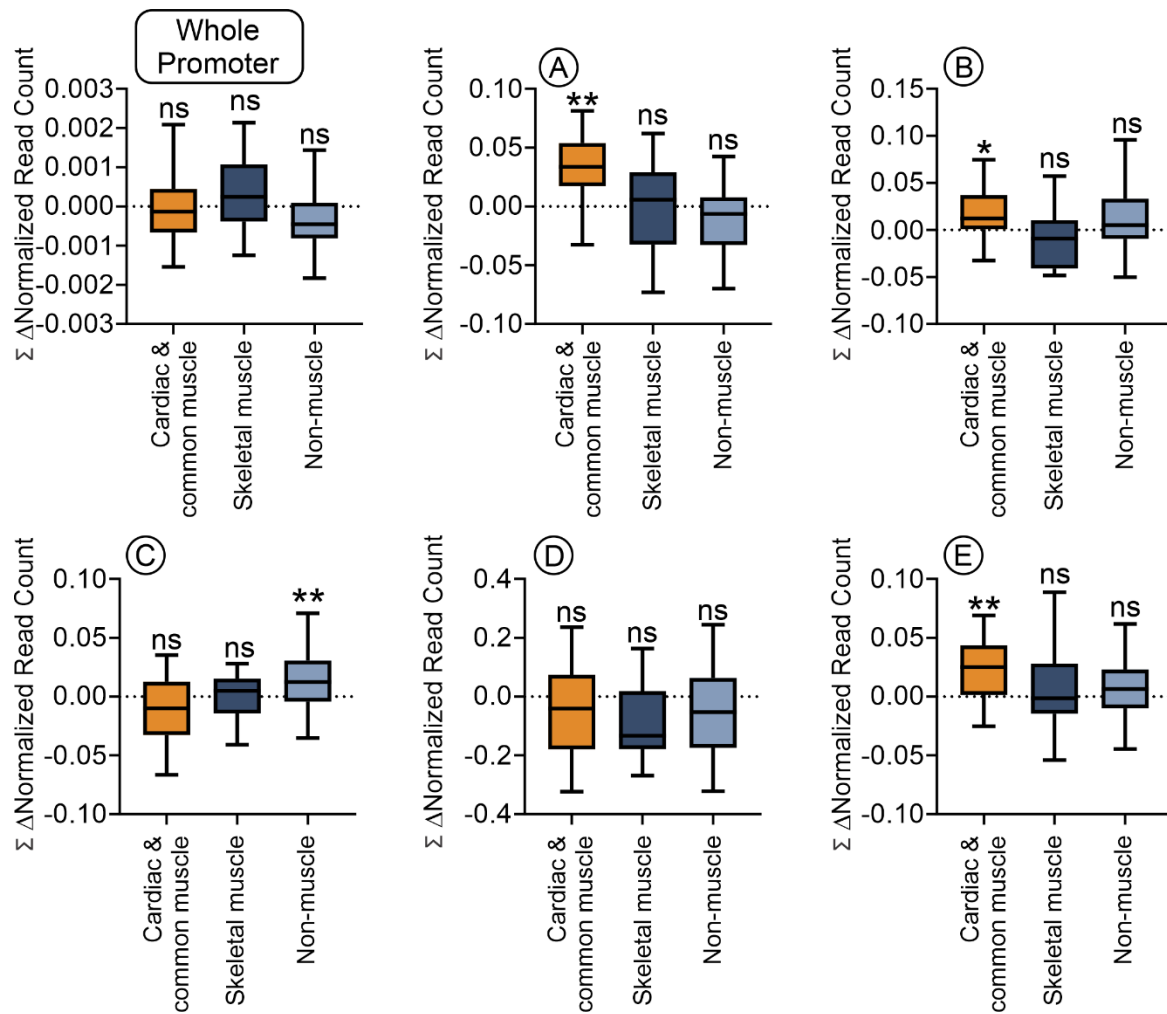
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Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 5

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