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Inferring human stem cell behaviour from epigenetic drift

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Abstract

Although conventional experimental manipulations are impractical, it may be possible to infer human stem cell fates by ‘reading’ histories recorded within their genomes. Genomes are almost perfect copies of copies, and ancestries may be surreptitiously recorded by replication errors that inevitably accumulate. The greater the number of divisions, the greater the number of replication errors (‘a molecular clock hypothesis’). Mutations rarely occur during a lifetime, but DNA methylation patterns are also copied after DNA replication and measurably drift with ageing at certain CpG sites in mitotic tissues, such as the colon. Such passenger methylation pattern variation may effectively function as ‘epigenetic’ somatic cell mitotic clocks. Replication errors can only accumulate in long-lived stem cell lineages, so methylation pattern drift largely records stem cell behaviour. How methylation patterns may encode stem cell ancestries is illustrated with two types of small reproductive units — colon crypt niches with continuous genealogies, and hair follicles with punctuated genealogies. Potentially, the genealogy of any human cell may be inferred by ‘reading’ its genome.

Keywords

colon; hair; stem cells; niche; methylation; molecular evolution; ageing

Introduction

This review discusses a relatively new approach to inferring human stem cell dynamics from variations in their genomes. The approach translates molecular evolutionary methods to the genealogies of somatic cells. Evolutionary studies commonly infer ancestries of many different species or populations by comparing differences between their genomes [1]. Molecular evolutionary approaches have a number of advantages and shortcomings, and these issues are outlined below.

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Teaching materials

Power Point slides of the figures from this Review may be found in the supporting information.

Problem — experimental manipulations to mark and follow human cells are impractical

Stem cell studies are facilitated by a host of clever methods (including DNA labelling, mutagenesis and genetic fate markers) that mark and subsequently follow stem cell fates. These experimental manipulations are impractical in humans and, even if possible, observations may require decades because humans are long-lived. Similar problems are also present in evolutionary studies and a now well-established solution is molecular evolution, or the reconstruction of the past from present-day genomes [1]. Although much of evolution cannot be directly observed, some of the best evidence of the past appears to be recorded within genomes. Genomes change through time. The greater the intervals since a common ancestor, on average the greater the numbers of differences between their genomes. Underlying this ‘molecular clock hypothesis’ is the neutral theory of molecular evolution [2], which suggests that the majority of changes within a genome have minimal selective value (ie passenger changes). These passenger changes do not influence survival but are ‘odometer-like’ clicks of a molecular clock.

A human somatic cell tree starts at the zygote and ends with present-day cells. In theory it should be possible to reconstruct this tree by comparing present-day genomes. This molecular ageing approach requires no prior experimental intervention because a lifetime of cell divisions may be surreptitiously recorded within somatic cell genomes.

Problem — somatic mutations are rare

Sequences change over thousands to millions of years and very few somatic mutations appear to accumulate during a lifetime. For example, the frequencies of somatic mutations in cancer genomes are <1 per 100 000 bases [3] and somatic mutation frequencies in normal cells are likely to be even lower. Interestingly, and consistent with the neutral theory of evolution, the majority of somatic mutations in cancer genomes appear to be passenger changes [3].

The low frequency of somatic mutations hinders a practical application of molecular phylogeny to human somatic cells because one would have to sequence millions of bases to find a handful of mutations. A potential solution is to measure epigenetic patterns because, by definition, epigenetic information is also usually inherited between generations [4]. Specifically, the 5' to 3' order of cytosine DNA methylation at CpG sites is generally copied by DNA methyltransferases after DNA replication (Figure 1). Methylation patterns can be read by conventional DNA sequencing after bisulphite treatment [5]. Instead of a four-base code, methylation patterns are binary strings (methylated or unmethylated at a specific CpG site).

Most CpG islands are unmethylated at birth [6] because most DNA methylation is removed early in development, before implantation [7]. DNA methylation is subsequently restored and has roles in development and differentiation. Methylation of promoter CpG islands is often associated with transcriptional silencing [8]. However, some methylation appears to represent passenger changes and, notably, age-related increases in methylation have been observed at certain CpG sites in mitotic human tissues such as the colon [9,10]. Cell division appears to be required for *de novo* DNA methylation [11]. The subset of CpG sites that

continue to accumulate passenger methylation throughout a lifetime may more effectively function as mitotic clocks (Figure 2).

A hallmark of selection is conservation — functional genome regions tend to be conserved across individuals and species. A similar paradigm is likely to apply to somatic methylation patterns. If methylation has a functional role at a particular stretch of CpG sites, its pattern should be similar between phenotypically identical cells. By contrast, if patterns differ between phenotypically identical cells, such diversity may indicate that this methylation represents passenger rather than functional changes. Passenger methylation patterns are unlikely to be spontaneously ‘created’ all at once, but may arise stepwise from rare random replication errors that accumulate over decades. In theory, such serial epigenetic passenger changes represent a trail that can be followed to reconstruct ancestry.

Problem — human stem cells are difficult to isolate

Any stem cell analysis would appear to depend on the ability to identify and isolate these unique cells. Their rarity and the lack of specific human stem cell markers might appear to preclude comparisons between their genomes because one cannot reliably isolate adult human stem cells. This ‘hurdle’ may be overcome with molecular phylogeny, which routinely detects long-lost ancestors from genomes in present-day progeny.

An ancestral tree is composed of four types of cells (Figure 3A). The start is the zygote and the ends are present-day cells. In between are many no longer present cells, which are either ancestors (with present-day progeny) or dead ends (with no present-day progeny). In an ancestral tree, stem cells are ancestors, whereas non-stem cells are dead ends. Therefore, it is possible to ‘detect’ stem cells by analysing the genomes of their more numerous and easy to isolate differentiated progeny.

Long human lifetimes facilitate a molecular clock analysis of their stem cells. Unlike shorter-lived mice models, the stem-cell phase dominates human somatic cell genealogies (Figure 3B). The genealogy of many human cells can be divided into three sequential phenotypic phases: development from the zygote, a stem cell phase, and differentiation [12,13]. Development (typically months) and differentiation (days to weeks) are usually programmed to occur with defined numbers of divisions. Any transient amplifying phase between a stem cell and its differentiated progeny is likely relatively short (weeks to months) compared to a human lifetime. Therefore, only the stem cell phase (decades-long) may vary through a lifetime.

One uncertainty is the mitotic activity of stem cells. The mitotic age of a differentiated cell is the total numbers of divisions since the zygote, or the sum of the divisions during development, a stem cell phase, and differentiation. Changes in the mitotic ages of differentiated cells during ageing are therefore a function of stem cell divisions, because divisions during differentiation and development are constant (Figure 3B).

The behaviour of the well-mannered epigenetic somatic cell mitotic clock

A somatic cell ‘clock’ is defined as a short stretch of CpG sites that exhibits random passenger methylation changes in somatic cells (Figure 4). To function as a mitotic clock, methylation should represent replication errors and therefore be a function of cell division [12,13]. Whereas mechanical clocks should look the same in different cells with similar mitotic ages, molecular clocks record divisions by becoming polymorphic. When first encountered, a well-mannered molecular clock may appear unruly and unreliable, because clock epialleles from phenotypically identical cells may have diverse methylation patterns. To reduce some of the mysteries of clocks, guidelines for extracting ancestry from seemingly random replication errors are outlined below.

Identifying a well-mannered clock requires a proper setting, a few tools and several assumptions. A proper setting is as uniform as possible because this allows variation to be attributed to a stochastic process. For example, a single colon is composed of multiple independent clonal units or crypts [14] that are essentially genetically identical, created at the same time, exposed to the same environment, and presumably their stem cells follow similar rules. Any two crypts from the same colon have similar mitotic ages but represent independent lineages — an ideal setting to unmask random replication errors, which should accumulate differently in different cells. Epi-allele diversity between crypts from the same colon is a strong indication of a stochastic clock.

A few quantitative tools convert epiallele diversity into stem cell behaviour (Figure 4). Because epialleles differ in a diverse population, multiple epialleles must be sampled to characterize their differences (typically eight epialleles per crypt and six to eight crypts per colon [10]). Each epiallele can be described by percentage methylation, and average percentage methylation can be calculated for each crypt and colon. A measure of diversity is the pairwise distance (or Hamming distance) between epialleles. Pairwise distance counts the minimum number of changes or errors that separate any two epialleles (Figure 4). The greater the pairwise distance, the greater the number of likely divisions that separate two epialleles. Another metric of diversity is the number of unique patterns observed in the sample (more diverse populations have more unique epialleles).

Clock behaviour over a lifetime can be examined by sampling the same tissue in different-aged individuals. The logic of this strategy is illustrated in Figure 3B and depends on the genome-wide demethylation that occurs early in development [7] — essentially, everyone starts with unmethylated clock epialleles, which facilitates comparisons between different-aged individuals. By examining average clock metrics (percentage methylation and pairwise distances) of colons from different-aged individuals, the predictability of a well-mannered but stochastic clock should emerge. Below we illustrate the behaviour of a relatively well-mannered clock in human colon crypts.

Human colon crypt niches: a continuous genealogy with stem cell clonal evolution

Age-related increases in methylation at certain CpG sites are commonly observed in the colon [9,10]. Data from one clock locus in BGN are illustrated in Figure 5. This locus is on the X-chromosome and in male individuals only a single epiallele is present per cell. Epialleles sampled from a 98 year-old male colon show that methylation patterns may be heterogeneous between and within crypts (Figure 5A). Average epiallele methylation from several different-aged colons exhibits an age-related increase (Figure 5B). An age-related increase in average epiallele methylation but heterogeneous patterns between crypts within the same colon can be explained by random replication errors (drift) that slowly accumulate over decades. As illustrated in Figure 3, errors can only accumulate in long-lived stem cell lineages, implying that human crypt stem cells divide throughout life. Recent evidence in the mouse suggests that crypt stem cells are mitotic [15].

Epialleles in independent mitotic lineages should drift apart because replication errors occur randomly, leading to progressively greater pairwise distances or diversity (Figure 4). As illustrated in Figure 5C, pairwise distances between crypts within a single colon increase with chronological age, consistent with the idea that each crypt is an independent mitotic clonal unit that progressively accumulates an independent set of errors. Within a single crypt there may be multiple different unique patterns, consistent with data suggesting that there are multiple stem cells per crypt [14,16]. However, unlike comparisons *between* crypts, pairwise distances *within* crypts are smaller and do not appear to increase with ageing. Numbers of unique epialleles per crypt also do not increase with ageing (Figure 5D).

The lack of increasing crypt clock diversity with chronological ageing implies a mechanism that counteracts the natural tendency of genomes to become polymorphic. In population genetics, ‘bottlenecks’ or the loss of individuals reduce diversity because alleles are lost. Similarly, crypt stem cell niches can reduce crypt epiallele diversity because niche stem cell numbers are constant but survival is probabilistic [17]. Although a stem cell almost always divides asymmetrically to produce one stem and one differentiated daughter, sometimes one stem cell will produce two differentiated daughters (‘extinction’), balanced by another stem cell that produces two stem cell daughters (‘expansion’). Even if stem cell lineage extinction is rare, over many years eventually all stem cell lineages except one will become extinct (Figure 5E). Such intestinal crypt clonal conversion is well known from mouse studies [14,18–20]. Morphologically, this replacement process or niche stem cell clonal evolution would be occult because numbers of stem cells remain constant.

Therefore, stem cell niches can be detected as an ongoing lineage–extinction mechanism that reduces diversity within a crypt versus between crypts. The production of new epialleles is linked to the loss of epialleles by stem cell division [Figure 5F] — rare new replication errors are constantly balanced by the loss of epialleles from rare stem cell lineage extinction events (both daughter cells exit the niche). Another study of human colons after therapeutic radiation also inferred stem cell turnover [21]. Much more information can be extracted from methylation patterns using quantitative tools, and modelling infers a human crypt niche scenario with about 64 stem cells that divide asymmetrically 95% of time, resulting in niche

stem cell clonal evolution about every 8 years [10]. This model implies that the average crypt in an 80 year-old colon has experienced ~10 sequential ‘bottlenecks’ of stem cell clonal evolution.

Human hair follicles: a punctuated genealogy

The value of a molecular clock approach is the potential to analyse many different cell types with similar algorithms. The same ‘reporter’ clock locus in the zygote is copied and passed into different cell lineages, and random replication errors may record mitotic ages regardless of cell phenotype. Scalp hair follicles are also highly mitotic and organized into small clonal bulb units that can be sampled by plucking individual hairs [22]. However, unlike the colon, average hair follicle epiallele clock methylation does not increase with chronological age (Figure 6). Both colon crypts and hair follicles contain mitotic cells, but hair cells somehow appear to ‘escape’ mitotic ageing. The mechanism that limits hair cell mitotic ages is massive extinction followed by re-expansion, or a punctuated genealogy (Figure 6), perhaps due to clonal succession [23].

Unlike the colon crypts, hair follicles have episodic cycles of growth and degeneration (Figure 6). Quiescent bulge hair stem cells divide briefly at the start of each hair cycle (about every 3–5 years) to produce differentiated progeny that migrate and reconstruct a new hair follicle bulb [24]. Differentiated bulb cells cannot divide forever and die at the end of the cycle when the hair falls out and the bulb degenerates. Therefore, the mitotic ages of differentiated bulb follicle cells are limited by the length of the hair cycle. The total mitotic age of hair bulb cells is again determined by numbers of bulge stem cell divisions, because numbers of divisions during development and differentiation are about constant. Because bulge stem cell divisions are limited to the start of each new hair cycle, essentially a new hair on an old head appears to have a mitotic age similar to that of a new hair on a young head.

Summary: imperfect but practical

The punctuated genealogy of hair follicle cells contrasts with the continuous genealogy of colon crypts. Although hair and crypt genealogies are consistent with many aspects of stem cell biology observed in experimental model systems, no-one has observed human adult stem cells over a lifetime. Therefore, it is difficult to validate epigenetic somatic cell clocks. Similar validation problems exist for more widely-accepted species clocks, because the exact genealogies of most species through millions of years are uncertain. Validation consists of empirical evidence that the same algorithms correctly infer likely ancestries of many different types of species. Similarly, the ability to reconstruct different genealogies provides empirical evidence for somatic cell clocks. Additional genealogies have been reconstructed for other human cell types. Observed are continuous genealogies for small intestinal crypts [25], endometrial glands [26] and T cells [27], punctuated genealogies for neutrophils and B cells [27], and static genealogies (no increase in mitotic age with chronological ageing) for heart and brain [28].

Analysing genome variation is difficult because a simple molecular clock hypothesis is incorrect and many aspects of molecular phylogeny are controversial [1,29]. Inferring

absolute mitotic age is difficult because error rates are uncertain and may vary [total errors = (error rate) $\times$ (mitotic age)]. Although most alterations do not accumulate in a simple clock-like fashion, appropriate algorithms can still extract ancestral information. Similar to sequence evolution, methylation is unlikely to follow a simple molecular clock hypothesis and the accumulation of methylation may depend on a number of factors. Indeed, the analysis of BGN methylation patterns in human colon crypts is consistent with a cooperative mechanism whereby methylation at one CpG site increases the probability of methylation at another CpG site [30].

Molecular evolutionary approaches are complex and difficult to validate, but their imperfections are balanced by the lack of practical methods to otherwise measure the past. The impracticality of human experimental manipulations provides motivation to further develop more powerful somatic cell molecular phylogeny tools. Mimicking species studies, more powerful somatic cell studies would require the analysis of more CpG-rich regions — conclusions are more certain when many different parts of a genome retell similar stories. In one sense, it is surprising that genome variation can reconstruct ancestry but, given that genomes are almost exact copies of prior copies, it would also be surprising if the past were not inscribed within genomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

DNA methylation patterns are usually copied after DNA replication by DNA methyltransferases that add methylation to hemi-methylated CpG sites

Figure 2.

During ageing in a mitotic tissue like the colon, some CpG rich regions remain unmethylated, whereas others rapidly reach a stationary or stable level of methylation (grey lines). To more effectively function as a mitotic clock, DNA methylation should continue to accumulate throughout a lifetime (black line)

Figure 3.

Somatic cell ancestry. (A) Stem cells are ancestors in a somatic cell tree. (B) Somatic cell genealogy can be divided into three sequential phenotypic phases: development from the zygote (months), a stem-cell phase (decades) and differentiation (days to weeks). Only the stem cell phase may vary during chronological ageing

Figure 4.

Example of passenger methylation replication errors. A single ancestral epiallele is duplicated and passed to two different lineages. The methylation pattern is copied with cell division, and random replication errors may independently accumulate. The greater the number of divisions, on average the greater the number of errors (a mitotic clock hypothesis). Because the ancestor epiallele was initially unmethylated, the increase in percentage methylation is a function of mitotic age. Pairwise distance, or the number of differences between two epialleles at each CpG site, also increases with mitotic age

Figure 5.

Epigenetic mitotic clock in the colon. (A) Methylation of a CpG rich region on the X-chromosome (BGN [10]) with eight CpG sites was sampled from six crypts taken from a 98 year-old male colon (single epiallele per cell). After bisulphite treatment and cloning of PCR products, eight epialleles were sampled from each ~2000 cell crypt. Epialleles are heterogeneous between and within crypts, and average crypt values can be summarized by average percentage methylation, numbers of unique tags per crypt and average pairwise distances between the epialleles. (B) Evidence of age-related increases in crypt methylation. Each circle is the average methylation of multiple crypts from each different aged colon. To test whether these experimental data match certain intestinal crypt dynamics, ~2000 cell human crypts can be simulated, starting with unmethylated epialleles at birth. Parameters are numbers of niche stem cells, methylation replication error rates and stem cell division rates. Simulated values are the solid (average) and dotted (95% simulation intervals) lines. This simulation assumes a crypt sniche containing 64 stem cells with 95% asymmetric divisions [10]. (C) Average pairwise distances increased with chronological ageing between crypts but were relatively constant within crypts. (D) Average unique epialleles per crypt also did

not appear to increase with chronological ageing. (E) Niches contain multiple stem cells and eventually undergo clonal evolution unless stem cell division is always asymmetrical. With rare symmetrical divisions, a stem cell lineage will become extinct when both daughter cells exist the niche, balanced by the expansion of another stem cell lineage (both daughter cells remain within the niche). Eventually clonal evolution will occur when all niche stem cells are the progeny of a single cell. This process is ongoing and estimated to recur on average every 8 years in human colon crypts [10]. (F) Average crypt diversity reaches equilibrium because the tendency for epialleles to become polymorphic (Figure 4) is balanced by epiallele losses from niche turnover. Cell division links the production of new epialleles from replication errors with epiallele losses from niche extinction

Figure 6.

Hair bulb versus colon crypts. (A) Unlike colon crypts, average hair bulb CSX epiallele methylation does not increase with chronological age (trend lines are illustrated). CSX is another short stretch of CpG sites that exhibits age-related methylation in the colon [10]. By inference, the average mitotic age of a hair bulb does not increase with ageing. (B) Colon crypts appear to have a continuous genealogy or mitotic stem cells. Hair bulb mitotic ages appear limited because at the end of the hair cycle the bulb disappears and mitotically older cells are lost. Hair bulge stem cells divide briefly at the start of a new hair cycle to create new bulb progeny, and newer hairs start with relatively lower mitotic ages. Therefore, average hair bulb mitotic age does not significantly increase with chronological ageing, because lifetime hair bulge stem cell divisions may be minimal