### Review Article Stress-triggered atavistic reprogramming (STAR) addiction: driving force behind head and neck cancer?

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Abstract: Recent results of the Cancer Genome Atlas on head and neck squamous cell carcinoma (HNSCC) revealed that HNSCC lacked predominant gain-of-function mutations in oncogenes, whereas an essential role for epigenetics in oncogenesis has become apparent. In parallel, it has gained general acceptance that cancer is considered as complex adaptive system, which evolves responding environmental selective pressures. This somatic evolution appears to proceed concurrently with the acquisition of an atavistic pluripotent state (i.e., "stemness"), which is inducible by intrinsic epigenetic reprogramming program as demonstrated by induced pluripotent stem (iPS) cells. This Nobel prize-winning discovery has markedly accelerated and expanded cancer stem cell research from the point of epigenetic reprogramming. Taken together, we hypothesize that stress-triggered atavistic reprogramming (STAR) may be the major driving force of HNSCC evolution. In this perspective, we discuss the possible mechanisms of STAR in HNSCC, focusing on recent topics of epigenetic reprogramming in developmental and cancer cell biology.

Keywords: Head and neck squamous cell carcinoma, epigenetic reprogramming, pluripotency, cancer evolution, stemness

#### Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [1]. Despite recent advances in multidisciplinary treatments, the overall survival and, more importantly, quality of life of patients with HNSCC have not significantly improved over the past decade. To solve this issue, growing interest has focused on the development of novel treatment strategies based on HNSCC cell biology. In this trend, recent identification of human papilloma virus (HPV)-positive HNSCC is a milestone discovery, because it is apparent that this type of cancer has a distinctive genetic and epigenetic profile, which can be cured within the framework of conventional organpreserving treatment [2-4]. However, with respect to HPV-negative HNSCC, the exponentially expanding information on the cellular biology remains segmental and has not lead to a holistic understanding of the dynamically evolving molecular circuitry of this dismal cancer [4], which we will mainly discuss about in this perspective.

In contrast, cancer research appears to have entered a new dimension during the last few years through a series of innovative technologies, fundamental discoveries, and novel conceptual frameworks. Recent whole-exome sequencing studies on HNSCC, achieved by next generation sequencing (NGS), has reveled that HNSCC lacked predominant gain-of-function mutations in oncogenes [5-7]. Considering these findings together with the rapidly accumulating evidence that epigenetics plays a critical role in the genesis and progression of cancer [8-12], the main driving force of HNSCC would appear to be epigenetic reprogramming rather than the stepwise accumulation of several genetic abnormalities. In addition, it is becoming a dominant concept to view cancer as a complex adaptive system comprising heterogeneous cell populations that evolve under selective pressures [4, 13-16]. Due to this Darwinian theory, the ultimate goal of cancer evolution must be survival in a harsh microenvironment, which apparently deteriorates in accordance with cancer evolution [17]. Consequently, the most advanced form of cancer cells



Figure 1. Epigenetic landscape of normal cell development (A), cancer evolution (B) and its implication in the current treatment strategy for head and neck squamous cell carcinoma (HNSCC) (C). (A) In normal cell development, developing cells flow down the surface of the rugged slope from the state of pluripotent embryonic stem cell (ESC) to differentiated cells. (B) As opposed to normal cell differentiation, the evolutionary trajectory of HNSCC is expected to be an uphill movement mainly driven by intrinsic epigenetic reprogramming system triggered by the environmental stressors. The goal of this trajectory is assumed to be the acquisition of an "atavistic" pluripotent state: i.e., generation of cancer stem cell (CSC). We postulate the term stress-triggered atavistic reprogramming (STAR) to describe this phenomenon. (C) An individual tumor is composed of a heterogeneous cell population, which resides in the stoichiometric equilibrium of the gene expression network called a "cancer attractor". As depicted in this Figure, a broader range of cancer cells in different cancer attractors may be eliminated through the intensification of the treatment modalities, which has been enthusiastically pursued during the last decade in the treatment of HNSCC. However, it is becoming apparent that this intensification strategy has reached the upper limit of human tolerance. In addition, it is evident that cancer cells in the higher attractor type can survive currently available combinations of conventional modalities (e.g., chemo/radiation), resulting in the selection of highly evolutionized clones. Moreover, a dose-intensification strategy may work as a strong selective pressure for STAR as illustrated by the green cell, which lie on the border line of the treatment effect. Thus, if we could reverse STAR, optimization of treatment intensity may be feasible.

have to acquire the highest plasticity and fitness. In reference to the current conceptual framework of cancer and developmental biology, cells with the capacity for unlimited selfrenewal and pluripotency (i.e., stemness) appear to fit this category [18, 19]. Thus, cancer evolution, particularly in the case of solid tumors, is likely to proceed in accordance with the acquisition of self-renewal and pluripotency. The Nobel Prize-winning discovery of induced pluripotent stem (iPS) cells clearly demonstrated that this "atavistic" phenomenon (i.e., de-differentiation) could be achieved through epigenetic reprogramming evoked by the introduction of only four core pluripotency factors (c-Myc, Sox2, Oct3/4 and Klf4) [18, 20]. This striking finding has markedly accelerated and expanded cancer stem cell (CSC) research from the standpoint of epigenetic reprogramming [10, 21]. Thus, it seems plausible to speculate that "stress-triggered atavistic reprogramming (STAR)" may play a critical role in HNSCC evolution, particularly promoting the production of CSCs. Based on this background, we discuss the perspective that HNSCC evolution may be highly dependent on (i.e., addicted to) the STAR phenomenon; therefore, STAR may be the Achilles' heel of HNSCC [22].

#### **Overview of HNSCC biology and treatment**

#### Genetic landscape

It has been generally accepted that the development and progression of HNSCC occurs through the stepwise and progressive accumulation of genetic and epigenetic alterations as depicted by several linear progression models proposed during the last two decades [1, 23]. Behind theses models, there seems to be a reductionist view that the causes of HNSCC could be empirically viewed as a few predominant genetic and epigenetic abnormalities, and thus normalization of these limited numbers of molecules may lead to the cure of this cancer. Accordingly many investigators have struggled to find a molecule to which HNSCC is addicted [1, 4, 24]. Three whole-exome sequencing studies on HNSCC tumor samples were conducted recently [5-7]. These studies revealed several important findings including the identification of NOTCH 1 as a novel tumor suppressive gene and the relatively frequent occurrence (30%) of genetic abnormalities, which accumulate in the PIC3K-Akt-mTOR axis. However, the most striking evidence confirmed by these studies was that HNSCC lacks major gain-of-function mutations in oncogenes, whereas the most prevalent mutations were loss-of-function mutations of tumor suppressive genes, TP53 and CDK-N2A. In parallel, the milestone FANTOM (Functional Annotation of the Mammalian Genome) and ENCODE (Encyclopedia of DNA Elements) project reveled that 80% of the human genome has functions at the level of RNA or chromatin [25, 26]. Encouraged and accelerated by this great discovery, it has become apparent that global epigenetic alterations play a fundamental role in the development and progression of cancer as well as genetic alterations [8-12]. Thus, the central dogma that cancer is a genetic disease is losing its predominant position. We may expect that HNSCC appears to be a disease that is mainly driven by constant "epigenetic reprogramming" rather than the stepwise acquisition of driver mutations in a limited number of oncogenes and/or tumor suppressor genes. This scenario robustly explains the reason why the identification of HNSCC-specific molecular targets has been unsuccessful so far, and more importantly, demands that we view HNSCC as an epigenetic disease.

#### HNSCC evolution and STAR

In parallel to this paradigm-shift from genetics to epigenetics, cancer evolution theory has gained wide acceptance, in which cancer is being recognized as a peculiar organ or a complex adaptive system that evolves in response to harsh selective pressures or signals generated in the tumor microenvironment [4, 13-16]. As demonstrated in our previous review and others, the evolutionary trajectory of cancer is often depicted borrowing the visual representation of the Waddington's epigenetic landscape [4, 15, 16, 27], which was originally applied to illustrate the physiological dynamics of normal cell differentiation (Figure 1A). In this evolutionary process, HNSCC cancer cells are thought to climb up "cancer attractors" (i.e., stoichiometric equilibrium of gene expression network) reversing the process of differentiation toward CSC attractors (Figure 1B). Due to the canonical Darwinian theory, evolution is propelled by a series of innovative emergencies caused by stochastic mutations in the germ cells, which facilitate phenotypic variations and survival of the fittest [28]. In contrast, "somatic" evolution of cancer appears to be a rewinding process of normal development triggered in response to environmental stressors rather than the accumulation of novel innovations. Thus, cancer cells evolve resuming sealed-memories of ancestral pluripotent cells (e.g., embryonic stem cell (ESC)), utilizing an intrinsic programmed system. This is consistent with the findings that the gene expression profiles of advanced cancer culminate in those of cells undergoing wound healing or ESC [29-31]. Accumulating evidence indicates that rather than the stochastic mutations of genes, epigenetic reprogramming may be a critical driving force of this atavistic phenomenon in cancerous cells as well as non-cancerous cells [18]. This is probably because epigenetic reprogramming allows cancer cells to adapt to a drastically changing cancer microenvironment much more rapidly

#### STAR addiction



**Figure 2.** Relevance of cancer stem cell (CSC), epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET). Recent studies indicate that as with the non-CSCs, CSCs may be a heterogeneous cell population. In addition, the CSC state is not consistent but rather a transient cellular state that is highly context-dependent, as exemplified by a recent study that clearly demonstrated that the bidirectional conversion of non-CSC and CSC can be inducible through the effect of a bivalent promoter of a single gene in response to micro-environmental signals [84]. It has been postulated that CSCs may exclusively have potential to undergo EMT and thereby to metastasize (migrating CSC theory). However, given that the EMT-MET program is an intrinsic program utilized during organogenesis and wound healing, it is also possible that stress-triggered atavistic reprogramming (STAR) may provide non-CSC with the potential to undergo EMT-MET plasticity in the process of tumor evolution. Interestingly, a recent study showed evidence that MET induced stemness in HNSCC [44]. Taken together there seems to be a cycling loop among cellular pool of CSCs, EMT cells, and non-CSCs.

than stochastic mutations [8]. In addition to the discovery of iPS cells, a recent study provided clear evidence that this atavistic reprogramming is indeed inducible in cancer via epigenetic modulation alone. Introduction of a core set of neurodevelopmental transcription factors (POU3F2, SOX2, SALL2, and OLG2) transformed differentiated glioblastoma into tumor-propagating stem-like cells [32]. In view of these findings, we speculate that the evolutionary process of epigenetic-driven HNSCC may be highly dependent on STAR.

# Controversial issues on CSC and its correlation with epithelial-mesenchymal transition (EMT)

In our STAR scenario, there are three caveats about the origin and identity of CSC and its relevance to epithelial-mesenchymal transition

(EMT) cells. Caveat 1; we do not support the hierarchy system, in which CSC is assumed to be the tumor initiating cells. In contrast, we propose pluripotent CSC is the product of clonal cancer evolution, which acquired high levels of biological robustness and plasticity. This is because a majority of early-stage HNSCCs can be cured by the chemo/radiation or surgery and seldom recurs or metastasizes, suggesting that these types of tumors lack CSC [4]. Caveat 2; during the last decade, several different molecules have been identified independently as the surface markers of head and neck CSC. including CD133, CD44 standard form, CD44 variant 9, CD44 variant 3, CD271, and CD10 [33-39]. These findings suggest that as with the non-CSCs, CSCs may be heterogeneous and transient cellular states, which are highly

context dependent. Thus, our Figure 1B seems overly simplistic, and there could be multiple CSC attractors in the trajectory of cancer evolution (Figure 2). Caveat 3; in addition to the heterogeneity of CSCs, the correlation of CSC and EMT appears to be controversial. It has been postulated that only CSCs may possess the potential to undergo EMT-MET (mesenchymalepithelial transition) conversion and thereby to disseminate and metastasize (i.e., EMT-MET cancer cells = migrating CSCs) [40, 41], based on the findings that EMT cells demonstrate CSC-like properties [42]. Similar close correlations between CSCs and EMT cells have been reported in HNSCC [4]. In a recent study, it was demonstrated that CSCs of HNSCC can switch between EMT CSC (CD44<sup>high</sup>ESA<sup>low</sup>ALDH1<sup>high</sup>) and non-EMT CSC (CD44<sup>high</sup>ESA<sup>high</sup>) states [43]. However, CSCs and EMT cells have fundamentally different characteristics; CSCs are slow cycling and static cells that retains epithelial cell lineage and stemness, whereas EMT cells are highly mobile cells that lack epithelial cell features with less stem-like properties [44, 45]. In addition, it is also possible that EMT-MET plasticity exhibited by cancer cells may also be the product of cancer evolution as depicted in Figure 2. This is because, as with the acquisition of pluripotency, the EMT-MET program is driven by the intrinsic epigenetic memory, which is essential during organogenesis and tissue repair processes [46]. Thus, although acquisition of stemness and EMT appear to occur at a relatively advanced phase of cancer evolution, it remains elusive whether only CSC has the potential to exhibit EMT-MET plasticity. Moreover, a recent study demonstrated that connective tissue growth factors could induce MET, as well as a stem-like phenotype in HNSCC cell lines through the up-regulation of NANOG, SOX2, POU5F1 and CDH1 [44]. Thus, the existence of a cycling loop is estimated among attractors of CSCs, EMT cells, and the remaining cancer cells that reside in relatively high cancer attractors (Figure 2).

#### Clinical significance of STAR targeting

During the last decade we have witnessed an intensification of the conventional chemo/ radiotherapy treatments for advanced HNSCC aimed mainly at organ preservation [47, 48]. By combining multiple chemotherapeutic agents and irradiation, sequentially and/or concurrently, several representative protocols (e.g., Tax 324 and RTOG91-11) demonstrated promising short-term results. In Figure 1C, we generated a schema to explain the efficacy of dose-intensified treatment using the cancer attractor model. Individual HNSCC tumors could be depicted as an aggregate comprising of heterogeneous cell populations that reside in different levels of cancer attractors. Presumably, a combination of dose-intensified modalities is expected to result in the elimination of a broader range of cancer cells, particularly cells in the higher attractor aspect, which are otherwise refractory to monotherapy or conventional moderate intensity combination therapy. However, it is apparent that this dose-intensification strategy has critical issues clinically and biologically [4, 49, 50]. Recent long-term results revealed that in addition to considerable acute toxicities, these regimens were associated with severe late toxicities causing functional loss of organs (e.g., laryngo-esophageal dysfunction) and treatment related death, indicating that these therapies have reached the upper limit of human tolerance. In addition, cancer cells in the higher attractors, particularly CSCs, are expected to survive ongoing dose-intensified treatments, and more importantly these therapies may work as a strong selective pressure which accelerates STAR in some cases [4] (Figure 1C).

Thus, if we could find a way to reverse or at least prevent the process of STAR, total cell killing may be feasible by more optimized-intensity treatment, and more importantly, STAR targeting may open up a novel treatment strategy for recurrent and /or metastatic tumors, which are predominantly composed of highly evolved cancer cells and are the main causes of cancer deaths in the order of months [4] (**Figure 2**).

#### Foundation for genome-wide epigenetic reprogramming

Loss of large organized chromatin K9 modifications (LOCKs): the opening of the Pandora's box?

During the last decade, we have gained remarkable insights into the mechanism of how genetic information is stored and translated. Genomic DNA is packaged in the form of chromatin using a winding system composed of a basic unit, the nucleosome, in which DNA is coiled around histone protein complexes. Essentially, accessibility of transcription-regulating molecules to DNA

#### STAR addiction



**Figure 3.** Estimating the basic principle of stress-triggered atavistic reprogramming (STAR) addiction in HNSCC. In normal cells, access to the genetic information is highly restricted by three-dimensional configuration of chromatin termed large organized chromatin K9 modification (LOCKs), which spreads to the genome-widely level. This H3K-9me2-marked heterochromatin (100 kb-5 Mb) expands in accordance with cellular differentiation and restricts totipotent genomic information to minimal repertoire required for the maintenance of specific cellular lineage. Although the mechanism is not known, LOCKs are lost in cancer. Analogous to the opening of Pandora's box, loss of LOCKs presumably liberate access to the forbidden genomic information. Intriguingly, the most frequent genetic abnormalities observed in HNSCC are the loss-of-function mutation of *TP53*, which encodes p53 protein, a strong barrier for epigenetic reprogramming. Thus, it is plausible HNSCCs take full advantage of this accessible and re-programmable DNA status and mainly utilize malignant epigenetic reprogramming as a driving force of cancer evolution. We speculate that HNSCC evolution is highly dependent on (i.e., addicted to) STAR, rather than the genetic mutations in the relatively small number of genes.

is determined by the three dimensional conformation of chromatin, which is regulated by a variety of chromatin and DNA modifying enzymes. In general, the loosened conformation, euchromatin, is open to transcription, while the compressed form, heterochromatin, is closed to transcription. For this basic principles of epigenetic reprogramming, see recent comprehensive reviews [9, 11]. Through a series of elegant studies, the group of Feinberg discovered that in the global genome of differentiated cells, there are large portions of a repressive histone mark, H3K9me2, enriched heterochromatin (100 kb-5 Mb), which they named large organized chromatin K9 modifications (LOCKs) [8]. LOCKs are not common in the ES cell (about 4%), and expand in accordance with cellular differentiation (e.g., 60% in normal liver cells). Furthermore, organ specific differences were observed in the formation pattern of LOCKs. These findings suggest that under normal cell physiology, the formation of LOCKs play a crucial role to restrict ("lock in") the totipotent genomic information to a minimal repertoire of functions. Consequently, the dynamic, flexible, and versatile state of ESC is transformed into the static, regulated, homeostatic states of the specific cell lineage commitment. As expected,

### STAR addiction



**Figure 4.** Epigenetic imbalance between differentiation and pluripotency and self-renewal. Factors related to differentiation are displayed in the blue circles or rectangles, whereas those related to pluripotency and self-renewal, are in red. Circles contain genes or proteins according to their function and rectangles designate non-coding RNA. For the acquisition of pluripotency, the Polycomb Group (PcG) protein, Trithorax Group (TrxG) proteins, and core pluripotency factors play critical roles. In contrast, squamous cell differentiation is regulated by a distinctive set of genes. To fine-tune the balance, non-coding RNAs are essential for exhibiting multifaceted interactions. Differentially methylated regions (DMRs) and bivalent genes in the black triangle also work as important fulcrums of the balance. miR represents micro RNA.

LOCKs are lost in cancer and its adjacent pathologically normal tissues. Although the main cause that leads to the loss of LOCKs in cancer remains to be elucidated, this ESC-like loosened chromatin state in the global genome appears to be a fundamental starting point of the malignant reprogramming of cancer (**Figure 3**). A recent study on HNSCC revealed the correlation between H3K9 demethylase KDMA4 and tumor progression, suggesting the relevance of loss of LOCKs in HNSCC [51]. Nevertheless, the map of LOCKs and its possible oncogenic roles in HNSCC remains to be elucidated.

# Prevalent TP53 mutation to elimination the barriers of epigenetic reprogramming

It is well know that loss-of-function mutations of *TP53* is the most prevalent (>80%) genomic

abnormality observed in HPV-negative HNSCC [5], which occurs at a relatively early phase of HNSCC carcinogenesis, because normal epithelium adjacent to HNSCC frequently harbors this mutation [52]. Through recent intensive studies on iPS cells, it has become apparent that wild type p53 protein functions as a strong barrier to epigenetic reprogramming [18]. Therefore, it is logical that HNSCC is liberated from this epigenetic barrier at a relatively early phase of carcinogenesis and mainly utilizes epigenetic reprogramming as a driving force of evolution rather than gain-of-function mutation of oncogenes (Figure 3). It is worth noting that squamous cell carcinoma of the esophagus and lung display similar mutation patterns: infrequent mutations in oncogenes and frequent mutations in TP53 [53]. These types of cancers may represent epigenetic-driven cancer.

#### Determinants of imbalance between differentiation and pluripotency

Due to the loss of LOCKs and the prevailed TP53 mutation, the genomic information for HNSCC is likely to become highly accessible and re-programmable. To resume the atavistic state, cancer cells are thought to alter the balance of two opposing sets of genes: differentiation and self-renewal and pluripotency in response to the cancer specific micro-environmental cues. Thus, genes required for differentiation and cell lineage commitment are inhibited, whereas those for self-renewal and pluripotency are up-regulated at a genome wide scale. Recently, several key components that regulate these reciprocal expressions have been identified through rapidly expanding information obtained from developmental and regenerative biology [8, 18]. These include Polycomb Group (PcG) proteins, Trithorax Group (TrxG) proteins, core pluripotency transcription factors (TFs), bivalent genes, differentially methylated regions (DMRs), and non-coding RNA (ncRNA). Overall, elucidation of the roles of these factors in cancer evolution, particularly with HNSCC, is in its infancy. In the following section, we will discuss possible roles and interactions of these components in HNSCC evolution and explore future directions of study (Figure 4).

#### PcG

The PcG proteins repress transcription of genes that are essential for cell fate determination [54]. In ESCs, PcG proteins are required for the maintenance of pluripotency, inhibiting the expression of a distinctive set of genes that promote differentiation. There are two major forms of Polycomb Repressive Complexes, PR-C1 and PRC2. PRC2 complex contains EZH2 that catalyzes trimethylation of H3K27 (H3k-27me3), a repressive histone mark, and causes the silencing of the targeted genes. PRC1 stabilizes H3K27me3 repressive chromatin marked by PRC2. In a variety of cancer, PcG proteins are overexpressed and are associated with an aggressive phenotype [54]. The expression levels of EZH2 inversely correlates with the survival of patients in HNSCC tumor samples [55] [56]. Inhibition of EZH2 by RNA interference or the EZH2 inhibitor, 3-dezanepllanocin A, inhibited the growth of HNSCC cell lines in vitro and in xenograft models and recovered the expression of squamous differentiation genes [56, 57]. Up-regulation of EZH2 caused by the loss

of micro RNA (miR)-101, an EZH2 repressor, resulted in an increase of the H3K27me3 and consequent promoter methylation and silencing of the RAP1GAP tumor suppressor gene [58]. The abundant expression of Bmi1, a member of the PRC1 complex, significantly correlates with poor outcomes of patients with oral carcinoma [59, 60]. RNA interference or pharmaceutical inhibition of Bmi1 deprived HNSCC cell lines of CSC-like properties [60]. Bmi1 and Twist cooperatively promote hypoxia-induced EMT in HNSCC through chromatin remodeling [61]. This is a typical example that explains how interactions of EMT transcription factors and epigenetic regulators cause EMT. In general, EMT TFs bind to the enhancer box of epithelial genes (e,g., CDH-1) and recruit suppressive chromatin regulators (e.g., PRCs and G9a) and silence the expression of the targeted genes [46].

#### TrxG

The TrxG, which mediates H3K4me3 active histone marks and gene activation, was originally identified as the counterpart molecule of PcG [18]. Wdr5, a core member of TrxG, plays a crucial role in ESC self-renewal and efficient formation of iPS cells, cooperating with Oct4, Sox2 and Nanog (OSN) [62]. In HNSCC, it was demonstrated that Wdr5 interacted with histone deacetylase 3 and activated mesenchymal gene expression and thereby induced EMT [63]. In a recent study of bladder cancer, it was shown that elevated levels of Wdr5 were associated with poor prognosis of patients and the parallel mechanistic studies in vitro and in vivo demonstrated that Wdr5 significantly promoted the cellular capacity of self-renewal by increasing the expression of several oncogenes including NANOG via tri-methylation of H3K4 [64]. Thus, it is of interest to investigate the roles of Wdr5 in STAR in HNSCC.

#### Core pluripotency transcription factors

The network of core transcription factors plays a fundamental role in the maintenance of pluripotency in ESCs [18]. Those factors include Oct4, Sox2, Nanog, c-Myc, Stat3, and Lin28. They are recognized as cancer reprogramming factors [10] and are often used as CSC markers of HNSCC and other solid tumors [65]. In a study of oral HNSCC, Chiu *et al.* reported that Oct4 and Nanog co-operatively induces stemness and triple-positive (Oct4, Nanog and CD133) tumors demonstrate the worst prognosis [66]. The interaction of the Oct4-Sox2-Nanog complexe and CD44 variant 3 promotes the expression of miR-302 and leads to the stem-like properties in HNSCC [37]. Increased expression of Nanog was observed in CD271positive hypopharyngeal CSCs [38]. Nanog and Stat3 promote miR-21 expression and cause stemness in CD44-standard-form-positive HN-SCCs [37]. Lin28, an RNA-binding protein, is required for the maintenance of pluripotency in ESCs through the inhibition of Let-7 miRNA that promotes cellular differentiation [67]. The Lin-28/Let-7 axis, thus, enhances efficiency of iPS reprogramming [68]. In HNSCC, forced expression of Lin28 promoted cellular proliferation in vitro and in vivo causing enrichment of genes related to cell migration, chromatin remodeling, and stress responses [69]. Increased expression of Lin28 was significantly associated with poor prognosis of patients with oral SCC [70]. The adverse prognostic value of Sox2 expression in HNSCC was confirmed in a recent metaanalysis [71]. In HNSCC cell lines, ectopic expression of Sox2 promotes CSC-like features, whereas genetic knockdown of Sox2 reduces capacities of self-renewal and in vivo tumorigenicity [72]. Among the pluripotency factors mentioned above, only the SOX2 gene is amplified in HNSCC, and this gene amplification is a common phenomenon observed in cancers of squamous cell lineage including the lung, esophagus, and cervix of the uterus, which demonstrate a relatively similar genetic landscape with HNSCC [5, 45, 73]. In addition to its relevance to pluripotency, Sox2 is known to possess interesting functions: the development and maintenance of squamous cell lineage that is retained in CSCs of squamous cell origin [44, 45]. Thus, Sox2 may play a distinctive role in the induction of pluripotency in SCCs. In a recent study, it was shown that in SCCs of the esophagus and lung, Sox2 preferentially binds to p63 and regulates the expressions of a specific set of genes [45]. Intriguingly, p63, a member of the p53 family protein, is known to be associated with the maintenance of normal epithelial stem cells and is frequently overexpressed in SCC [74]. Therefore, as with Sox2, p63 appears to possess dual functions: commitment to squamous cell lineage and maintenance of stmeness. Moreover, the TP63 locus is located on 3q28 proximal to the SOX2 locus

(3q26), and thereby these two genes are frequently co-amplified in the above-mentioned SCCs [5, 45, 74]. Through several recent studies, *NOTCH1*, one of the p63-targeted genes via p53, has been identified as a putative tumor suppressor gene in cancers of squamous cell origin including HNSCC [5, 75]. This is because Notch1, which is required for the normal differentiation of squamous epithelium [74, 76], is frequently silenced in SCCs. Taken together, it appears to be of great importance to elucidate how the interactions of Sox2, p63 and Notch1 regulate the balance between pluripotency and differentiation in HNSCC.

The discovery of super-enhancers (SEs) is a recent topic of interest in both developmental and cancer cell biology. Master transcription factors, including OSN and Mediator, assemble a large (spanning from several to ten thousand bases) enhancer complex called super-enhancers, which strongly drive expression of a small set of select genes that define specific cell identity (e.g., ESC) [77, 78]. SEs and their targeted set of genes have been identified in several types of cancers [78]. A recent study discovered a unique oncogenic function of SEs in colon and breast cancer [79]. SEs of these cancers are enriched with the terminal transcription factors of tumor specific signaling, TCF in colon cancer and ER in breast cancer, causing substantial amplification of the Wnt and estrogen signals, respectively. This finding implies SEs may be a strong transmitter and amplifier of oncogenic signal (input) to the pluripotency genes (output). Given that pharmaceutical inhibition of transcriptional co-activator BRD4 by JQ1, leads to the selective inhibition of the MYC gene that is regulated by SE in multiple myeloma [80], SE may be a promising molecular target for the inhibition of STAR. Thus, the identification of HNSCC-specific SEs appears to be an urgent priority.

#### Bivalency

In ESCs, promoters of key developmental and lineage-specific genes are marked by repressive H3K27me3 (plus PRC) and active H3K4me3 histone marks, simultaneously, which are repressed [81]. This poised condition, referred to as the bivalent state, is essential for regulatory plasticity of ESC by keeping these genes quiescent to maintain pluripotency, but enabling rapid activation through the removal of H3K27me3 mark in response to differentiating cues [11]. It is of note that about 50% of bivalent domains in ESC coincide with binding sites with at least one of the core pluripotency core factors, OSN [81], implying that these OSN factors exert dual functions for the maintenance of ESC states: activation of genes related to pluripotency and self-renewal (e.g., OSN themselves) and inhibition of genes related to developmental regulation [82].

Chapman-Rothe et al. investigated the bivalent state in high-grade ovarian cancer and found that there were sets (580) of bivalent marked genes that were repressed [83]. Among them 215 (37%) were bivalent genes in ESCs, whereas the remaining 365 genes were not. Interestingly the latter set of genes was significantly enriched for PI3K and TGF-beta signaling. These findings indicate that, in cancer, ESC bivalent genes are maintained at a low level, presumably providing cancer cells with stemness features, whereas the mechanism of bivalency itself contributes to tumor evolution due to cancer-specific bivalent genes. In addition, a recent study showed striking evidence that the bivalent promoter of a single specific gene plays a pivotal role in bidirectional conversions of CD44<sup>low</sup> non-CSCs to CD44<sup>high</sup> CSCs in the basal cell type of breast carcinoma [84]. Thus, transcriptional activation of the bivalent ZEB1 promoter by TGF-alpha causes rapid transition of the cellular phenotype from CD44<sup>low</sup> non-CSC to CD44<sup>high</sup> CSC. These findings indicate that there is sub-population of non-CSCs that can readily transit to a CSC state in response to micro-environmental signals, utilizing epigenetic reprogramming. This phenomenon appears to be a good example of STAR and suggests a significant role of bivalency for the acquisition of stemness (Figure 2). Taken together, the status and roles of bivalent genes in HNSCC evolution should be elucidated.

#### DNA methylation signature

DNA methylation is the most intensively investigated epigenetic alteration in cancer. In the normal cell, a majority of methylation occur at repetitive CpG sites (i.e., CpG islands) except for gene promoters [21]. In contrast, the cancer epigenome is characterized by global alterations of two major DNA methylation patterns: CpG island hypermethylation in gene promoters and widespread hypomethylated blocks

(mean 144 kB) in gene bodies and in non-coding repetitive elements such as LINE (longer interspersed nucleotide elements), or Alu sequences [8, 21]. It is well known that the hypermethylated CpG island is associated with the silencing of genes, including tumor suppressors (e.g., CDKN2A). Whereas the role of hypomethylation in gene expression remains controversial, the overall level of gene expression in hypomethylated blocks remains low. However, in some cases, hypomethylation causes the overexpression of oncogenes (e.g., RAS), suggesting that hypomethylated blocks are functionally unstable and dysregulated. It is also known that these lesions are structurally fragile and provide DNA break hotspots that fuel cancer progression [21]. Due to the advancement of whole-genome epigenetic analysis, the range explored in methylation assays has been extended considerably. For example, a new concept of "CpG island shore", the 2-kb region on either side of a CpG island, has been proposed by the Feinberg group [8, 85]. Through the comparison of paired samples (fibroblast vs iPS cells and normal vs cancer tissues). they discovered reprogramming and cancerspecific differentially methylated regions (designated as R-DMRs and C-DMRs, respectively), 70% of which were found in CpG island shores. As 16% of C-DMRs overlaps R-DMRs, it is likely that CpG island shores play fundamental roles in epigenetic reprogramming of both normal and cancer cells. It is of particular interest that the majority of hypomethylated R-DMRs were found at bivalent genes with OSN binding sites, i.e., developmental and cell lineage regulators, as described above.

Frequent promoter methylation and consequent silencing of CDKN2A is observed in HNSCC [52]. Several types of cancers display distinctive profiles a of CpG island methylator phenotype (CIMP) that was first identified in colon cancer [86]. CIMP reflects biological aggressiveness of the individual tumor. In HN-SCC, Shaw et al., first examined the CIMP using ten empirically selected genes and found that their criteria of CIMP correlated with less aggressive tumor phenotypes [87]. Following this random target study, several investigators conducted genome wide high-throughput methylation assays on HNSCC samples. Each investigator found unique CIMP patterns that were associated with parameters of tumor aggressiveness [88-91]. Shaw et al., found a novel

CIMP that was associated with poor recurrence-free survival of patients [88]. The Kelsey group first found that hypermethylation of a distinct subset of genes is significantly associated with LINE-1 hypomethylation, albeit the clinical significance of this finding was obscure [89]. In the following study they demonstrated a hypermethylation profile of 13 CpG loci characterized by PcG targeted genes, mammalian interspersed genes, and transcription factor binding sites that were associated with poor survival of patients [90]. This result is at least partly consistent with the concept of a "DNA-hypermethylation module" in CpG islands proposed by the laboratory of Baylin. In a series of cancers, many promoter-hypermethylated genes are PcG targets in the context of bivalent chromatin in ESCs, and they are enriched by developmental regulators [92]. Jung et al. found an omics profile through the combination of gene expression (transcriptome), DNA methylation (methylome), and miRNA (miRNome) that was a predictor of shorter metastasis-free survival [91]. Teh et al. revealed that oncogenic FOXM1 promoted a cancer-specific methylation signature in HNSCC by modulating DNA helicase and DNA methyltransferase 1 and 3B [93, 94]. In a recent study, a team from John Hopkins and MD Anderson Cancer Center conducted the analysis of "greater promoter" methylation that included CpG island shore and shelf and identified ten key tumor suppressor genes with an emphasis of the PAX gene family [95]. However, there is one puzzling point about these results. These methylation signatures in HNSCC did not overlap each other and were rather mutually exclusive. The reasons for this discrepancy remain elusive. In addition, the role of genomewide DNA methylation such as the C-DMR has to be elucidated with relevance to STAR in HNSCC. The topic of HPV-positive HNSCC is beyond the scope of this review. However, in brief, it is becoming apparent that HPV-positive HNSCC displays a clearly different methylation signature from HPV-negative HNSCCs, which resembles that of cervical cancer, another representative HPV-related tumor [5, 96-98].

#### Non-coding RNA

ncRNA has been termed the "RNA continent" or the "dark matter of the genome", due to the discovery by the FANTOM project that the number of transcribed ncRNA (23, 218) was greater than that of coding RNA (20, 299) [25]. Recent rapid progress in epigenetic studies has shed light on this dark matter and revealed that ncRNAs are not junk or noise but fine-tuners of transcription [99]. Structurally, ncRNA is categorized into long ncRNA (lncRNA, >200 bp) and small ncRNA (<200 bp, e.g., miRNA) [100, 101]. Through interactions with mRNA, DNA and chromatin modulators and each other (e.g., lncRNA-miRNA) [100-102], ncRNAs are likely to orchestrate the stoichiometric equilibrium of gene expression depending on dynamically changing cellular context, thus playing pivotal roles in embryonic development, reprogramming and cancer progressions.

In Figure 4, we schematically summarized proposed interactions of representative ncRNAs with factors that are associated with cellular differentiation, reprogramming, and pluripotency. Briefly, we describe here the functions of these ncRNAs. Of note, the partial oncogenic roles of miR-21, miR-101, miR-302 and Let-7 were already described in studies of HNSCC mentioned before. miR-21 inhibits several tumor suppressor genes (e.g., PTEN) and promotes EMT [21]. miR-302 up-regulates OSN thereby inducing pluripotency [103]. miR-101 negatively regulates the expression of Ezh2. Let-7, a critical inducer of differentiation, downregulates Ezh2 and promotes the expression of differentiating genes [21]. There is a negative feedback loop between Lin28 and Let7 [21]. miR-200 reduce the expression of master EMT regulator, ZEB1, and PcG proteins, Suz and Bmi1 [21]. miR-34 is a target of p53 and represses the expressions of NANOG, SOX2 and N-MYC and serves as a barrier for somatic reprogramming [104]. A IncRNA, HOTAIR, works as a scaffold on PRC2-targeted genes and recruits PRC2 complexes thereby silencing these developmental and cellular lineage commitment genes. The HOTAIR promoter has an OSN binding site. HOTAIR is, therefore, essential for the maintenance of pluripotency in ESC and caner development [105]. miR-34 downregulates HOTAIR [106].

In HNSCC, several investigators have identified tumor specific miRNAs or modules, which were differentially expressed between normal and tumor tissues using microarray- or RT-PCRbased comprehensive assays. These studies emphasized the clinical significance of several miRNAs including miR-21, miR-221, miR-375, miR-106b-25, and miR-451 [107-111]. The

TCGA analysis found frequent Let-7c inactivation (40%) [5]. This is consistent with the results of the above-mentioned two studies [109, 111], suggesting the importance of prevalent Let-7c inactivation in HNSCC oncogenesis. However, as observed with the results of methylation signature, these results overlap only partially. In a mechanistic study with HNSCC cell lines, a tumor suppressive role of miR-34a was demonstrated in relevance to angiogenesis [112]. The clinical significance of HOTAIR was confirmed in three recent studies [113-115]. An interesting finding was demonstrated in a recent study that the increased levels of serum exosomal miR-21 and HOTAIR were useful in distinguishing patients with cancers or with benign polyps in the larynx, suggesting the feasibility of liquid biopsy by exosomal ncRNA [116]. Through the comparison of the paired normal and cancer tissues of larynx, Shen et al. found two differentially expressed IncRNAs, AC026166.2-001 (decreased) and RP11-169D4.1-001 (increased), which were significantly associated with clinicopathologic parameters [117]. Overall, studies of ncRNAs that focused on the acquisition of pluripotency have not yet been conducted in HNSCC.

#### Stumbling blocks for STAR studies

In this session, we have discussed the possible mechanisms of STAR based on the findings obtained in general reprogramming and HN-SCC-specific epigenetic studies. However, there seems to be a large stumbling block to further advance STAR studies in cancer; it is not feasible to exclusively identify and analyze the pluripotent cells, which comprise only a small portion of the heterogeneous bulky tumor. As a result, the critical information relating to the molecular circuitry of pluripotent cells are embedded in and obscured by the results of the remaining bulky tumor cells in the current quantity-oriented assays, which usually compare the paired tissue samples (e.g., normal vs tumor). This may, at least in part, explain the reasons why the some of the major players in normal cell reprogramming (Figure 4) did not show up in the afore-mentioned HNSCC epigenetic studies. The development of sophisticated methods to isolate pluripotent cells from the bulk tumor samples, which are usually stocked as frozen or formalin-fixed and paraffin-embedded tissues. is a challenging but critical mission to gain insight into the molecular background of the STAR phenomenon. In addition, technical and

financial problems associated with genome wide epigenetic study (e.g., chip-sequencing), particularly when handling with tissue samples, appear to be another significant hurdle to overcome.

#### Conclusions

Recent remarkable progress in CSC research indicates that the true therapeutic targets of solid cancer are pluripotent cells, which comprise only a small population of the bulky tumor. In this perspective review, we have explored a possible mechanism that is responsible for the acquisition of pluripotency in the evolutionary trajectory of HNSCC and have postulated that STAR may be the critical regulator of this event. We believe that this perspective review will be of help to fellow investigators eyes with respect to epigenetic reprogramming studies, which appear to have great potential for the development of clinically efficient treatments for HNSCC.

#### Disclosure of conflict of interest

None.

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