Exercise-associated DNA methylation change in skeletal muscle and the importance of imprinted genes: a bioinformatics meta-analysis

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ABSTRACT

Background Epigenetics is the study of processes—beyond DNA sequence alteration—producing heritable characteristics. For example, DNA methylation modifies gene expression without altering the nucleotide sequence. A well-studied DNA methylation-based phenomenon is genomic imprinting (ie, genotype-independent parent-of-origin effects).

Objective We aimed to elucidate: (1) the effect of exercise on DNA methylation and (2) the role of imprinted genes in skeletal muscle gene networks (ie, gene group functional profiling analyses).

Design Gene ontology (ie, gene product elucidation)/meta-analysis.

Data sources 26 skeletal muscle and 86 imprinted genes were subjected to g:Profiler ontology analysis. Meta-analysis assessed exercise-associated DNA methylation change.

Data extraction g:Profiler found four muscle gene networks with imprinted loci. Meta-analysis identified 16 articles (387 genes/1580 individuals) associated with exercise. Age, method, sample size, sex and tissue variation could elevate effect size bias.

Data synthesis Skeletal muscle gene networks including imprinted genes reported. Exercise-associated effect sizes were calculated by gene. Age, method, sample size, sex and tissue variation were moderators.

Results Six imprinted loci (*RB1*, *MEG3*, *UBE3A*, *PLAGL1*, *SGCE*, *INS*) were important for muscle gene networks, while meta-analysis uncovered five exercise-associated imprinted loci (*KCNQ1*, *MEG3*, *GRB10*, *L3MBTL1*, *PLAGL1*). DNA methylation decreased with exercise (60% of loci). Exercise-associated DNA methylation change was stronger among older people (ie, age accounted for 30% of the variation). Among older people, genes exhibiting DNA methylation decreases were part of a microRNA-regulated gene network functioning to suppress cancer.

Conclusions Imprinted genes were identified in skeletal muscle gene networks and exercise-associated DNA methylation change. Exercise-associated DNA methylation modification could rewind the 'epigenetic clock' as we age.

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INTRODUCTION

British developmental biologist Sir Conrad H Waddington introduced the term 'epigenetics' as a science of development from genotype to phenotype.[1] However, the term 'epigenetics' had an independent origin and meaning, which led to a conflation of terms.[2] Recall Waddington's use of the term 'epigenetics' to refer to the causal processes of development, with an emphasis on interactions among genes and between genes and the environment. In contrast, Nanney[3] used 'epigenetic' in 1958 to describe a system of cellular heredity that was not based on DNA sequence.

Most molecular biologists use the term epigenetic to mean the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence—hence the name epi- (Greek for 'over', 'above' or 'outer') -genetics. Examples of such changes are DNA methylation and histone modifications (figure 1), both of which can regulate gene expression without altering the underlying DNA sequence. One well-studied epigenetic phenomenon based on DNA methylation in mammals (and notably humans) is genomic imprinting.[4] One purpose of this review is to introduce this term to the sports and exercise medicine/physiotherapy community and explain its importance.

Genomic imprinting

Genomic imprinting is defined as genotype-independent parent-of-origin gene expression. Specifically, for most genes we inherit two working parental copies. However, in the case of imprinted genes, an epigenetic tag (via DNA methylation) is placed on either the maternal or paternal copy rendering the other inactive. Such parent-of-origin gene expression is mediated by epigenetic modifications which differ between the two parentally derived chromosomes.[5] Approximately 1% of the human genome is imprinted.[6 7] Despite their rarity, imprinted genes are of great medical importance. Studies of metabolic growth and neurodevelopmental disorders have shown that imprinted genes are absolutely essential for healthy development.[8–13] Furthermore, epigenetic dysregulation in imprinted genes—which often have growth-enhancing and tumour suppressor functions—predict disease and cancer outcomes.

Once epigenetic mechanisms emerged during mammalian evolution (eg, genomic imprinting), a source of environmental information (ie, parent-of-origin of a gene) was transmitted transgenerationally. Imprinting machinery (eg, DNA methylation, see figure 1—a type of silencer at a gene's promoter—places marks on a gene when the gametes are produced) allowed for biased gene expression in the subsequent generation (ie, monoallelic gene expression).

The realisation that the environment has profound influences on the epigenome has led to a strong hypothesis that exercise can also affect DNA methylation and have long-term health outcomes.

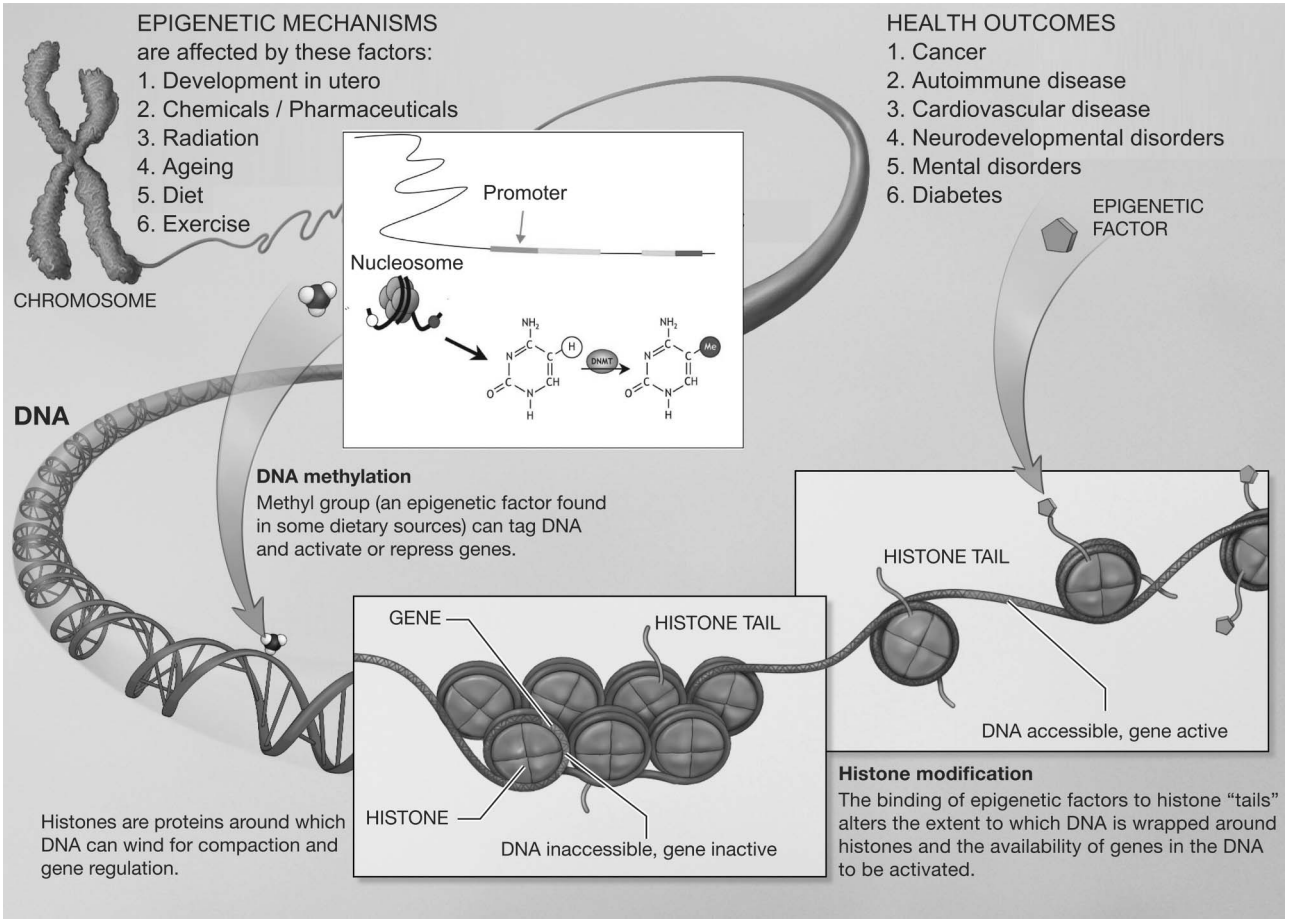

Figure 1 DNA methylation: DNA methylation occurs when methyl groups 'tag' DNA and activate or repress gene expression. DNA methyltransferase is a catalyst, which transfers methyl groups to DNA. Silencing of a gene activity can occur if the hydrogen (H) molecule of cytosine (C) is replaced by a methyl (Me) group at a gene's promoter. *Histones*: Histones are proteins around which DNA can wind for compaction and histone modification can regulate gene activity. Both epigenetic processes (ie, DNA methylation and histone modifications) affect health resulting in cancer, autoimmune disease, neurological disorders or diabetes. Image modified from the National Institutes of Health, Benjamin I. Laufer and Forluvoft.

Specifically, we propose that the same mechanism that controls genomic imprinting in mammals (ie, DNA methylation) allows for phenotypic modification and the possibility of multiple sources of environmental information (eg, exercise, nutrition) to be transmitted to the next generation. Muscle and nerve cells share the property of responding to electrochemical/environmental stimuli and thus are ideal epigenetic interfaces for transgenerational phenotypic modification, which may explain in part why imprinted genes are particularly involved in neural development.

DNA methylation

DNA methylation refers to the adding of a methyl group on a cytosine base. It occurs primarily in the context of CpG dinucleotides, which cluster in regions called CpG islands.[14] 'CpG' refers to regions of DNA where a cytosine nucleotide appears next to guanine nucleotide interconnected by phosphate. When cytosines in CpG dinucleotides are methylated to form 5-methylcytosine, a gene can be turned off. CpG dinucleotides are rare in mammals (~1%), but ~50% of gene promoters are linked to CpG islands which are often unmethylated in healthy cells. Cells become methylated in a tissue-specific and age-specific manner during development. Where DNA methylation occurs can be critical for its effect. DNA methylation (ie, adding methyl groups to a cytosine base, figure 1) at a gene's promoter is linked to silencing (ie, less gene expression); in contrast, DNA methylation outside the promoter region (eg, gene body) is sometimes associated with increased gene expression. In the case of genomic imprinting, hypermethylation of one of the two parental alleles leads to monoallelic expression (conceptually similar to gene-dosage reduction in X-inactivation, see figure 2). DNA methylation has been implicated in cancer, neurodevelopmental disorders and autoimmune diseases.[15] Thus, if exercise can influence DNA methylation, it may be the mechanism that underpins the lower cancer rate in those who are physically active.

DNA methylation, health and diverse disease states

Cancer cells are characterised by a global loss of DNA methylation among growth enhancers; and the coordinated acquisition of hypermethylation at the CpG islands of tumour suppressor genes. Global hypomethylation occurs primarily at parasitic DNA regions of the genome. For example, the LINE family member L1 is hypomethylated in a variety of cancers, such as those of the breast and colon.[16]

Neurological disorders are also associated with epigenetic dysregulation (ie, reversed patterns of a normal DNA methylation profile). Specifically, dysregulation of DNA methylation occurs in several neurological diseases, giving rise to hypermethylated and hypomethylated CpG sites. *FMR1* promoter hypermethylation occurs among individuals diagnosed with Fragile X syndrome. Rett syndrome, an X-linked neurological disease, is

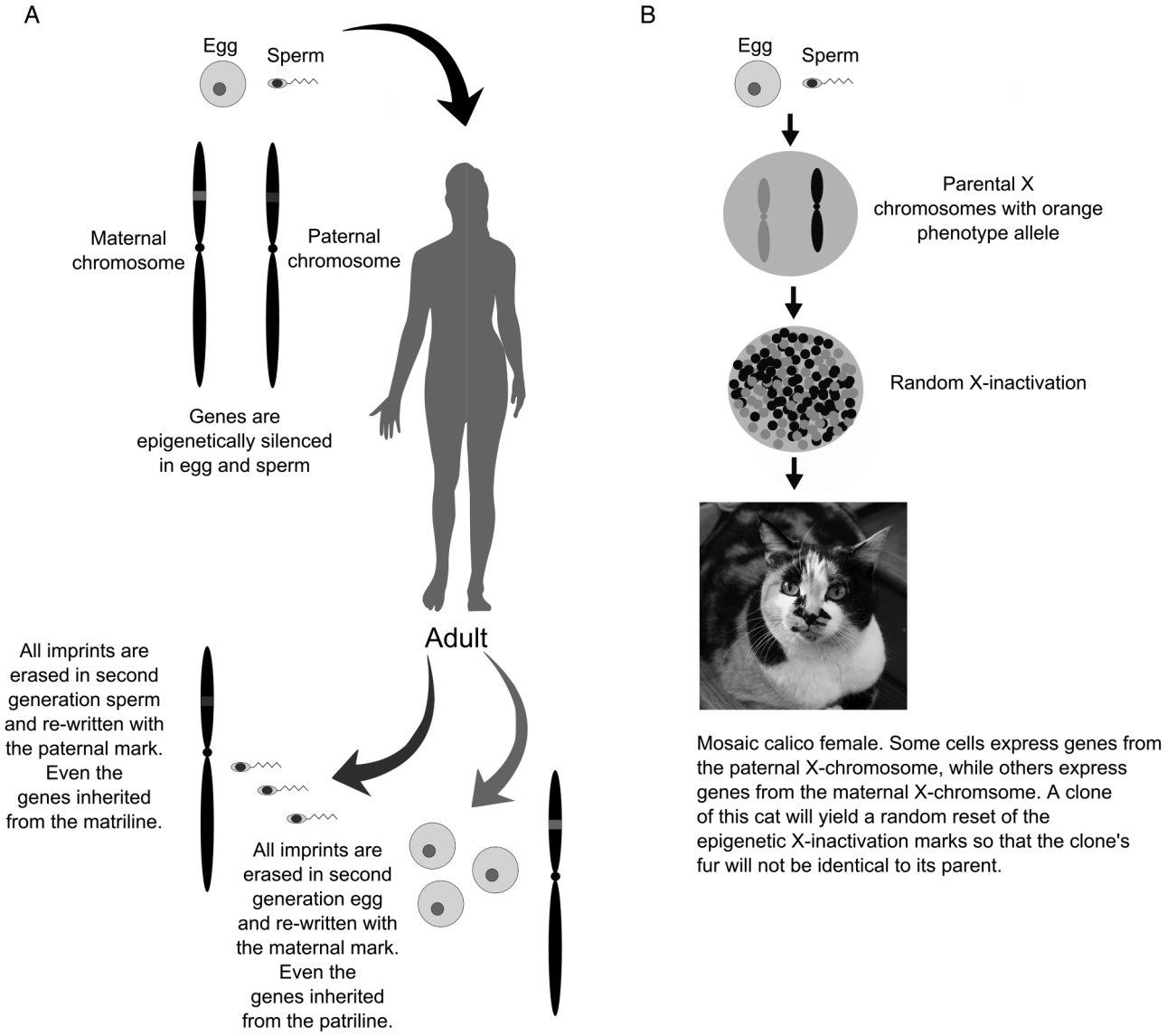

Figure 2 (A) Genomic imprinting: Genomic imprinting is parent-of-origin (but genotype independent) gene expression. When males and females produce gametes (ie, sperm or eggs) an epigenetic mark (eg, DNA methylation, which silences one of the parental alleles) is placed on the DNA to indicate parent-of-origin. Regardless of sex of offspring imprinted genes affect growth and neural development differentially by parent-of-origin. Once the child produces its own gametes the imprints are erased and new parent-of-origin marks are established. Imprinted genes are rare but have profound effects on growth and neurodevelopment. (B) X-inactivation: X-inactivation is a process by which one parental copy of the X chromosome in women is randomly deactivated. X-inactivation prevents females from having twice as much X chromosome gene production as males (which only have one copy of the X chromosome). Once the X chromosome is deactivated, it remains silent throughout the cell's lifetime. Compared with transcriptionally active X chromosome, the inactive X has higher levels of DNA methylation, which is associated with gene silencing. One difference between imprinting and X-inactivation is the former is not random process with respect to which parental allele is epigenetically silenced.

caused by point mutations in *MECP2*, which encodes a methyl binding protein and is proposed to be a gene silencer.[17]

DNA methylation dysregulation is associated with autoimmune disease. For example, the Immunodeficiency Centromeric Instability and Facial Anomalies (ICFA) syndrome is caused by heterozygous mutations in *DNMT3B*. Individuals with ICFA show DNA hypomethylation among Alu repeats. Interestingly, despite patients with ICFA having normal global DNA methylation profiles, key developmental regulatory and immune function genes show loci-specific epigenetic dysregulation.[15]

Whether it is cancers, neurological disorders or autoimmune disease, DNA methylation and imprinted genes are emerging causal factors. Some imprinted genes are involved in multiple phenotypes,[18] suggesting that imprinting performs a regulatory function during ontogeny (eg, regulation of other genes).

The current paper argues that imprinted genes are important for skeletal muscle development and their phenotypic effects reflect an underlying ancestral tug of war between parental genomes over offspring growth and developmental trajectories.

Imprinted genes and skeletal muscle gene networks: growth suppression and enhancement

Imprinted genes are genes whereby an epigenetic mark is laid down during gametogenesis, indicating a key environmental source of information, the parental origin of a particular gene. There are few imprinted genes in the human genome, but they are often associated with growth, neural functioning and

behaviour. Parental antagonism theory[19] is currently the best theory for the phenotypic effects of growth regulators (eg, the paternal genome within offspring fosters growth at a cost to the maternal genome, while the maternal genome attempts to minimise these costs by suppressing growth).

Haig's model[19] proposes that imprinting evolved as a result of opposing fitness interests of parental genomes. For example, in polygamous species, patrigenes (genes expressed within offspring inherited from the father) favour fetal growth at the expense of depleting maternal resources and disadvantaging future offspring. Meanwhile, matrigenes (genes expressed within offspring inherited from the mother) will oppose the paternal effect and conserve resources to optimise inclusive fitness of the mother and future offspring. Haig's theory[19] predicts that paternally expressed imprinted genes will often promote growth, while maternally expressed genes will have opposite effects to reduce costs on matrilineal inclusive fitness.

IGF2—an imprinted gene and paternally expressed in humans—regulates muscle development. *IGF2* is upregulated early in *MyoD*-induced in myocyte differentiation and *IGF2* inhibition leads to reduced expression of *MyoD* target genes, which suggests that *IGF2* is essential for amplifying and maintaining *MyoD* efficacy.[20] *IGF2*'s role as a paternally derived skeletal muscle growth enhancer is consistent with the theoretical orientation of this paper.

Germane to sports medicine, some imprinting disorders affect muscle growth. For example, Angelman (AS) and Prader-Willi syndromes (PWS) are imprinting disorders affecting muscle development and health. PWS is caused by an overexpression of maternal genes on chromosome 15, while AS is due to an overexpression of paternal genes. Muscle biopsies of 11 PWS children have been investigated using histochemical and morphometric methods.[21] The phenotypic abnormalities included (A) fibre size variation of both type 1 and 2 fibres, (B) type 2 fibre atrophy, (C) increased numbers of type 2C fibres and (D) decreased numbers of type 2B fibre. This finding is consistent with the overexpression of maternal genes suppressing skeletal muscle growth. In addition to their low muscle tone, PWS individuals experience chronic hunger, potentially leading to overeating and obesity.

DNA methylation and imprinted loci influence muscle hypertrophy—extremely muscled hindquarters—in callipyge sheep.[22] Hypomethylation of *Clpg1* causes muscle hypertrophy, in part due to the overexpression of *Dlk1*,[22] a paternally expressed imprinted gene associated with muscle precursor cell (myoblast) differentiation.[23] DNA demethylation promotes skeletal myotube maturation.[24] Early experiments in the 1970s showed that DNA methyltransferase inhibitors (eg, 5-azacytidine) induced transdifferentiation of fibroblasts into myoblasts.[25] More recently, in C2C12 culture, Hupkes *et al*[24] noticed that on treatment with the methylation inhibitor (ie, 5-azacytidine), myotubes spontaneously acquired repetitive membrane activity, intracellular calcium transients and contractility. Hupkes *et al*[24] suggested that DNA methylation may pose an epigenetic barrier to C2C12 myotubes reaching maturity. However, when imprinted genes are involved in skeletal muscle development, the so-called 'DNA methylation barrier' will likely be parent-of-origin dependent. Beyond the distinct possibility that imprinted genes coordinate mammalian skeletal muscle development (eg, regulating skeletal muscle gene networks), it remains to be investigated whether the DNA methylation of imprinted loci are responsive to human exercise.

Exercise epigenetics and DNA methylation

Traditionally, exercise biologists envision biological systems changing by the regulation of protein synthesis (eg, alteration of receptor expression or intracellular signalling). Since transcription precedes translation, it is often at the level of the 'transcriptome' that adaptations can be tracked at the molecular level. Subtle changes in gene transcription occur through epigenetic regulatory machinery. A variety of epigenetic mechanisms allow for transcriptional activation and specification of cell identity, maintaining homoeostasis and responding to environmental conditions. These epigenetic mechanisms encompass DNA methylation, post-translational histone modifications and microRNA (figure 3). Much of the previous research on environmental epigenomics involves nutrition; however, exercise physiology is coming to the forefront. There is evidence that DNA methylation can change due to short bouts of exercise (eg, exercising to exhaustion[26]) and longer, more sustained exercise regimens (eg, 6 months of controlled walking[27]). For example, a high-intensity interval walking regimen increased DNA methylation of the proinflammatory gene *ASC* (apoptosis-associated speck-like protein containing a caspase recruitment domain) among older adults nearly to the levels of healthy younger adults.[28] Among breast cancer sufferers, DNA methylation changes in *L3MBTL1* (an imprinted and possible tumour suppressor gene) due to exercise (eg, brisk walking on treadmill) has been demonstrated.[29]

Exercise epigenetics: research questions

What is the average effect of exercise on changes in DNA methylation and does age, research design, sample size, sex or tissue heterogeneity influence the size of the effect? Are imprinted genes implicated in skeletal muscle gene networks and exercise-associated DNA methylation changes in humans? Since imprinted genes regulate adiposity, energy expenditure and glucose homoeostasis, it was hypothesised that imprinted genes will be involved in human skeletal muscle gene networks and targets of exercise-associated DNA methylation change. To test these hypotheses, gene ontology and meta-analytic methodologies were utilised.

METHODS

A human skeletal muscle gene network: testing the importance of imprinted genes

It is hypothesised that human skeletal muscle growth is regulated by imprinted genes. A gene ontology networking web server called g:Profiler[30 31] was used to assess the functional involvement of imprinted genes for skeletal muscle gene networks. First, human skeletal muscle genes were determined using the Xavier laboratory gene enrichment profiler[32 33] (figure 4). To test the hypothesis that imprinted genes are implicated in skeletal muscle gene networks, g:Profiler[30 31] was used. Human imprinted genes (29 maternally expressed and 57 paternally expressed) were selected from http://www.geneimprint.com. Imprinted genes were combined with 26 skeletal muscle genes (figure 4).

Meta-analysis on exercise-associated DNA methylation change

This meta-analysis was limited to English as no foreign language results were found. Only published papers measuring DNA methylation and exercise in humans were used. The following search strings were entered into PubMed (1968 to 2 May 2014): (1) 'DNA methylation and exercise'; and (4) 'DNA methylation and physical activity (human-only)'. Search results finalised by the author (figure 5). Both PRISMA (see online supplementary file 1) and MOOSE (see online supplementary file 2) guidelines

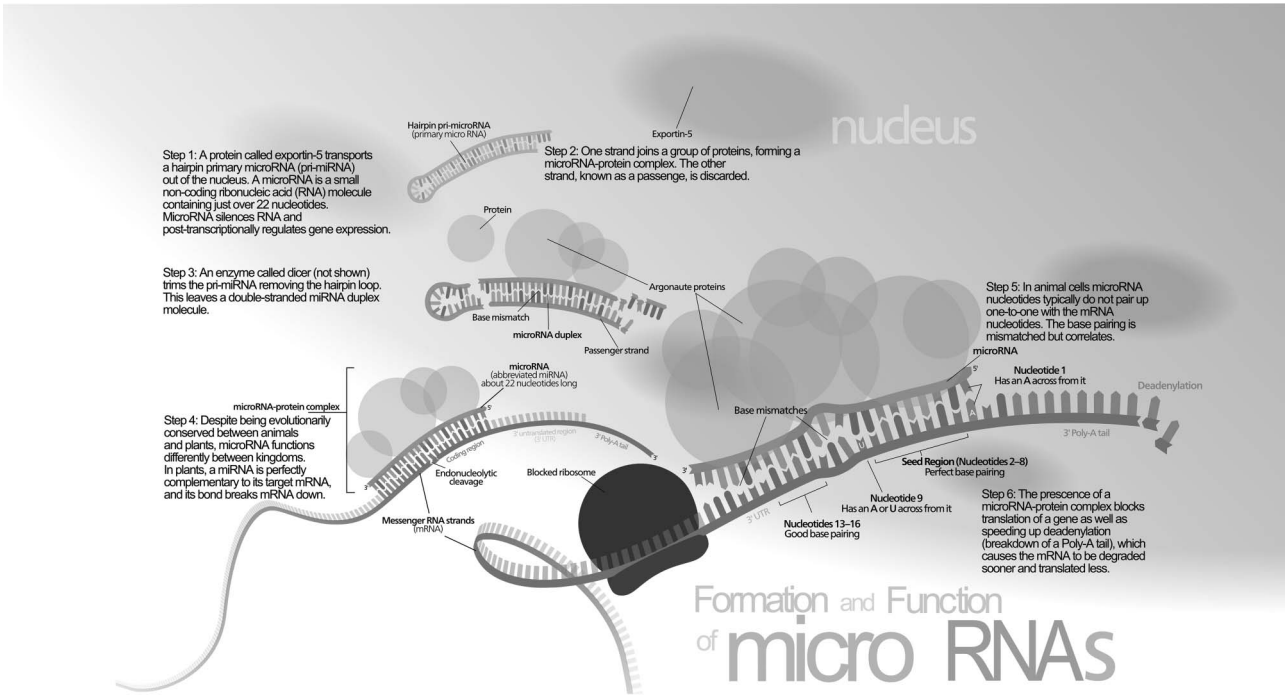

Figure 3 MicroRNAs (miRNAs): A miRNA is a small (ie, approximately 19 to 25 nucleotides in length) non-coding RNA molecule functioning to silence RNA and involved in the post-transcriptionally modification of gene expression. miRNA should not be confused with messenger RNA. miRNAs are another important molecular epigenetic regulator. miRNAs can result in small, but important reductions in physiologically relevant gene expression by blocking translation. Modified from Kelvin Song.

followed. One author did not respond to a direction of effect query. Reference lists did not yield additional articles.

RESULTS

Imprinted genes and skeletal muscle gene networks

Table 1 shows the imprinted genes associated with skeletal muscle gene networks. Six imprinted genes (ie, three maternally expressed genes *RB1*, *MEG3* and *UBE3A* and three paternally expressed genes *INS*, *PLAGL1* and *SGCE*) were revealed to be part of the gene networks of highly enriched skeletal muscle loci. Considering the rarity of imprinted genes in the human, this is biologically significant. Below is a description of each imprinted gene involved.

Paternally expressed genes linked to muscle-related phenotypes

1. Pleomorphic adenoma gene-like 1 (*PLAGL1*): As seen in table 1, *PLAGL1* is part of two gene ontology networks: 'muscle organ development' (GO: 0007517) and 'skeletal muscle tissue development' (GO: 0007519). *PLAGL1* encodes a zinc finger protein with transactivational and DNA-binding functions. *PLAGL1* has antiproliferative properties making it a candidate for functioning as a tumour suppressor gene. Overexpression of this gene during fetal development underlies transient neonatal diabetes mellitus (TNDM). In most tissues (eg, skeletal muscle), *PLAGL1* appears to be expressed from the paternal allele.[34]
2. Sarcoglycan, epsilon (*SGCE*): As seen in table 1, *SGCE* is part of a gene ontology network called 'muscle organ development' (GO: 0007517) and a human phenotype gene network called 'abnormality of the musculature of the neck' (HP: 0011006). *SGCE* encodes the epsilon member of the sarcoglycan family (ie, transmembrane proteins which are part of the dystrophin-glycoprotein complex linking the actin cytoskeleton to the extracellular matrix). Epsilon sarcoglycan is more broadly expressed (ie, not just restricted to striated muscle). Mutations in this gene are associated with the myoclonus-dystonia syndrome and it is imprinted (preferentially expressed from the paternal copy).[34]
3. Insulin (*INS*): As seen in table 1, *INS* is part of a human phenotype gene network called 'motor delay' (HP: 001270). The *INS* gene encodes for proinsulin (a prohormone precursor to insulin), which is post-translationally cleaved into three peptides. Binding of insulin to the insulin receptor (*INSR*) stimulates glucose uptake. A multitude of mutant alleles with phenotypic effects have been identified. Notably, *INS-IGF2*, a read-through gene, aligns to the *INS* gene, whereby *INS* is at the *5'* region and *IGF2*—an extremely well-studied growth regulatory imprinted gene—is at the 3' region.[34]

Maternally expressed genes linked to muscle-related phenotypes

1. Retinoblastoma 1 (*RB1*): As seen in table 1, *RB1* is part of two gene ontology networks: 'muscle organ development' (GO:0007517) and 'skeletal muscle tissue development' (GO:0007519). *RB1* encodes a protein that negative regulates cell cycle and was the first tumour suppressor gene discovered. The encoded protein maintains overall chromatin structure. Defects in *RB1* cause childhood retinoblastoma (RB), bladder cancer and osteogenic sarcoma.[34]
2. Maternally expressed 3 non-protein coding (*MEG3*): As seen in table 1, *MEG3* is part of two gene ontology networks: 'muscle organ development' (GO: 0007517) and 'skeletal muscle tissue development' (GO: 0007519). *MEG3* is expressed in many healthy tissues, but expression is lost in multiple cancer cell lines of various tissue types. Notably, *MEG3* suppresses tumour cell proliferation in vitro and

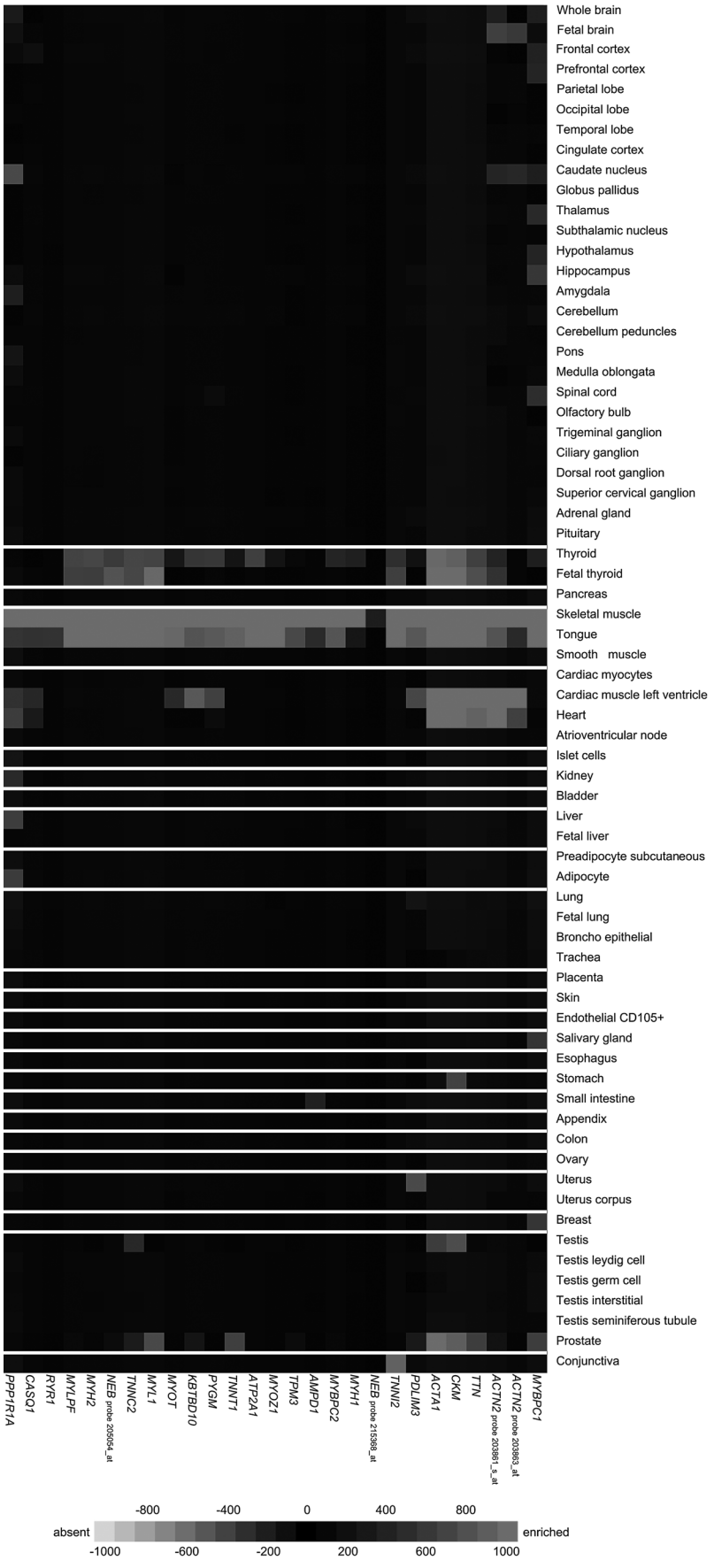

Figure 4 Enrichment profile for selected human skeletal muscle genes (defined as genes that have high enrichment scores within tissue and between tissues). Embryonic stem cells, t cells, b cells and myeloid gene loadings have been removed from heat map.[32 33]

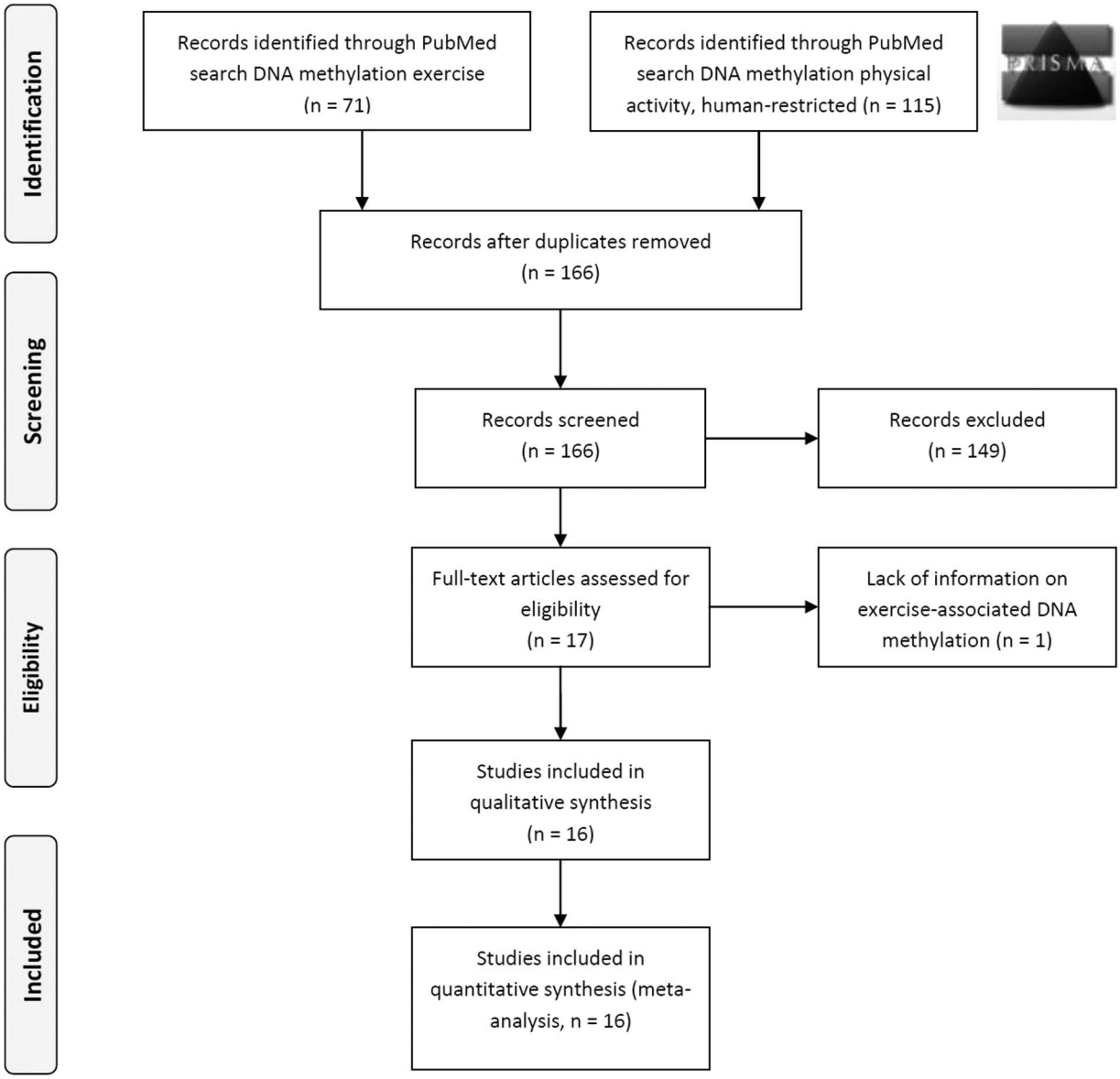

Figure 5 PRISMA flow diagram for meta-analysis component of paper. Search terms used were 'DNA methylation exercise' or 'DNA methylation physical activity'. Human in vivo studies only (May 2014).

interacts with tumour suppressor p53. Deleting *MEG3* enhances angiogenesis in vivo. Many studies show that *MEG3* is a long non-coding RNA tumour suppressor.[34]

3. Ubiquitin protein ligase E3A (*UBE3A*): As seen in table 1, *UBE3A* is part of a human phenotype gene network called 'motor delay' (HP:001270). *UBE3A* encodes an E3 ubiquitin-protein ligase. This imprinted gene is maternally expressed in the brain and most likely biallelically expressed in skeletal muscle. Maternally inherited deletion of this gene causes a neurodevelopmental disorder AS which is characterised by severe motor and intellectual impairments, ataxia, hypotonia, epilepsy and absence of speech. *UBE3A's* protein in part causes ubiquitination and proteolysis of tumour protein p53.[34]

Meta-analysis of exercise-associated DNA methylation change

To determine if there is a directional bias in exercise-associated methylation change, a binomial signed test was conducted. Two-hundred and eighty-seven genetic elements (out of 478) showed significantly decreased DNA methylation after exercise (binomial test $p<0.001$). Online supplementary table S2 reports the 478 genetic elements showing exercise-associated DNA methylation change, 5 of which are imprinted genes (maternally expressed (*GRB10*, *KCNQ1*, *MEG3*) and paternally expressed (*PLAGL1*, *L3MBTL*)). Despite appearing like a small percentage of imprinted loci, this is much higher than the expected number of 1–2 (ie, assuming there are ~90 imprinted genes in a human genome containing ~22 300 genes): binomial test $p<0.03$. All imprinted genes showed a decrease in DNA methylation after exercise, except for *GRB10* and *KCNQ1* (adipose tissue only).

Table 2 provides location, ontology and growth-related effects for the imprinted genes showing exercise-associated DNA methylation change. Unfortunately, it was not possible to determine if exercise-associated DNA methylation change in the imprinted loci was near differentially methylated regions (DMRs) as each imprinted gene in table 2 has clinically relevant single-nucleotide polymorphisms. Among the five imprinted

Table 1 Imprinted genes networked with human skeletal muscle genes and network function

Function	ID	p Value	Genes	PAT	MAT
Muscle organ development	GO: 0007517	0.0005	*PLAGL1, SGCE, RB1, MEG3, MYL1, NEB, ACTA1, TTN, MYLPF, RYR1, CASQ1*	*PLAGL1, SGCE*	*RB1, MEG3*
Skeletal muscle tissue development	GO: 0007519	0.05	*RB1, MEG3, PLAGL1, ACTA1, MYLPF, RYR1, CASQ1*	*PLAGL1*	*RB1, MEG3*
Motor delay	HP: 001270	0.03	*UBE3A, INS, NDN, NEB, ACTA1, TTN, TNNT1, TPM3, RYR1*	*INS*	*UBE3A*
Abnormality of the musculature of the neck	HP: 0011006	0.05	*SGCE, NEB, ACTA1, TTN, TPM3*	*SGCE*	–

Networks determined by g:Profiler.[30 31] Static url: http://tinyurl.com/imprinting-skm.
GO, gene ontology; HP, human phenotype; MAT, maternally expressed gene; PAT, paternally expressed gene.

genes revealed by the meta-analytic search in table 2, DNA was extracted from diverse tissues (ie, skeletal muscle, adipose and blood).

Degree of exercise-associated DNA methylation change: effect of age and confounded factors

The effect size of exercise on DNA methylation for the 478 genetic elements (387 were unique genes) across 16 different publications and 1580 people—see online supplementary table S2)—is large (mean Cohen's d=1.20±1.20; 95% CI of the mean 1.10 to 1.31) and significantly different from a test value of zero (ie, no effect of exercise on DNA methylation): one-sample t(477)=22.77, p<0.001. Analysis of covariance (ANCOVA) revealed that the effect size of exercise-associated DNA methylation change was significantly greater for people over 40 years of age (Cohen's d=2.89±1.97) compared with those under 40 years of age (Cohen's d=0.90±.51): (F(1,471) =197.26, p<0.001, partial η^2=0.30). In this model, the effect of age was independent of research design (experimental designs' larger effect size, p<0.001), sample size (smaller studies' larger effect sizes, p<0.001), sex (larger effect size among females than males, p<0.03) and tissue specificity (larger effect sizes in tissue with more cell types, p<0.001). The fact that sample size and effect size are significantly and negatively correlated suggests the presence of publication bias: r (477)= −0.11; p<0.02. Sample size has been included as a covariate in analyses.

Online supplementary table S2 shows that most of the genes decreased in DNA methylation percentage after exercise (238/387 different genes). ANCOVA (sample size controlled) found a direction of change by age interaction: F(1,381)=20.14, p<0.001, partial η^2=0.05. Specifically, among older people (people older than 40 years of age), the effect size was significantly larger (p<0.05) when DNA methylation increased with exercise (3.85; 95% CI of the mean 3.32 to 4.38) compared with when DNA methylation decreased with exercise (3.04, 95% CI of the mean 2.45 to 3.63). However, the reverse was true for people under 40 years of age. Specifically, among younger people (people less than 40 years of age), the effect size was significantly smaller (p<0.05) when DNA methylation increased with exercise (0.90; 95% CI of the mean 0.83 to 0.97) compared with when DNA methylation decreased with exercise (1.00, 95% CI of the mean 0.95 to 1.05).

To elucidate the possible function of this interaction among older people, the genes showing increases and decreases after exercise were exposed to g:Profiler[30 31] for ontology analysis by age. As seen in table 3, among older people the genes that increased in DNA methylation after exercise were associated with growth regulation (GO:0022603), and the genes that become less methylated after exercise are targets of a putative tumour suppressing microRNA *miR-519B*. Two imprinted genes (*L3MBTL1*, *PLAGL1*)—both of which are tumour suppressors—are associated with *miR-519b*'s microRNA network.

Among younger individuals, a microRNA-regulated gene network involved in stem cell activity was implicated. Specifically, as seen in table 4, genes that increased in DNA methylation among younger people were part of a microRNA-regulated (hsa-miR-130b*) gene network that suppresses stem cell activity. Statistically significant (all p values <0.04) gene networks were uncovered for the genes that decreased in DNA methylation after exercise among younger people. Specifically, these gene networks are important for the biological processes of the extracellular matrix, skeletal muscle and cartilage development (table 4).

Tissue heterogeneity

Tissue type is a moderator of the degree of exercise-associated DNA methylation change. To further elucidate this apparent moderator, a one-way ANCOVA (controlling for sample size) was conducted and found significant: F(4,471)=137.03, p<0.001, partial η^2=0.54 (figure 6A). As seen in figure 6A, exercise-associated DNA methylation change was greater in

Table 2 Imprinted genes that showed DNA methylation changes associated with exercise

Gene	Ontology	Expression	Chromosome	Start	End	Growth
PLAGL1	Cell differentiation skeletal muscle; apoptosis	Paternal	6	144 328 445	144 328 885	Enhancer
GRB10	Insulin receptor pathway (negative regulation)	Maternal skeletal muscle γ splice variant	7	50 850 662	50 851 107	Suppressor
KCNQ1	Cardiovascular system development; negative regulation of insulin secretion; gene silencing	Maternal	11	2 465 914	2 870 339	Suppressor
MEG3	Negative regulation of angiogenesis; cell proliferation, positive regulation of skeletal muscle fibre development	Maternal	14	101 293 947	101 294 390	Suppressor
L3MBTL	Regulation of megakaryocyte differentiation	Paternal	20	42 142 508	42 142 820	Enhancer

Function, parent-of-origin, differentially methylated region (DMR) location and hypothesised growth effects. Location of DMRs courtesy of Randy Jirtle. For *KCNQ1*, the DMR[48] resides in intron 10. Genome Reference Consortium Human Build 37 (GRCh37).

Table 3 Genes that showed exercise-associated increases (http://tinyurl.com/methincreased) and decreases (http://tinyurl.com/methdecreased) in DNA methylation among older people

Change	Function	ID	p Value	Genes
Increased DNA methylation after exercise	Regulation of anatomical structure morphogenesis	GO:0022603	0.018	*CXCL10,DCC, PPP2R3A, RASA1,SULF1,TMEM100, WNT7A*
Decreased DNA methylation after exercise	microRNA 519 inhibits cell proliferation and decreases tumour growth	MI:hsa-miR-519b-3p	0.016	*GAB1,L3MBTL1, PLAGL1, WNK3,BCL2L11,CACNA2D3*

Gene networks courtesy g:Profiler.[30 31]
GO, gene ontology; MI, computationally predicted microRNA target sites from the MicroCosm database (formerly miRBase).

blood samples compared with all tissue types (ie, buccal and salvia, breast and adipose, skeletal muscle and gastric tumour). For three out of five tissue types, the effect sizes are large. For buccal cells and saliva and gastric tumours, the effect sizes were medium.

Exercise type

Exercise type is a potential moderator of the degree of DNA methylation change. To further elucidate the effect of exercise type on the degree of DNA methylation change, a one-way ANCOVA (controlling for sample size) was conducted and found significant: $F(2,453)=126.11$, $p<0.001$, partial $\eta^2=0.36$ (figure 6B). As seen in figure 6B, DNA methylation change was significantly greater among those engaged in Tai Chi and walking compared with those engaged in cycling. Walking and Tai Chi were not significantly different from one another ($p=0.25$). Regardless of the specific type of exercise, effect sizes were large.

DISCUSSION

Imprinted genes and skeletal muscle gene networks

As predicted, imprinted genes were implicated in skeletal muscle gene networks. Table 1 is consistent with the hypothesis that parental genomes act simultaneously to suppress and promote skeletal muscle growth. The argument is that for skeletal muscle the maternal genes in table 1 (*RB1*, *MEG3* and *UBE3A*) suppress growth, while paternal genes (*INS*, *PLAGL1* and *SGCE*) perform antagonistic functions (ie, growth enhancement).

Some imprinted genes expected based on non-human animal research were conspicuously absent from the human skeletal muscle gene networks. For example, in sheep, imprinted gene *H19* regulates muscle development.[35] *H19* is also known as *ASM* for 'adult skeletal muscle'. *H19* is a negative regulator of prenatal growth and bovine muscle development.[35] *H19* is maternally expressed at high levels in embryonic and fetal skeletal muscle and is located closely downstream of paternally expressed *IGF2* performing antagonistic functions (ie, growth enhancement).

Beyond *H19*, other imprinted genes studied in non-human animals were absent from the human skeletal muscle gene networks, such as *DLK1*, which is a well-known imprinted gene and paternally expressed muscle growth enhancer. In mouse skeletal muscle cultures, the genetic ablation of Delta-like 1homolog (*Dlk1*) causes reductions in skeletal muscle mass, in part due to myofiber number loss and myosin heavy chain IIB gene expression.[36] *GRB10* was another imprinted gene missing from g:Profiler's human skeletal muscle gene networks. In mice, *Grb10* has a tissue-dependent imprinting status (ie, paternally expressed in the brain and maternally expressed in muscle, see Garfield *et al*[37] for its links to behaviour). Holt *et al*[38] have found evidence that when *Grb10* is deleted, hypermuscularity overgrowth occurs, suggesting that maternal gene expression functions to suppress muscle growth. The same pattern occurs in human skeletal muscle.[39]

Considering the rarity of imprinted genes, it is remarkable that six imprinted genes were discovered as part of skeletal muscle gene networks. Imprinted genes most likely repress, maintain and induce muscle-specific transcription during myogenesis. Future studies should investigate epigenomic antagonisms between paternally and maternally derived genes during myogenesis, as opposed to assuming that decreased methylation invariably leads to growth. Owing to the importance of

Table 4 Genes that showed exercise-associated increases and decreases (http://tinyurl.com/methdecreases-young) in DNA methylation among younger people

Change	Function	ID	p Value	Genes
Increased DNA methylation after exercise	Regulation of stem cell activity	MI: hsa-miR-130b*	0.008	*SNCG,NCOA6, MRPS26, SPINT4,HDACC3,ESR2,TSTD1,RGS6,FHL1, ANO2*
Increased DNA methylation after exercise	Negative regulation of cell cycle	GO:0045786	<0.02	*FHL1,RB1,RPTOr,ZFHX3,CAB39,CDK9,HDAC3,MED25, PSMC5, BRCA1, RUNX3*
Decreased DNA methylation after exercise	Extracellular structure organisation; extracellular matrix organisation	GO:0043062, GO:0030198	<0.02	*LTBP4, COL15A1, COL18A1,COL4A1,LAMA2,NID1, NRXN1, OLFML2A,PTK2,SFRP2,SULF2,COMP,FBLN2*
Decreased DNA methylation after exercise	System development; skeletal system development; cartilage development	GO:0048731, GO:0001501, GO:0051216	<0.04	*PRKG1,ALDH1A2, ANK3,ANKS1A, BATF,CAMK2B, CASP8,CD74, CENPF,CHL1, COL15A1, COL18A1,COL4A1,DKK3,EHD1,EYA1,FLG, HDAC9,HYAL2,IL7,IPMK,KCNQ1, KERA,LAMA2, LFNG,LILRB1, LMO4, LY6D, MBNL1,MEF2A, MEPE,MITF,NFIB, ND1,NNMT, NPR2,NRXN1, PLXND1, PTK2,RUNX1,SCIN,SERPINI1,SFRP2,SIX6,SLC35D1, SULF2, TRPV4, TTLL7,CHRDL2, COMP,PAX6, RUNX3,SIM1*

Gene networks courtesy g:Profiler.[30 31]
GO, gene ontology; MI, computationally predicted microRNA target sites from the MicroCosm database (formerly miRBase).

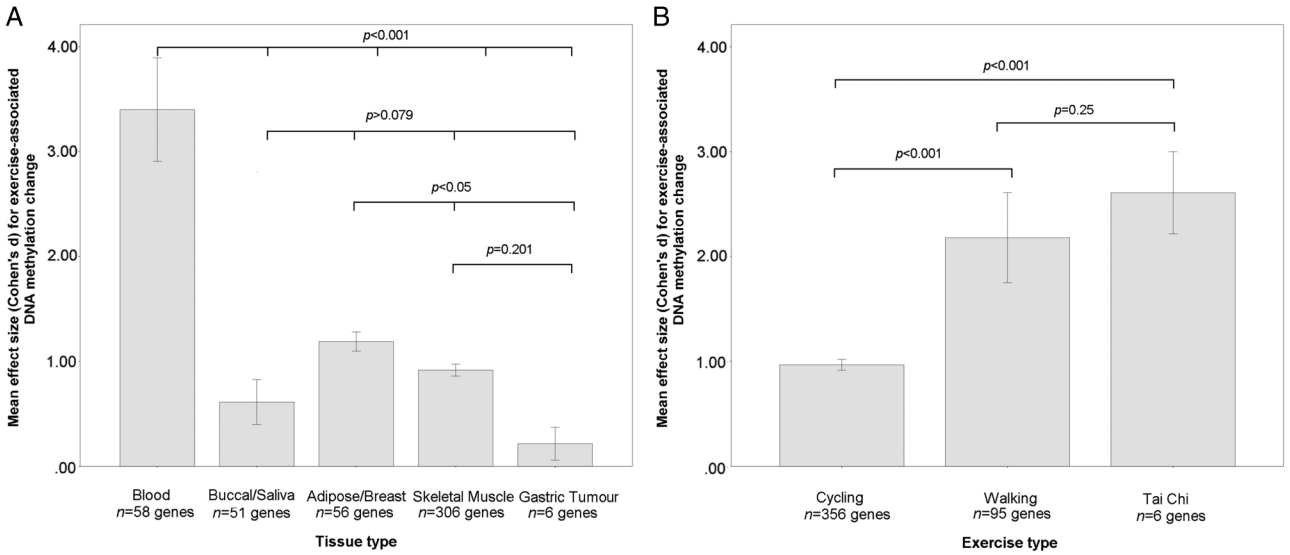

Figure 6 (A/B) Effect of tissue and exercise variation on exercise-associated DNA methylation change. Error bars represent Bonferroni-corrected 95% CIs.

imprinted genes for skeletal muscle development (table 1), it was hypothesised that imprinted genes would be implicated in exercise-associated DNA methylation changes. These findings are discussed below.

Exercise-associated DNA methylation change

Human exercise has medium to large effect sizes on the DNA methylation of genes extracted from different tissues across sex and lifespan. The effect sizes are strong among older people above and beyond the independent effects of research design, sample size, sex and tissue type. Publication bias is possible and difficult to rule out. However, the correlation between effect size and sample size was small (r=−0.11) and driven in part by two studies, both of which were tightly controlled molecular exercise physiology experiments with small sample sizes and large effect size. The large effect size in these studies could be due to methodological rigour. Nonetheless, sample size and other moderating factors were included in the analyses, suggesting that exercise has substantial effects on DNA methylation. Since this work was limited to published studies from western cultures, file drawer artefacts are a potential source of bias. Future work will need to investigate cultural and geographical effects, which could bias the findings. Given the plasticity of human development and population genetic variation, we may expect regional variation in the size of exercise-associated DNA methylation change.

As expected, imprinted genes—a DNA methylation-based transgenerational epigenetic phenomenon—are responsive to exercise exposure. Recall the skeletal muscle gene network analyses revealed both maternally expressed (*RB1*, *MEG3*, *UBE3A*) and paternally expressed (*PLAGL1*, *SGCE*, *INS*) imprinted genes likely played growth regulatory functions. Likewise, the meta-analysis imprinted genes showed changes in DNA methylation associated with exercise. Specifically, maternally expressed (*GRB10*, *KCNQ1*, *MEG3*) and paternally expressed (*L3MBTL1*, *PLAGL1*) genes were represented in the meta-analysis. Sixty per cent of the 478 genetic elements uncovered in the meta-analysis showed decreased DNA methylation after physical exercise. Among older people, the genes that *increased* in DNA methylation were involved in growth, while the genes that *decreased* in DNA methylation were part of the cancer-suppressing microRNA gene network. This strongly suggests that exercise may have a protective function among older people, perhaps shielding them to a degree from the age-related diseases and decline.

It is notable that two of the six genes that decreased in DNA methylation among older people after exercise (ie, *L3MBTL1*, *PLAGL1*) are imprinted targets of tumour suppressor microRNA *miR-519b*. Considering that imprinted genes (eg, hypermethylation of tumour suppressors) are often associated with diverse forms of cancer, this is both a biologically and medically important finding. Exercise-associated decreases in *L3MBTL1* DNA methylation are associated with decreased mortality among patients with breast cancer.[29] Exercise-associated decreases in *CACNA2D3* (also included in the microRNA network reported here) DNA methylation may help reduce gastric tumorigenesis.[40] These findings suggest that, at least for older people, exercise could have a protective effect against a variety of cancers in both sexes. More experimental approaches (ie, a mouse transfection model) likewise suggest that *miR-519b* suppresses breast cancer.[41] The ability of the *miR-519b* network to inhibit cell proliferation and decrease tumour growth[42] makes it potentially an epigenetically labile network for clinical researchers interested in exercise. In contrast, among younger people (less than 40 years of age), a microRNA-regulated (hsa-miR-130b*) gene network, which functions to suppress stem cell proliferation, increased in DNA methylation after exercise.

The fact that exercise-associated DNA methylation change was stronger in older compared with younger people indicates that exercise could alter an organism's 'epigenetic age' by warding off senescence. Why would exercise have more profound effects on older compared with younger epigenomes? One possibility is that as organisms age, epigenetic errors accumulate, and because there are more to correct or reset, older people experience greater (and more positive) DNA methylation change compared with younger people (who have experienced fewer epigenetic errors). Since physically active grandparents were probably a characteristic of the majority of human evolution, the pronounced age effect could also be an example

of an age-dependent adaptive epigenetic response to antagonistic pleiotropy. Antagonistic pleiotropy is the hypothesis that genes with multiple effects can be beneficial at younger ages and costly later in life. In the context of growth effects, older individuals' tumour suppressor genes were becoming demethylated (possibly more expressed), but growth-related loci were becoming methylated (presumably less expressed). Thus, rebuilding new tissue would become increasingly costly for older people relative to younger people. To reiterate, stem cells and tissue regeneration adaptive gene complexes in younger people may well be costly among older age groups due to cancer proliferation and premiums on cancer suppression as we age. Please note that reverse antagonistic pleiotropic effect may be operating here, that is, growth suppression among younger individuals may be particularly costly (eg, inability to regenerate or develop costly secondary sexual characteristics) relative to older postreproductive individuals. The effect of age was independent of tissue type effects. Specifically, it was found that exercise-associated DNA methylation changes are greater in blood compared with breast, adipose and skeletal muscle tissues. This latter result is consistent with the hypothesis that epigenetic dysregulation in more heterogeneous samples (eg, blood)—compared with a single tissue sample—may be a better proxy for the accumulation of environmental stress as we age. Beyond repeated exposure of physical exercise across the lifespan being important for a healthy epigenome, in utero maternal epigenetic effects are likely to be important.

There is recent work in mice showing that maternal exercise during pregnancy can reverse the deleterious epigenetic effects of poor maternal diet on newborn pups' *Pgc-1a*.[43] The beneficial effects of maternal exercise during pregnancy on *Pgc-1a* DNA methylation levels in the next generation suggest that a transgenerational mechanism exists for long-lasting epigenetic changes and is consistent with a fetal origins of disease approach.[44] There is no evidence of transgenerational DNA methylation effects of exercise in humans.[44] However, it is noteworthy from the meta-analysis that *GRB10* (γ isoform)—maternally expressed in human fetal skeletal muscle[39]—showed greater exercise-associated DNA methylation change among those *without* a type 2 diabetes family history. Relaxation of the growth-inhibiting effects of maternal genes could be dependent on genetic, ecological conditions or fetal exposure to maternal exercise. For example, individuals from families exhibiting more sedentary behaviour (ie, characterised by a history of type 2 diabetes) have less silencing of maternally expressed *GRB10* in skeletal muscle. Such differentially epigenetic responses of *GRB10*'s γ isoform depending on a family history of type 2 diabetes would be extremely interesting if reliable. A powerful interface between family history and offspring epigenetic could be signalled during gestation. Specifically, maternal exercise during gestation could produce dose-dependent epigenetic responses in offspring.[43]

Despite using two different methods (ie, g-Profiler gene ontology network analysis vs meta-analysis), multiple imprinted loci appear to be missing. This raises the distinct possibility that additional imprinted genes will be found to be associated with muscle adaptation and exercise adaptation in humans. For example, in newborns with transient neonatal diabetes, the loss of an epigenetic mark at the TNDM locus on chromosome 6q24 in the mesodermal lineage causes abdominal muscle hypoplasia, the so-called prune belly sequence.[45 46] When Laborie *et al*[47] investigated a family with prune belly that included one discordant set of MZ twins, the twin with prune belly (relative to the normal co-twin) had extensive loss of methylation at the TNDM locus, as well as at the following imprinted loci *IGF2R*, *DIRAS3* and *PEG1*. Future work in humans and other animals should be able to develop a more comprehensive list of imprinted genes regulating skeletal muscle and associated with exercise. One reason that some imprinted genes may be missed from these analyses could be due to the fact that imprinted genes are often involved in neural systems,[18] which, unlike skeletal muscle, cannot be extracted from healthy human participants.

Future work needs to be conducted to test whether or not imprinted DMRs[48] are modified by exercise. Once again, given the relevance of imprinted genes for human cancers, one long-standing conundrum in medicine could be resolved. Specifically, why does exercise treatment appear to reduce the incidence of cancers? One answer is that tumour suppressor genes are 'reactivated' at promoters on long-term exercise treatment and the corresponding reduction in DNA methylation.[29] Given these possible medical benefits, future research should look at the relationship between exercise stress and regulation of imprinted genes in order to understand more fully the underlying mechanisms.

It is worth noting that exercise-associated DNA methylation changes for imprinted genes occurred *only* in studies where participants were exposed to longer term exercise (ie, 6 months) as opposed to short bouts of exercise. This interpretation should be taken with caution as it is biased by the fact that fewer genes were studied in the acute study by Barrès *et al*[26]. Specifically, Barrès *et al*[26] selected genes from a previous study of DNA methylation in patients with type 2 diabetes, while the long-term exercise studies revealing the imprinted genes (see online supplementary table 2) screened many more genes using Illumina's Infinium HumanMethylation450 BeadChip (San Diego, California, USA).

CONCLUSIONS

Modern epigenomics helps to end nature-nurture debates over health and disease. The genome is sensitive to the environment and environmental information is encoded into the epigenome transgenerationally (eg, imprinted genes). Rather than argue which is more important, nature or nurture, we can now measure the interface between the two directly. Measuring the interface between genes and the environment (eg, DNA methylation) will have ramifications for health and human disease due to an ageing and an increasingly physically inactive population. Given the increasing amount of research from multiple independent laboratories[26–29 49–40] indicating that human exercise has varied associations and effects on DNA methylation, it is a reasonable hypothesis that long-term exercise throughout the lifespan (or exposure during sensitive periods of in utero development) could have profound effects on the epigenome.[44] Future work should determine the optimal exercise types, timing and duration for ameliorating epigenetic-based disease outcomes. The strength of exercise-associated DNA methylation change could be an overestimate due to publication bias (eg, unpublished studies not included). Genetic background could affect the associations reported here and was not ruled out. Future work should sample from monozygotic twins reared together and apart to elucidate the importance of genetic background. To rule out publication bias, a collection of unpublished exercise epigenetics papers will need to be collected and analysed.

Uncovering epigenetic biomarkers is likely to be *more* clinically relevant than looking for 'disease genes' because epigenetic changes *can be reversed* and also since disease variants are

expected to be at low frequency due to the power of natural selection to remove deleterious genetic variants from a population. Techniques are being developed to remove epigenetic marks (ie, DNA demethylation), which can radically change disease phenotypes (eg, tumour progression). Exercise medicine should work alongside clinical epigenetics to investigate how exercise shapes the human epigenome. An applied goal would be to adaptively decouple chronological and epigenetic age by using exercise interventions. The analyses presented here suggest that exercise-associated DNA methylation change reduces epigenetic age (eg, cancer reduction[29 40]). Controlled exercise interventions could help the ageing epigenome, especially among older hospitalised patients. For example, one study of older people (aged 58–90 years) with cerebrovascular disease suggests that physical function improvements during hospitalisation covary with subtelomeric methylation of long telomeres.[59] If exercise alters the epigenome to reduce age-related disease outcomes, it could be a relatively inexpensive treatment option within hospital environments. In conclusion, *human* studies in exercise epigenetics are required not only because of the impact on health of ageing populations, but also because key epigenetic elements (ie, imprinted genes) responsible for regulating adiposity, energy expenditure, glucose homoeostasis and hunger are differentially imprinted (or read differently) between mice and man.

What is already known on this subject

Recent empirical studies suggest that physical exercise modifies the human epigenome. Specifically, DNA methylation—an important regulator of gene expression and correlate of diverse disease states—is altered by physical activity. No systematic review has been conducted to elucidate these effects and associations.

What this study adds

This study isolates imprinted genes—known to be important for health and disease—as important for muscle growth and clinical targets of exercise. Further, older people received significant benefits from exercise in terms of the adaptive epigenetic regulation of tumour suppressor genes.

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