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REVIEW

Histone variants: critical determinants in tumour heterogeneity

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Abstract **Malignant cell transformation could be considered as a series of cell reprogramming events driven by oncogenic transcription factors and upstream signalling pathways. Chromatin plasticity and dynamics are critical determinants in the control of cell reprograming. An increase in chromatin dynamics could therefore constitute an essential step in driving oncogenesis and in generating tumour cell heterogeneity, which is indispensable for the selection of aggressive properties, including the ability of cells to disseminate and acquire resistance to treatments. Histone supply and dosage, as well as histone variants, are the best-known regulators of chromatin dynamics. By facilitating cell reprogramming, histone under-dosage and histone variants should also be crucial in cell transformation and tumour metastasis. Here we summarize and discuss our knowledge of the role of histone supply and histone variants in chromatin dynamics and their ability to enhance oncogenic cell reprogramming and tumour heterogeneity.**

Keywords cancer-testis; TH2B; TH2A; H1T; H1.0; H1F0; linker histones

Introduction

Malignant transformation of normal somatic cells is a multistep process eventually leading to the selection of deadly metastasis-prone cancer cells. Within this context, the molecular mechanisms that generate genetic and epigenetic heterogeneity are essential to provide the indispensable ground for the selection of such cells [1]. The selective pressure here operates on genetic/epigenetic elements [2] to stably support the acquisition of the so-called cancer hallmarks [3]. The transformation of a normal cell into a cell with malignant characteristics could therefore be qualified as an oncogenic reprogramming of cells.

This process could be compared to the induced cell reprogramming, first defined by Yamanaka [4], with a major difference: malignant reprogramming relies on the aberrant activation of intrinsic reprogramming events [5]. Successful malignant transformation should therefore obey to the same laws as those underlying the induced reprogramming of differentiated somatic cells [6].

Indeed, not only there is a need to activate driver transcription factors and appropriate cell signalling, but there is also a requirement to break reprogramming barriers, mostly of epigenetic nature [7]. Among these reprogramming barriers important ones are the Suv39H1 enzyme, H3K9 tri-methylation and other factors responsible for heterochromatin formation [8,9].

In addition to the erasure of repressive epigenetic marks, a critical factor for successful cell reprogramming is increased chromatin dynamics [10]. Factors capable of sustaining enhanced chromatin dynamics seem to also improve cell reprogramming [11]. Chromatin dynamics could be directly modulated by canonical replication-dependent histones through the control of their assembly during DNA replication, as well as through induced histone degradation and histone under-dosage or independently, through the assembly of a specific class of histone variants [12–16].

Regulated replication-dependent nucleosome assembly is in fact an important element in defining the dynamic states of chromatin. Accordingly, two studies based on interference with CAF1 activity, a histone chaperone

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required for the replication-dependent chromatin assembly [17], highlighted the critical role of this factor in modulating cell reprogramming. Down-regulation of CAF1 leads to an increased chromatin accessibility, a shift in the cell gene expression programs and the acquisition of new characters [18,19].

Histone variants that are tissue-specific or whose expression depends on specific regulatory signals, also play their role by conferring specific states to chromatin, either globally in a particular cell type, or locally on defined genomic regions. Some of these histones are associated with more stable and transcriptionally repressed chromatin such as the H2A variant, macroH2A [20], while others, in contrast, are associated with unstable nucleosomes such as H2A.B.3 and H2A.L.2 in spermatogenic cells [21,22]. Interestingly, somatic cell reprogramming assays showed that macroH2A expression is associated with a resistance to reprogramming [23], while the ectopic expression of testis/oocyte-specific histone variants of the H2A and H2B types (THA, TH2B) in fibroblasts, greatly stimulates the process of reprogramming [24].

All these data highlight the importance of histone metabolism, histone dosage and histone type synthesis in the capacity of cells to be reprogrammed. Mechanisms controlling histone turnover and the expression of histone variants are largely affected in cancer [25]. More specifically, cancer cells express almost systematically tissue-restricted genes [26], among which, genes encoding histone variants [27] as well as mutated histones known as "oncohistones" [28].

Here we develop a discussion on the roles of specific types of histone variants and of histone dosage in cell epigenetic reprogramming that could be essential for the establishment of malignant transformation and tumour heterogeneity (Fig. 1).

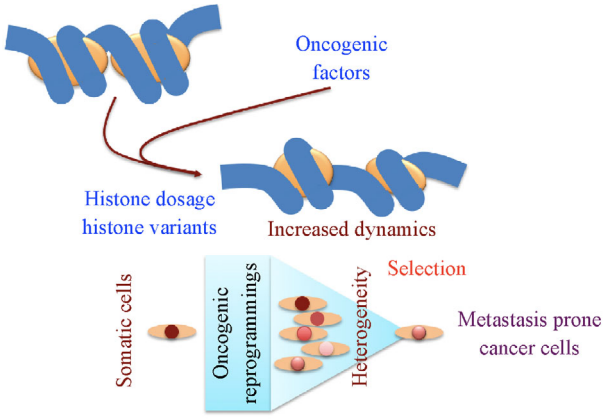

Fig. 1 Histone-based malignant transformation, tumour heterogeneity and selection of aggressive characters. Pro-oncogenic events could lead to aberrant activation of silenced histone variants-encoding genes or histone assembly defects or histone under-dosage, leading to increased chromatin dynamics and enhanced genome reprogramming by oncogenic factors. The resulting heterogeneity would create a window of opportunity for the selection of newly reprogrammed oncogenic cells capable of surviving and disseminating.

Male germ cells express the largest set of histone variants

In addition to canonical histones that are the building blocks of nucleosomes, the human genome encodes a number of histone variants of the H3, H2A and H2B types. Many of these variants are predominantly or exclusively expressed in spermatogenic cells [14]. The reason is that the final stages of male germ cell differentiation involve one of the most dramatic chromatin remodelling events, characterized by an almost genome-wide eviction of histones and their replacement by protamines [29]. Functional, biochemical and structural analyses of these histone variants showed that many of them present the ability to generate unstable nucleosomes. This is true for H3, H2B and H2A variants [14]. Taking into account the final dismantlement of chromatin in post-meiotic spermatogenic cells, before the generation of mature spermatozoa, one can easily understand why most of these variants confer nucleosome instability. Indeed, a more open and dynamic chromatin should lower the energy supply required for the genome wide removal of histones.

There are data strongly supporting the idea that most of these variants are in fact nucleosome-destabilizing elements. Structural studies of the testis-specific histone H3, H3T/t, showed that both the human and the mouse members create unstable nucleosomes [30,31]. More particularly, in H3t, a single amino acid, H42, is critical to generate a flexible linker DNA at the entry and exit of the nucleosome [30]. This ability to open the nucleosome with flexible DNA ends is also shared by several H2A variants that are expressed at different stages of spermatogenesis, namely H2A.B.3 [21] and H2A.L.2 in mouse [22], as well as H2A.Bbd (H2.B.1 and H2A.B.2) in human [32]. Finally, the major testis-specific H2B variant, TH2B, also induces nucleosome instability, especially when it is paired with TH2A [24,33].

Since nucleosome instability underlies chromatin dynamics, and that the latter is essential for efficient cell reprogramming, the question arises on the role of these histone variants in enhancing cell reprograming. A positive answer then invites to consider if this histone driven chromatin dynamics could also induce malignant cell reprogramming and thereby contribute to tumour heterogeneity.

TH2B/TH2A enhances somatic cell reprogramming

Among the male germ cell histone variants, TH2B and

TH2A show very particular characteristics, since they act during two unique periods of the male genome life. At the time of commitment of male germ cells into meiotic divisions, these variants accumulate and gradually become the major nucleosomal H2A and H2B histone types until their genome-wide eviction and replacement, prior to the generation of mature spermatozoa [33,34]. Interestingly, these histone variants also accumulate in maturing oocytes and are stored until fertilization. Upon removal of protamines from the male genome, maternal TH2A/TH2B become again associated with the male genome [35], and spread over the zygote's genome at a period critical for epigenetic programming in pre-implantation embryonic cells. During later embryonic cell divisions, TH2A/TH2B are gradually replaced by somatic type H2A/H2B histones [33,34]. Their presence at two critical periods of general genome programming, first during the male germ cell differentiation in preparation of histone-to-protamine exchange and again during protamine-to-histone replacement and embryonic cell genome programing, strongly suggests that the properties conferred by these histone variants to chromatin are of critical importance to make the genome programmable.

This hypothesis has been actually elegantly confirmed by Ishii's group, who showed that the ectopic expression of TH2B/TH2A in somatic cells strongly enhances induced pluripotent stem cells (iPS) formation by Yamanaka factors. Additionally, in the absence of both TH2B and TH2A (double KO mice), fertilized eggs do not develop properly, again indicating the importance of these histone variants in early development [24].

Taking into account the essential role of these two histone variants in genome reprogramming, we also hypothesized that their ectopic activation could help oncogenic cell transformation or create a heterogeneity among tumour cell populations, which would facilitate the evolution of cancer cells toward increased aggressiveness.

To test this hypothesis the expression of TH2A and TH2B was monitored in transcriptomic data available from several cohorts of cancer patients. Fig.2A shows that in human both TH2A and TH2B encoding genes are indeed tissue-restricted genes specifically expressed in testis. The analysis of a cohort of breast cancers as well as of two series of lung tumour samples shows that both genes could become aberrantly active in a subset of tumours (Fig. 2B). However, these data do not allow us to conclude on a

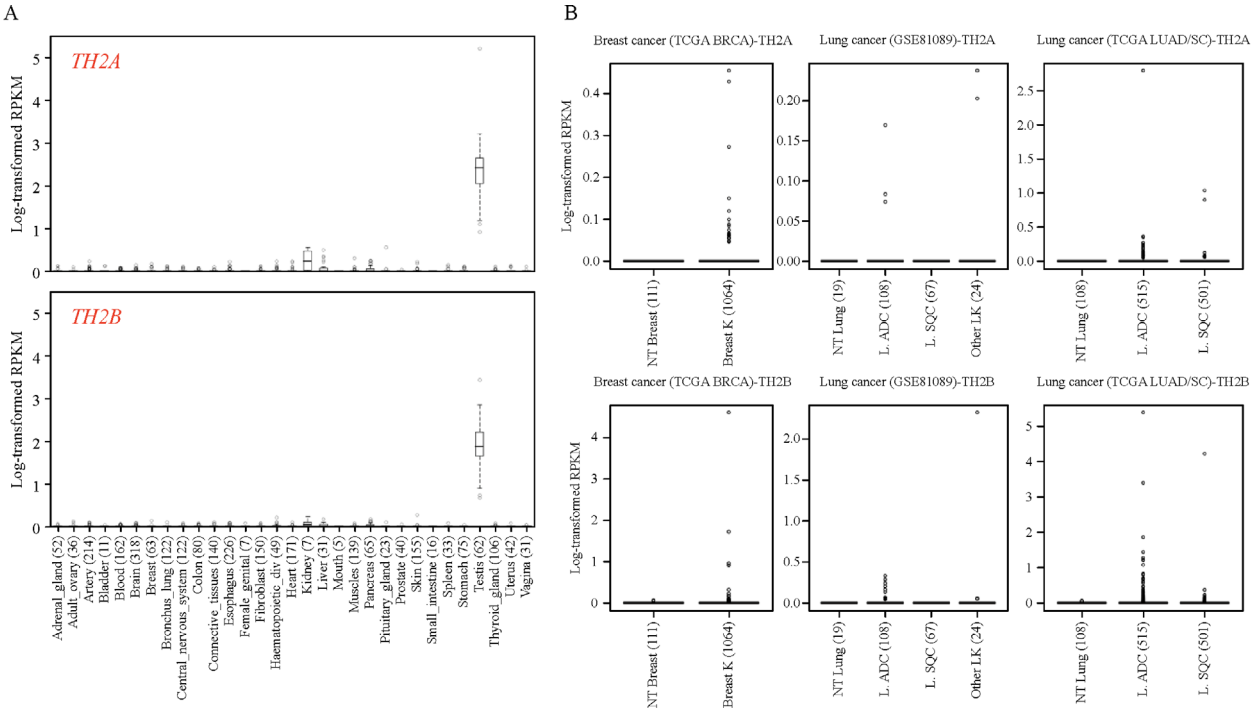

Fig. 2 Aberrant activation of testis-oocyte specific TH2A/TH2B in various cancers. (A) Expression of TH2A and TH2B genes in normal human tissues samples from RNA-seq data, provided by the Genotype-Tissue Expression (GTEx) project [56]. (B) Expression of TH2A and TH2B genes in breast and lung cancer samples. Breast cancer RNA-seq data are provided by the TCGA-BRCA project [57]. Lung cancer RNA-seq data are provided by the NCBI GEO GSE81089 [58], TCGA-LUAD and TCGA-LUSC projects [57]. For all plots, the expression level of genes is represented as a distribution of log-transformed RPKM values, after addition of a pseudo count of 1 (log2 (1 + RPKM)). Breast cancer: NT Breast = non tumoral breast; Breast K = breast cancer. Lung cancer: NT Lung = non tumoral lung; L. ADC = Lung adenocarcinoma; L. SQC = Lung squamous cell carcinoma; other LK = lung tumours of other histological subtypes.

relationship between the activation of these genes and the process of oncogenesis. Indeed, in most cases, the tumours analysed here were harvested long after the initial pro-oncogenic crisis and the malignant transformation process. It is therefore likely that a counter-selection against the expression of both or either of these genes is required for tumour cells to survive beyond the initial events, with a stable gene expression program (Fig. 1). In support of this hypothesis, in the normal frame of TH2A/TH2B expression, it has been shown that embryonic development is associated with a sharp decrease in both TH2A and TH2B in embryonic cells, indicating a requirement for the silencing of these genes after the initial reprogramming period. The sporadic expression of these genes in established cancer cells in tumours would merely be part of the vestiges of their full activation at the time of oncogenic transformation.

Short H2A variants

One of the most remarkable characteristics of the canonical histone H2A is the presence of an acidic patch, which locates at the surface of nucleosomes and was shown to mediate the compaction of the chromatin fibre [21,32,36]. The other important contribution of H2A to the nucleosome structure is through its C-terminal region, known as the "docking domain," involved in the stabilization of H3 a-N helix. The H2A docking domain also contributes to the stability of the H2A-H2B dimer–H3-H4 tetramer interaction [37]. Indeed, H2A variants with a short docking domain [38] are unable to form stable histone octamers. Within a nucleosome, a H2A with a short docking domain disrupts the ability of the H3 a-N helix to stabilize nucleosomal DNA and leads to the release of unwrapped DNA ends [39,40].

Taking into account the ability of the H2A variants to open and destabilise nucleosomes, it is expected that their incorporation into nucleosomes should increase chromatin dynamics and genome reprogramming ability. Unfortunately, however, the role of this category of short H2A variants in cell reprogramming has not yet been investigated.

Interestingly, although most of the short H2A variants, including human H2A.Bbd, are testis-specific [38], several investigations pointed to their contribution to oncogenic cell programming in specific sets of cancers. Indeed, the H2A.Bbd-encoding gene was found de-repressed in Hodgkin lymphoma [41]. The analyses of various Hodgkin lymphoma cell lines showed that the expression of this histone variant is remarkably variable [42], suggesting that the ectopic activation of this gene could have been important in the process of malignant transformation and that a counter-selection against its expression operates after the establishment of the transformed state.

Ectopic testis-specific linker histone expression as a measure of tumour heterogeneity

Similar to core histones, linker histones are also encoded by a variety of canonical replication-dependent as well as replication-independent and tissue-specific genes [43].

There are testis-specific linker histones named H1T and HILS1 in human. Here we first verified their tissue-restricted pattern of expression in human adult tissues. Fig.3 shows that while, as expected, H1T shows a strict testis-specific expression, HILS1 is also expressed in other tissues such as in muscle (not shown). The questions are whether the H1T gene could be aberrantly activated in cancers, whether its activation would reflect tumour heterogeneity and whether its expression could be associated with tumour types, sub-types and prognosis.

To answer these questions the expression of H1T was monitored in breast and lung tumours where the ectopic activation of genes was observed in a significant number of tumours (Fig. 3). Since HILS1 did not show the expected testis-specific expression pattern, its expression in cancer was not considered. The ectopic activation of H1T, as in the case of TH2A/TH2B, could be an important event during malignant transformation and the presence of H1T in some of the analysed tumours could be a vestige of this initial oncogenic reprogramming process (and the subsequent counter-selection required to stabilize the epigenome of the established malignant cells).

However, in the case of H1T, this hypothesis remains highly speculative since, in contrast to the TH2A/TH2B situation, the role of H1T in genome reprogramming has not been shown. Therefore additional experimental data on the reprogramming capacity of H1T and its expression in oocytes and early development are required to support the hypothesis.

Linker histone variants and tumour heterogeneity

Among linker histone variants genes, *H1F0* encodes histone H1.0, which presents a differentiation-dependent expression.

H1.0 is a conserved linker histone present in all vertebrates, except in birds. The corresponding gene is also present in some invertebrates such as sea urchin [43]. Interestingly, not only the H1.0 protein shows conserved features, but also the regulatory circuits that control the expression of its gene is conserved in different species [44]. These data highlight the fact that the differentiation-dependent nature of H1.0 expression is also an evolutionary conserved characteristic of cell differentiation and hence should probably contribute to the epigenetic stability of differentiated cells.

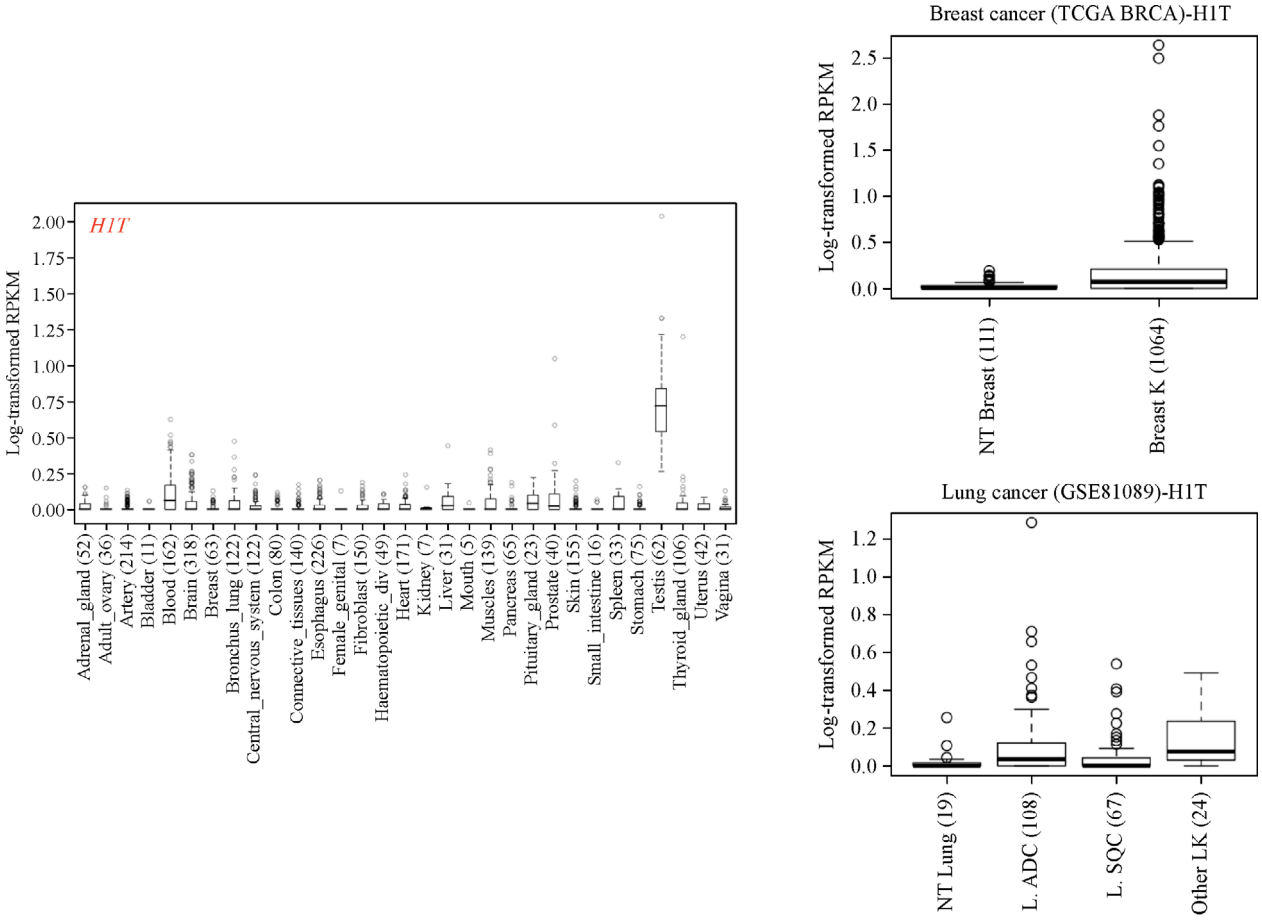

Fig. 3 Aberrant activation of testis-specific H1T in various cancers. Expression of H1T gene in normal (left panel) and tumour (right panels) samples from RNA-seq data, provided by the GTEx [56], TCGA-BRCA [57] and NCBI GEO GSE81089 [58] datasets. The expression level of genes is represented as a distribution of log-transformed RPKM values, after addition of a pseudo count of 1 (log2 (1 + RPKM)). Breast cancer: NT Breast = non tumoral breast; Breast K = breast cancer. Lung cancer: NT Lung = non tumoral lung; L. ADC = Lung adenocarcinoma; L. SQC = Lung squamous cell carcinoma; other LK = lung tumours of other histological subtypes.

The gene is repressed during early embryonic development and in non-differentiated stem cells as well as in various cancer cell lines but is induced upon the commitment of cells into differentiation [43]. The study of established cancer cell lines such as Friend murine erythroleukemia [45] and murine melanoma cells [46] showed that the induced differentiation of these cells is associated with the activation of H1.0-encoding gene.

Additionally, in contrast to other linker histone-encoding genes, *H1F0* expression is potently induced by histone-deacetylase inhibitors in cultured cells in all vertebrates [43] and in developing embryos in a stage-specific manner [47–50].

A recent study showed that heterogeneous H1.0 expression in tumour cells directly reflects tumour heterogeneity, with more differentiated cells expressing higher levels of H1.0. An absence of H1.0 in tumour cells correlates with the stem type nature of the cells [51]. The degree of H1.0 expression could therefore be considered as a measure of the level of differentiation of tumour cells and hence reflect tumour aggressiveness. This work also shows that H1.0 restricts self-renewal and favours differentiation. These data are in agreement with data we previously published on H1.0 expression in hepatocytes after partial hepatectomy. Indeed, the H1.0 content dramatically decreases after partial hepatectomy corresponding to the natural reprogramming of hepatocytes and the induction of cell proliferation [52]. Altogether, these data show that reprogramming of adult differentiated cells should be associated with a decrease in H1.0 content, either in a physiological setting, after partial hepatectomy, or in the pathological condition of malignant cell transformation [51]. In addition, we do not expect the oncogenic transformation of stem types of cells to be associated with any change in H1.0 gene expression, since non-transformed adult stem cells do not express high levels of

H1.0. However, the induced expression of H1.0 in cancer cells could reflect the proliferation and differentiation of malignant stem cells.

Following these data, we analysed the relationship between *H1F0* gene expression and patient survival data taking into account published cancer data used by Torres and colleagues [51], as well as some other cancer transcriptome series not considered by these authors (Fig. 4).

Unexpectedly, we observe that, in all the considered cases, *H1F0* expression was lower in normal control tissues compared to the corresponding cancer series (Fig. 4). Two additional observations could help explaining this observation. First, the *H1F0* gene promoter bears regulatory elements which are shared with the replication-dependent H4 encoding genes [53]. Second, in cells in culture, *H1F0* shows an increased expression in S phasis [54]. Therefore, the higher expression of *H1F0* genes in cancers compared to their non-transformed counter-parts could actually reflect the presence of proliferative sub-populations in the considered tumours.

The capacity of the *H1F0* gene promoter to be responsive to both cell differentiation and cell proliferation signals could appear contradictory. The higher levels of *H1F0* gene expression in tumour cells compared to their non-transformed counterparts could be explained by the higher proportion of proliferative cells in tumours. However, taking into account the heterogeneity of H1.0 in a given tumour [51], it can be proposed that, within the context of proliferative cancer cells, a higher level of differentiation would lead to an even higher expression of this gene therefore allowing the identification of less aggressive more differentiated cancer cells.

However, in contrast to the reported data [51], when we looked for a correlation between the level of *H1F0* expression and survival in a cohort of breast cancer patients and in three cohorts of patients with lung cancer and we found no significant association between expression of *H1F0* and survival probability (Fig. 4).

In conclusion, although a variable expression of H1.0 might be used as a measure of tumour heterogeneity [51], its level of expression cannot be reliably used as an indicator of prognosis.

In addition to H1.0, other tissue-restricted linker histones are also expressed in vertebrates. Birds express a linker histone known as H5 only in erythrocytes, a gene, which is probably related to an ancestral H1.0-encoding gene, uniquely expressed in amphibian erythrocytes [55]. The specific/high expression of H1.0/H5 in amphibian/birds erythrocytes is certainly linked to the functional inactivation of the nucleus in these cells, which in contrast to mammals, remain nucleated. There are however no data on the expression of H5 and cancer in avian.

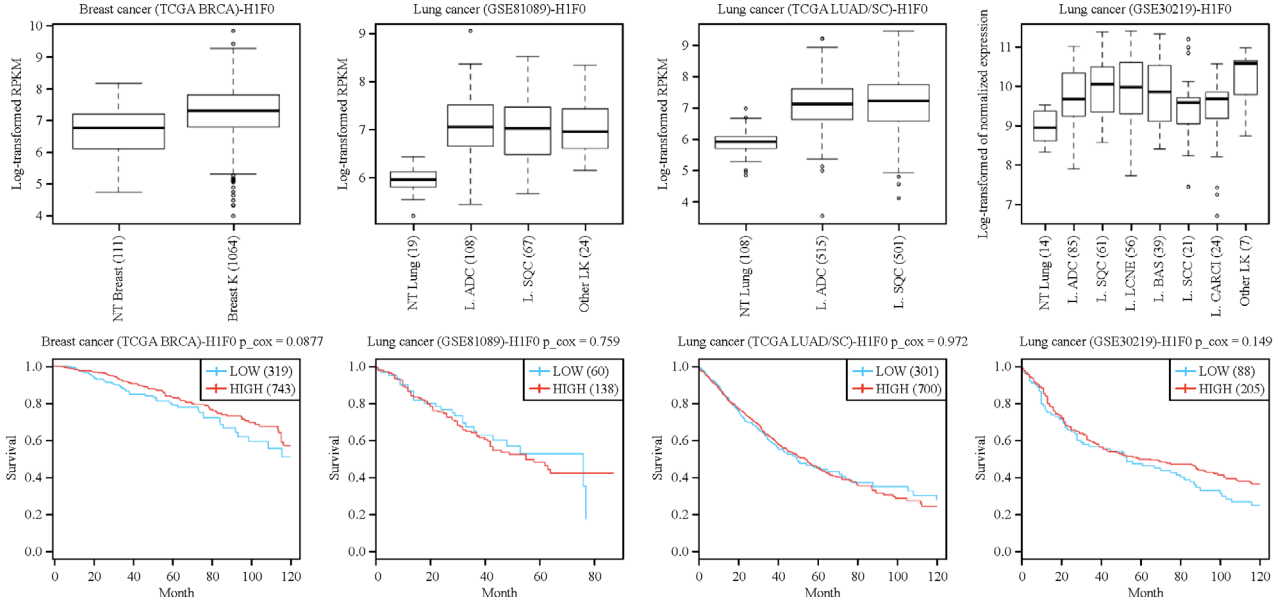

Fig. 4 *H1F0* gene expression is activated in different cancers. Expression of *H1F0* gene in breast and lung tumour samples with corresponding Kaplan–Meyer survival curves. Breast cancer RNA-seq data are provided by the TCGA-BRCA project [57]. Lung cancer RNA-seq data are provided by the NCBI GEO GSE81089 [58], TCGA-LUAD and TCGA-LUSC projects [57]. For all plots, the expression level of genes is represented as a distribution of log-transformed RPKM values, after addition of a pseudo count of 1 (log2 (1 + RPKM)). Breast cancer: NT Breast = non tumoral breast; Breast K = breast cancer. Lung cancer: NT Lung = non tumoral lung; L. ADC = Lung adenocarcinoma; L. SQC = Lung squamous cell carcinoma; L. LCNE = Lung large cell neuroendocrine tumours; L. BAS = Lung basaloid tumours; L. SCC = Lung small cell carcinoma; L. CARCI = Lung carcinoid tumours; other LK = lung tumours of other histological subtypes.

Discussion

Epigenetic stability is a critical barrier to physiological and pathological cell reprogramming. The establishment of specific strategies to induce reprograming in adult differentiated cells also enabled to highlight the epigenetic determinants that prevent cell reprogramming. Histone dosage seems to be an essential barrier in hindering reprogramming factors' action. Further investigations suggest that increased chromatin dynamics could explain the effect of histone under-dosage and under-assembly in facilitating reprogramming. Indeed, we know that histone loss increases chromatin dynamics [13] and increased chromatin dynamics is associated with enhanced reprogramming capacity of the cells [11]. Therefore, histone assembly defects or histone under-dosage as well as the expression of specific classes of histone variants should break the adult cell resistance to reprogramming and facilitate malignant transformation and tumour cell heterogeneity.

Here by analysing cancer transcriptomic data we show that malignant cells express histones that are normally either expressed in a tissue-restricted manner (H1T, TH2A, TH2B) or expressed during a particular physiological state such as differentiation (H1.0). We also show that the aberrant activation of these genes could give a clear measure of tumour heterogeneity. Additionally, they may also functionally impact the cells and facilitate oncogenic cell reprogramming as they do in reprogramming assays (TH2A/TH2B).

Taken together we can conclude that, beyond the specific emerging role of oncohistones [28], the general concept of histone-driven oncogenesis should be considered and promises to increase our understanding of malignant transformation and the molecular basis of tumour cell heterogeneity.

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Compliance with ethics guidelines

Tao Wang, Florent Chuffart, Ekaterina Bourova-Flin, Jin Wang, Jianqing Mi, Sophie Rousseaux, and Saadi Khochbin declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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