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Assessing the achievements and uncertain future of paleoepigenomics

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Authors

Smith, Rick WA

Non, Amy L

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INTRODUCTION

Paleoepigenomics is an emerging area of research that focuses on generating epigenomic data from ancient or extinct organisms with the goal of understanding their regulatory, environmental, and evolutionary significance [1-3]. We are excited about the prospects of learning more about epigenetic regulation in the past, which may help us understand environmental plasticity, drivers of inter- and intraspecies variation, and the evolution of modern and ancient diseases, among other phenotypes. However, we are also concerned about the potential for speculation in an area of research that has often been overhyped. Given the complex combination of methods involved in this work as well as the significant gaps that remain in our understanding of regulatory mechanisms, there are reasons to maintain a healthy skepticism about what paleoepigenomics may achieve. The history of paleogenomics has been no stranger to the speculative and the far-fetched, with multiple early claims of DNA from the Mesozoic turning out to be contamination [4]. Similarly, epigenomics has sometimes been home to the sensational and the brash, perhaps most notably in the ongoing debates regarding transgenerational inheritance and the significant gaps that remain between identifying epigenomic changes and understanding their functional roles in health outcomes [5, 6].

Paleogenomics and epigenomics are both still relatively new areas of science, and their respective histories of overreach and overhype have led to the development of rigorous methodological, authentication, and analytical standards by which to guide and scrutinize research [4, 7-10]. Paleoepigenomics is the integration of these two fields, which not only brings a double burden of credibility by existing standards but also raises new questions of plausibility. Understandably, there has been substantial enthusiasm around the potential of using paleoepigenomics to elucidate ancient environments, lifeways, and evolutionary events. However, we question whether the field is ready to dive into fully interpreting the epigenomes of the past when conventional epigenomics is still figuring out fundamental questions of the relevance and role of the epigenome for diseases and other phenotypes among living organisms in the present. Here we briefly assess paleoepigenomic work to date in humans and closely related hominins and consider the exciting advancements that have been made in the field. In addition, we consider the known limitations of paleoepigenomic research today and urge caution if – and until – the science catches up with the hype.

PALEOEPIGENOMICS: METHODS, APPLICATIONS, AND ACHIEVEMENTS

Early on, much of the work in paleoepigenomics was necessarily methodological and proof-of-concept based. While epigenetic studies in extant organisms focus on a variety of epigenetic regulations at the DNA, RNA, and protein levels, the majority of paleoepigenetic/omic research has focused on cytosine methylation. To our knowledge, the first evidence demonstrating that cytosine methylation status could be detected in ancient DNA (aDNA) came from the

observation that enzymatic removal of deaminated cytosines depended on dinucleotide context [11]. DNA degrades rapidly after an organism dies and is characterized by highly fragmented nucleotide chains and spontaneous hydrolytic deamination of cytosines [1]. The presence of random post-mortem transitions of cytosines into other pyrimidines (i.e., thymine or uracil) results in higher sequencing errors for aDNA relative to non-degraded sources of DNA [11]. To increase sequencing accuracy, enzymatic repair techniques such as uracil-DNA-glycosylase (UDG) treatment have been used to remove cytosine-to-uracil transitions and have become common in aDNA library preparations. However, Briggs et al. [11] found that some deaminated cytosines resisted enzymatic removal and that this resistance depended strongly on dinucleotide context, with cytosine-guanine (CpG) dinucleotides retaining a higher fraction of deaminated cytosines after UDG treatment than other dinucleotide contexts (i.e., CpA, CpC, and CpT). Because cytosines degrade differently after death, with unmethylated cytosines degrading to uracils and methylated cytosines degrading to thymines [12], the persistence of deaminated cytosines in CpG dinucleotides after repair reflects the presence of deaminated 5mCs (i.e., thymines) which are not removed by UDG treatment.

This finding led to the development of computational methods for deamination-dependent cytosine methylation prediction, which have become the most widely used techniques in paleoepigenomics. Here we focus our discussion on these deamination-dependent methods but note that some paleoepigenomic studies have used conventional bisulfite-sequencing approaches as well [12, 13]. The application of deamination-dependent approaches led to the first methylome reconstructions for an ancient human [14], Neandertals and Denisovans [15], and the inference of methylation in an increasing variety of other ancient and extinct organisms [14-16]. These computational methods have since been formalized into computational pipelines and open-source software programs for methylome prediction in aDNA [17]. In addition, methods for methylated binding domain (MBD) enrichment and array-based methylation detection have also been evaluated for use in aDNA [16, 18].

One of the greatest promises of paleoepigenomics was that it might provide novel insights into major evolutionary changes and adaptive transitions in humans [2, 3]. Some progress has been made towards these goals, with a few studies beginning to identify morphological and potentially behavioral differences between humans and our closest primate and hominin relatives. Following the sequencing of the complete Neandertal genome, studies began to identify coding and regulatory differences between living humans and Neandertals. One such difference is a polymorphism identified in the miRNA miR-1304, which is derived in living humans, and is predicted to increase the number of putative regulatory targets by more than 10-fold. Among the predicted regulatory targets are genes involved in neurodevelopment and enamel formation, and it has been suggested that this polymorphism may play a role in the evolution of dental and behavioral distinctions between living humans and Neandertals [19]. Building on the analysis of sequence-based regulatory differences, the reconstruction of Neandertal and Denisovan methylomes allowed for the identification of thousands of potentially differentially methylated regions (DMRs) between contemporary and archaic humans, including differences in HOXD cluster methylation which might explain morphological differences such as limb proportions between living humans and Neandertals [3, 15].

More recently, methylation maps have been used to predict the anatomy of Denisovans which are currently known only from fragmentary and very limited subfossil remains. These analyses suggest that Denisovans likely shared features with Neandertals such as robust jaws, long and low craniums, low foreheads, thick enamel, large ribcages, wide pelvises, and large femoral articulations. However, this analysis also identified 11 features potentially distinct in Denisovans, including an elongated dental arch, changes in the dimensions of the mandible, and lateral expansion of the parietal bones in the cranium, among others [20]. In the case of the Denisovan mandible, Gokhman et al. [20] report that 7 out of 8 of their *a priori* morphological predictions based on epigenetic differences matched the description of the first confirmed Denisovan mandible. Subsequently, epigenetic changes have been linked with potential changes in the facial, vocal, spinal, and mandibular anatomy of living humans since our geographic distancing from Neandertals and Denisovans [2, 21].

Some of the first epigenetic studies of past human lifeways have identified potential epigenetic differences related to diet and immune function between groups which vary in their subsistence patterns, including across the Mesolithic to Neolithic transition. For example, Gokhman et al. [22] identified hypermethylation of LOC654433, RBM46, and EXD3, and hypomethylation of BOLA3 in ancient hunter-gatherers compared with sedentary peoples. More recently, Seguin-Orlando et al [23] detected some evidence of differential methylation in C1Qb and LCK – genes which are related to humoral immune complement response and T cell maturation (respectively) between Mesolithic and Neolithic peoples in France. These types of studies can provide intriguing hints of past life experiences and their regulatory impacts, but also are relevant for understanding variation in methylation related to diets and disease states among living people.

Taken together, work in paleoepigenomics has demonstrated not only that epigenetic marks can be reconstructed, but they can also lend some insights into ancient environments and evolutionary processes. The field has therefore accomplished some of its original goals. However, it is also important to be cognizant of the technical limitations of this work, and consequently what questions are – and may remain – out of reach.

LIMITATIONS

The demonstrated correspondence between *a priori* morphological predictions of Denisovan anatomy and newly reported subfossil remains is one of the strongest pieces of evidence to date supporting the viability of paleoepigenomics for predicting unknown phenotypic outcomes [20]. However, it is important to keep in mind the authors' own note of caution that their study relied on predicting broad directional changes in anatomical features rather than precise phenotypic outcomes. In addition, while this represents an incredibly exciting result, the emergence of corroborating subfossil evidence represents a fortuitous and perhaps non-replicable set of circumstances. How might we vet paleoepigenomic predictions in other contexts where there may be no possibility of corroborating evidence of phenotypes and no means to assess the regulatory pathways that help shape them? This would at a minimum require a more robust understanding of methylation and its specific regulatory consequences among extant peoples, ideally living in very similar conditions. Unfortunately, we have yet to fully arrive at such an understanding and this places constraints on what can be inferred from methylation patterns in the past. In paleoepigenomics, we are often left with only the baseline epigenetic marks – with

greatly diminished access to or knowledge of the downstream biology – and therefore little to no reliable way to mechanistically link epigenetic patterns to phenotypic outcomes.

The relationship between methylation and expression is remarkably complex. Even in studies of living organisms it is difficult to meaningfully link epigenetic marks with their regulatory impacts, and correlations can vary by genomic context, cell, or tissue type [24]. Because of this, our ability to interpret the specific function of ancient methylation patterns is very limited. We should therefore be exceedingly cautious to not overinterpret results. This is true of any paleoepigenetic/omic study no matter the phenotype under study but is perhaps especially true in the case of predicting cognitive or behavioral distinctions between groups, because the linkage between epigenetic marks and behavioral outcomes is especially tenuous.

In addition, constraints caused by gaps in the archaeological record, and uneven DNA preservation in the available archaeological contexts, place profound limits on both the types of questions that can be addressed and the sample sizes and statistical power available to analyze them. While ancient methylation from a small number of Neanderthals provides a unique glimpse into the past, these few individuals do not represent all the variation across an entire group, nor allow us to search for subtle trait- or disease-related methylation patterns in a way that satisfies the current analytical standards of the field. For example, statisticians have recently indicated that sample sizes upwards of 1000 people may be necessary to ensure adequate power to detect small disease-associated epigenetic differences when using array-based epigenome-wide approaches [8]. Further, fragmentary skeletal preservation, even in a rich archaeological context, may hinder interpretations of any detected methylation differences. If a past population experienced famine, violence, and deprivation – all of which could potentially influence methylation in similar and overlapping ways – it can become impossible to disentangle effects. We face similar limitations in studies with living people even when we can directly measure these simultaneous exposures, but these problems are exacerbated in ancient contexts where exposures can only be inferred from limited remains.

Paleoepigenomics analyses are also limited to the tissues that preserve most often in the archaeological record – bone, teeth, and hair. It is thus somewhat unsurprising when studies of ancient methylomes highlight skeletal differences of Neandertals and Denisovans, or when methylome data from hair demonstrate patterns characteristic of that tissue [14, 15, 20]. Because the epigenome varies substantially across tissues, this raises the question of what insights can be achieved when we lack some of the most physiologically relevant tissues in the archaeological record. For example, if a goal is to understand a phenotype such as stress response in archaeological communities, but the most commonly studied tissues of blood and buccal cells are not preserved, can reconstruction of methylation in less physiologically relevant tissues such as bone, teeth, and hair suffice? These issues have been thoughtfully considered in Gokhman et al. [3], but continue to represent significant hurdles to the reconstruction of ancient environments and lifeways, a core goal of the field.

The ability of paleoepigenomics to address more specific questions requiring fine-scale specificity will necessitate the development of methods for enhanced methylome resolution in aDNA. While the deamination-dependent technique has become the most widely used method, it

is very limited in resolution, typically providing regional methylation estimates in genomic windows of a few hundred base pairs. This approach is therefore constrained to analyses of DMRs rather than individual CpGs, and only in individuals and genomic regions where sufficient deamination has occurred to infer methylation patterns. Thus, a DMR approach may determine regional differences in methylation but is not well suited to finer-scale epigenomic questions, such as determining the methylation status of transcription factor binding sites in the promoter regions of dietary or stress-related genes, which sometimes entails determining the methylation state of single cytosines. Bisulfite pyrosequencing (BS-Seq) of aDNA is the most direct method for detecting ancient methylation patterns at single-cytosine resolution. However, this method is highly destructive to already heavily degraded aDNA, and thus far has only been successfully applied to reconstruct methylation of repetitive elements rather than single-copy loci that would be necessary for specific analyses of diet and stress, for example [12, 13]. Importantly, even with this direct measurement method, post-mortem deamination can still bias methylation estimates. Methylation levels may be artificially reduced or show increased variability, depending upon the amount of cytosine deamination in the genome [12].

FUTURE POTENTIAL

Keeping these limitations in mind, we recognize the potential value of paleoepigenomic techniques, particularly to answer questions that can only be addressed in ancient or archaeological contexts. Such questions could include some of the impacts of historical traumas, such as those resulting from slavery and forced removals of African and Indigenous peoples from their homelands. Events such as these may carry forward to impact the health of living descendants today via the perpetuation over generations of harmful social and environmental conditions that maintain epigenetic states, the potential (yet undemonstrated) mechanisms of transgenerational inheritance through the germline, or other mechanisms. In order to distinguish historic effects from those of more recent experiences, methylation levels assessed close to the time of the historic trauma would be necessary. As previously noted, however, this would require either remarkable aDNA preservation such as in recent historical archaeological contexts and/or the development of higher resolution methods for methylome reconstruction. In addition, while paleoepigenomic data may elucidate some of the molecular mechanisms associated with historical traumas, it is important for researchers working in this area to be cognizant that many impacts of historical traumas are already well characterized by multiple lines of historical, biomedical, and community knowledge, among others. Epigenomics researchers of the past, like those of the present, should also be careful not to introduce or reinforce new forms of biomedical reductionism by overemphasizing a deterministic role of the environment, or ‘victim blaming’ of parents or marginalized groups. Given the combination of paleogenomic and epigenomic approaches involved in this work, future research should also be mindful of the necessity for ethical and decolonial approaches to archaeological and aDNA research with historically marginalized people [e.g., 25, 26], as well as critical scholarship considering the potential and pitfalls of epigenomics as a means to elucidate historical traumas [27].

Another area of future potential for paleoepigenomics is the opportunity within ancient contexts to detect effects of longer-term exposures than can be measured among living people, such as the stress of living through lengthy sociopolitical transformations, climatic disruptions, environmental degradations, or generations of exposure to lead poisoning or other toxins. It is

also now possible to detect epigenomic effects of past exposures in living adults which may be no longer observable or measurable in other ways, such as smoke exposure *in utero,* remarkably over 20 years prior [28]. Perhaps paleoepigenomics may ultimately enable inference of other unmeasurable exposures, such as past extreme climates, with potential to predict epigenomic consequences of current and future climate change. Finally, it is clear that paleoepigenomics may be able to provide additional morphological guidelines for identifying new subfossil evidence from species which are only known from fragmentary remains.

RECOMMENDATIONS

The future of paleoepigenomics will depend on advances in our knowledge of contemporary epigenomics, which can refine our approach on where to look and exactly what to look for in the past, i.e. tissues and regions of the genome most affected by particular exposures. Inferences of past cellular phenotypes could be improved once we have more extensive documentation of the relationship between DNA methylation and expression across genomic regions in contemporary studies, and the role of other epigenetic mechanisms, such as histone modifications, miRNAs, or availability of transcription factors, that also influence expression patterns. These advances can be aided by the expansion of existing public databases that document epigenetic associations (EWAS Atlas [29]), and tissue-specific gene expression (Genotype-Tissue Expression project [30]).

In terms of study design and implementation, we recommend that paleoepigenomic reconstructions only be considered in circumstances where DNA preservation and genome coverage is exceptionally high. This constrains analyses to the occasional ancient individual, to a limited set of environmental contexts (e.g., cold, arid) that especially facilitate aDNA preservation, or to more recent historic archaeological contexts where adequate DNA preservation may be more common. Because of the high risk and ethical stakes of this research, we recommend that paleoepigenomic research be considered on the backend of other, lower-risk genomic work such as analyses of population history or natural selection – if and when the DNA preservation allows. We further recommend ancient epigenetics studies be limited to questions that cannot be answered by studies of living people, to preserve precious samples and ancestral remains. For example, just as with genomics research, many questions about our species' deep evolutionary history can be inferred with comparative approaches between modern human and non-human primates [31], and by using complex computational modeling techniques.

CONCLUSIONS

The history of early research in both paleogenomics and epigenomics alike have been fraught with speculative claims, with subsequent and ongoing developments of methodological, authentication, and analytical criteria. Merging these two often over-hyped fields entails a double burden of credibility. We urge caution and suggest that researchers avoid conclusions that over-reach their data. Finally, we emphasize that just as with paleogenetic data, there are limits to the resolution that can be gained by paleoepigenomic data, as samples and ancestral remains are scarce and unevenly preserved, and evolutionary and historic events can never be directly observed. While ancient epigenomic data can provide exciting hints of the past, this research is at its best when it integrates data from ancient and living human genomics/epigenomics, history,

and archaeology, which creates a more holistic view of human evolutionary history and our past environments.

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