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Metal carcinogen exposure induces cancer stem cell-like property through epigenetic reprograming: A novel mechanism of metal carcinogenesis

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Abstract

Arsenic, cadmium, nickel and hexavalent chromium are among the most common environmental pollutants and potent carcinogens. Chronic exposure to these metals causes various types of cancer in humans, representing a significant environmental health issue. Although under active investigation, the mechanisms of metal carcinogenesis have not been clearly defined. One common feature of these metal carcinogens is that they are all able to cause various epigenetic dysregulations, which are believed to play important roles in their carcinogenicity. However, how metal carcinogen-caused epigenetic dysregulation contributes to metal carcinogenesis remains largely unknown. The evolution of cancer stem cell (CSC) theory has opened exciting new avenues for studying the mechanism of metal carcinogenesis. Increasing evidence indicates that chronic metal carcinogen exposure produces CSC-like cells through dysregulated epigenetic mechanisms. This review will first provide some brief introductions about CSC, epigenetics and epigenetic regulation of CSCs; then summarize progresses in recent studies on metal carcinogen-induced CSC-like property through epigenetic reprograming as a novel mechanism of metal carcinogenesis. Some perspectives for future studies in this field are also presented.

Keywords

arsenic; cadmium; nickel; chromium; metal carcinogenesis; cancer stem cell; cancer stem cell-like cell; epigenetics; DNA methylation; histone posttranslational modification; microRNA; non-coding RNA

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8. Conflicts of interest

All authors declare no conflicts of interest.

1. Introduction

Metals are common environmental and occupational pollutants causing a variety of adverse health effects on humans, representing a significant environmental and occupational health issue. Among many metal contaminants that humans are frequently exposed to, arsenic, cadmium, nickel and hexavalent chromium were classified as Group 1 carcinogens to humans by the International Agency for Research on Cancer (IARC) [1-3]. This classification is based on the findings from both human epidemiology studies and laboratory *in vitro* and *in vivo* animal model studies showing that chronic exposure to these metal pollutants cause various cancers in humans and animals. The carcinogenicity is the primary adverse health effect concern of human long term exposure to these metal carcinogens. Although it is under active investigation, the mechanism of metal carcinogenesis has not been clearly defined.

The classic model explaining mechanism of carcinogenesis is the clonal evolution model [4], which proposes that multiple and accumulated genetic changes occurring in somatic cells give the cells survival and proliferation advantage leading to uncontrolled cell growth and eventually development of tumors (Fig. 1A). With increasing evidence showing the important role of epigenetic dysregulation in cancer initiation and progression, it is also proposed that multiple and accumulated epigenetic alterations happening in somatic cells is capable of providing cells survival and proliferation advantage resulting in tumor development (Fig. 1A). While the clonal evolution model lines up well with the observations of numerous mutations in tumors, it does not well explain the distinct feature of heterogeneity inside tumor tissues. Alternatively, a newer model for the mechanism of carcinogenesis is the cancer stem cell (CSC) model (Fig. 1B), which proposes that cancer is initiated by CSCs or CSC-like cells or tumor initiating cells [5,6].

Unlike many other carcinogens, metal carcinogens (arsenic, cadmium and nickel) are usually non-mutagenic or weakly mutagenic and do not cause many mutations or strong genotoxic effects. Instead, accumulating evidence indicates that metal carcinogens are capable of triggering various epigenetic changes, which may play important roles in metal carcinogenesis [7-11]. It is now well-recognized that epigenetic mechanisms play critical roles in producing and maintaining CSCs leading to cancer initiation and progression [12-15]. Therefore, a new trend in the endeavor of dissecting the mechanism of metal carcinogenesis is investigating the capability of metal carcinogen exposure inducing CSCs or CSC-like cells and the underlying mechanism through epigenetic reprograming. This review will first provide some brief introductions about CSC, epigenetics and epigenetic regulation of CSCs, then summarize recent progresses in this exciting area of metal carcinogenesis study.

2. Cancer stem cells

The somatic stem cell concept was originated from findings in the eighteenth century showing that lower organisms are capable of regenerating multiple tissues/organs [5]. The initial clues leading to the development of cancer stem cell (CCS) concept came from the nineteenth century observations revealing the histologic similarities between tumors and

embryonic tissues, which suggests that cancers might be caused by cells with similar characteristics to early embryonic cells [5]. By definition, it is now generally accepted that CSCs refer to a small population of cancer cells possessing characteristics associated with normal stem cells, especially the capability of self-renewal and generation of different types of cells found in a tumor. The CSC concept proposes that cancers are originated from CSCs although it remains to be determined where CSCs come from. It has been postulated that CSCs (i) may come from adult tissue stem cells that are malignantly transformed through genetic mechanism or epigenetic reprograming; (ii) may be converted from the “ordinary” cancer cells; (iii) may come from cells residing in a special compartment termed stem cell or cancer stem cell niche [16,17].

The first evidence demonstrating the tumor initiating capability of CSCs came from human acute myeloid leukemia (AML) studies by Dr. John E. Dick’s group [18,19]. It was reported that human AML originates from a primitive hematopoietic cell termed the SCID leukemia-initiating cell, which is exclusively $CD34^{++}CD38^{-}$ possessing the differentiating and proliferative capacities and the potential for self-renewal [19]. Subsequently, the milestone findings from Drs. Michael F. Clarke and Peter B. Dirks’s groups revealed the existence of tumor initiating cells or CSCs in solid tumors [20,21]. Dr. Clarke’s group first reported that only a very small portion of human breast cancer cells is capable of forming new tumors in immunocompromised mice. Further characterization of this tumor initiating cell population revealed they are $CD44^{+}CD24^{-/low}Lineage^{-}$ cells. While injection of 100 cells with this phenotype produced tumors in mice; in contrast, injection of tens of thousands of breast cancer cells lacking this phenotype were unable to grow tumors. Moreover, tumors from injection of $CD44^{+}CD24^{-/low}Lineage^{-}$ cells contained additional $CD44^{+}CD24^{-/low}Lineage^{-}$ cells and other type of cells and non-tumor initiating tumor cells, demonstrating the self-renewal and differentiating capabilities of CSCs [20]. Similarly, Dr. Dirks’ group identified a $CD133^{+}$ minority of cells from human brain tumor tissues as tumor initiating cells. Injection of 100 $CD133^{+}$ cells was able to grow a tumor phenocopying the patient's original tumor. However, injection of tens of thousands of $CD133^{-}$ cells failed producing tumors in mice [21].

In addition to these pioneer studies, numerous further studies demonstrated the existence of CSCs in many other types of solid tumors as well. Moreover, many studies provided compelling evidence showing that CSCs not only produce the initial primary tumor, they also play critical roles in tumor recurrence, metastasis and resistance to various therapies [22]. It has been proposed that developing novel therapeutic strategies targeting CSCs may hold the hope of curing cancer [23-25].

3. Epigenetics

Epigenetics refers to heritable alterations in the pattern of gene expression that are not caused by changes in DNA sequences, but are mediated by DNA methylation, histone posttranslational modifications (acetylation, methylation, etc.), and non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). The first identified epigenetic dysregulation in human cancer tissues was reported by Drs. Andrew P. Feinberg and Bert Vogelstein in 1983 [26]. The authors found increased hypomethylation in genes of

cancer cells compared with their normal counterparts. At the same time, Dr. Melanie Ehrlich's group determined the over-all 5-methylcytosine (5mC) content of DNA from 103 human tumors consisting of benign tumor, primary malignant tumor and metastatic tumors [27]. It was found that the majority of metastatic tumors displayed much lower global 5mC contents than the benign tumors or normal tissues; and the content of hypomethylated DNA in primary malignant tumors was higher than metastatic tumors but lower than benign tumors [27]. These findings suggest a role of global DNA hypomethylation in tumor malignancy progression. Indeed, it was found that DNA hypomethylation in cancer cells activates the expression of genes that are usually silenced in normal tissues such as oncogene H-Ras [28].

Interestingly, after initial reports showing DNA global hypomethylation in tumor tissues, DNA hypermethylation at specific genomic locus was soon identified. In fact, DNA hypermethylation at specific sites that are usually not methylated in normal tissues was frequently observed in almost all tumor types [29]. Studies showed that DNA hypermethylation usually occurs at a gene promoter CpG islands causing transcriptional repression, silencing gene expression such as tumor suppressor genes, leading to tumor development and progression [12].

Histone posttranslational modifications (PTMs) have a significant impact on chromatin thus affecting gene expression. At least 17 kinds of PTMs on more than 30 amino acid residues of histone nuclear proteins have been identified such as acetylation, methylation, phosphorylation, etc. [30]. In general, histone acetylation is linked with activation of gene expression. However, the effect of histone methylation on gene expression is complex depending on what type of amino acid is methylated and where the amino acid is located in the histone tail. For example, methylation of histone H3 at lysine 4 (H3K4), 36 (H3K36), and 79 (H3K79) are correlated with euchromatin activating gene transcription; whereas methylation of histone H3 at lysine 9 (H3K9) and 27 (H3K27) is associated with heterochromatin repressing gene transcription [31,32]. While DNA hypomethylation was the first common epigenetic mark found to be associated with tumor progression, subsequent studies revealed that a variety of abnormal histone PTMs are linked with tumor development and progression, serving as additional common epigenetic marks in cancer [33,34].

DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs) play major roles in establishing and maintenance of DNA and histone methylation patterns, respectively. Interestingly, abnormal DNA methylation and dysregulated histone PTMs in cancer do not occur alone. Studies showed that the cross-talk exists between the DNA methylation and histone methylation pathways, which is mediated by the interaction between DNMTs and HMTs. For example, certain tumor suppressor gene promoter CpG island hypermethylation is usually associated with gain of repressive H3K9 methylation and H3K27 trimethylation [34-36]. Indeed, studies showed that H3K27 methyltransferase EZH2 (enhancer of zeste homolog 2) and H3K9 dimethylation are able to interact with and recruit DNMT1 or DNMT3b to targeted genomic regions enhancing gene silence [37,38].

The non-coding RNAs (ncRNAs) are RNA transcripts without significant protein-coding capacity, which are generally classified as small ncRNAs (<200 bp in length) and long

ncRNAs (lncRNAs) (>200 bp in length) [39,40]. The ncRNA is a relatively newly recognized epigenetic mechanism regulating gene expression. One group of most extensively studied small ncRNAs is known as microRNAs (miRNAs), which is ~22 nucleotide long and regulates gene expression by imperfect base pairing with 3'-UTRs (3'-untranslated regions) in mRNAs (messenger RNAs), down-regulating protein-coding gene expression post-transcriptionally [41]. Many studies showed that miRNAs play essential roles in almost all biological processes including carcinogenesis [42,43]. While global miRNA and tumor suppressive miRNA expression levels are reduced in cancer cells and tissues; oncogenic miRNA expression levels are often increased in cancer [44]. Based on the reported important roles of miRNAs in tumor initiation, growth, metastasis and resistance to various therapies, miRNAs are explored to serve as biomarkers for cancer diagnosis and prediction of prognosis as therapeutic targets as well [45].

The first lncRNA was identified before the discover of miRNAs, however, the biological importance of lncRNAs is much less studied compared to miRNAs, which is partially due to its lower conserve nature, tissue-specific expression patterns and lower expression abundancy [46-48]. With the advance of sequencing technologies and bioinformatics analytic tools, the lncRNA study is re-gaining attention. While miRNAs down-regulate gene expression at posttranscriptional level, lncRNAs exert their gene expression regulation functions at both transcriptional and posttranscriptional levels and could up-regulate or down-regulate gene expression. Moreover, the mechanism of lncRNA regulation gene expression is much more complex and lncRNAs mainly function as scaffold molecules recruiting additional epigenetic regulators to fine tune gene expression [49]. Increasing evidence demonstrates that aberrant lncRNA expression plays important roles in carcinogenesis and has a significant impact on CSC as well [50].

4. Epigenetic regulation of CSCs

Since the first report showing altered DNA methylation in human cancer in 1983 [26], studies over past 35 years provided compelling evidence demonstrating that epigenetic dysregulations play crucial roles in cancer initiation and progression [12,51]. There are studies showing that epigenetic alterations are capable of largely, if not completely, replacing genomic instability to cause cancer development in the absence of extensive mutations. For example, McKenna ES et al. found that epigenetic dysregulation due to disruption of a chromatin remodeling complex causes cancer without genomic instability [52,53]. Mack SC et al. reported that a very aggressive type of infant ependymoma exhibits a CpG island methylator phenotype but has extremely low mutation rate and zero significant recurrent somatic single nucleotide variants [54]. These findings suggest that cancer may be initiated by non-mutational mechanisms such as epigenetic mechanisms.

Alternatively, Vicente-Dueñas C. and colleagues recently proposed an epigenetic priming model for cancer initiation, emphasizing that "contribution of oncogenes to cancer development is mediated mainly through epigenetic priming of cancer-initiating cells" [55]. This new cancer initiation model argues that "stem or progenitor cells have a specific epigenetic program that renders them more susceptible to genetic alterations"; and "specific

developmental stages with their associated epigenetic states may be more susceptible to oncogenes and progression to cancer" [55].

The methylation patterns of gene promoter CpG islands in differentiated somatic cells are largely different from that of normal stem cells and CSCs; and abnormal DNA methylation events are thought to play important role for cancer cells to gain stemness [25]. A common DNA methylation feature across almost all types of human cancer is the promoter CpG island hypermethylation of many tumor-suppressor genes [56]. Mechanistically, it was proposed that a 'bivalent' promoter chromatin pattern with the recruitment of two additional key histone repressive marks (H3K9me2 and H3K9me3) in stem or progenitor cells may predispose these tumor suppressor genes more vulnerable to DNA hypermethylation causing gene silencing during tumor initiation and progression [57]. Other studies provided compelling evidence showing that increased level of DNMT1 and DNA methylation is essential for producing CSCs and cancer initiation [58,59]. On the other hand, it was also found that deletion of DNMT3A in mouse hematopoietic stem cells (HSCs) predisposes mouse HSCs to malignant transformation developing a spectrum of myeloid and lymphoid malignancies, which show distinct methylation aberrations [60]. Together, these findings demonstrate the importance of abnormal DNA methylation patterns in producing CSC property and cancer initiation.

Similarly, appropriate histone PTM patterns are also important for maintaining cell fate and identity and dysregulated histone PTMs could contribute to cancer initiation and progression. In addition to above mentioned the important role of repressive histone methylations in silencing tumor suppressor genes and cancer initiation, other histone PTMs such as acetylation and deacetylation regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) have also been shown to play critical roles in CSCs and cancer development [13,61,62]). Liu C et al. found that HDAC3 is highly expressed in liver CSCs participating in self-renewal of liver CSCs via regulating expression of pluripotency factors [63]. Lee JS et al. reported that SIRT1, a nicotinamide adenine dinucleotide–dependent HDAC, plays a critical role for oncogenic transformation of neural stem cells and for the survival of "cancer cells with neural stemness" [64]. Indeed, many studies showed HDAC inhibitors could restore dysregulated histone acetylation patterns in cancer cells to a normal state, promoting cancer cell differentiation and apoptosis [65,66]. Various HDAC inhibitors are currently being tested in clinic for cancer treatment in hope of suppressing CSCs when used alone or in combination with other therapies [67].

In addition, many studies also showed that dysregulation of non-coding RNAs (miRNAs and lncRNAs) plays important roles in producing and maintaining CSCs and cancer initiation as well [50,68]. The important role of miRNAs in regulating stem cell property was evidenced by the findings showing that knockout of Dicer 1, a critical enzyme in miRNA biogenesis, causes mouse embryonic lethality with Dicer1-knockout embryos having no stem cells, suggesting a critical role for Dicer and miRNAs in maintaining the stem cell population during mouse early development [69]. Further mechanistic study revealed that many defects in Dicer-deficient embryonic stem (ES) cells could be rescued by transfection with miR-290 family miRNAs, indicating that the miR-290 family plays an important role in maintaining undifferentiated ES cell functions [70]. Similarly, miRNA expression dysregulation was

found to play critical roles in regulating CSC functions and a detailed list of abnormally-expressed miRNAs in a variety of CSCs was included in a recent review [68]. Mechanistically, abnormal miRNA expression leads to aberrant expression, activation or inhibition of key signaling molecules or pathways critical for CSC generation and maintenance, promoting cancer initiation and progression [68]. Similarly, lncRNAs have also been shown to play important roles in CSCs through regulating the expression of classic stemness-related factors or pathways [50]. Moreover, some lncRNAs contribute to CSC generation and maintenance by regulating the expression of CSC-related miRNAs [50].

5. Induction of CSC-like property by metal carcinogens

5.1 Arsenic

Arsenic is a ubiquitously-presented environmental contaminant and human carcinogen affecting millions of people worldwide; and general population arsenic exposure is mainly through arsenic-contaminated drinking water and food. Many human epidemiology and laboratory studies showed that long-term arsenic exposure increases the risk of developing multiple types of cancer such as skin, lung, and bladder cancer [71-76]. Many studies also demonstrated that arsenic does not cause strong genotoxic effects; instead, arsenic exposure causes various epigenetic alterations. It is well-accepted that epigenetic mechanisms may play critical roles in arsenic carcinogenesis. The hypothesis that metal carcinogen exposure induces CSC-like property was first originated, proposed and tested in arsenic carcinogenesis studies [77-83].

The successful establishment of a mouse arsenic transplacental carcinogenesis model by Dr. Michael P. Waalkes's group provided the initial critical clues implying that embryonic stem cells may be the target of arsenic carcinogenesis [77,78,83]. Dr. Robert H. Rice's group first reported that low micromolar concentrations of sodium arsenite (0.1-2.0 μM) treatment is capable of maintaining germinative state in cultured human epidermal cells by slowing down epidermal stem cell exit into differentiation pathways [79,80]. These findings showing that arsenic exposure increases the relative proportion of the epidermal stem cells in culture and successful establishment of mouse arsenic transplacental carcinogenesis model led to the hypothesis that stem cell could be a key target cell population for arsenic carcinogenesis [77-83]. This novel hypothesis was first tested in an animal model by Dr. Michael P. Waalkes's group using a mouse strain (Tg.AC mouse) sensitive to skin carcinogenesis via activation of the v-Ha-ras transgene likely in keratinocyte stem cells (KSC) [82]. The pregnant Tg.AC mice were exposed to arsenic (0–85 ppm arsenite in drinking water) during gestation day 8 to 18; the offspring were given topical 12-O-tetradecanoyl phorbol-13-acetate (TPA) from weaning through adulthood [82]. While arsenic exposure alone had no effect and TPA treatment alone only induced papillomas and some squamous cell carcinomas (SCC); however, arsenic exposure plus TPA treatment increased SCC multiplicity 3-fold more than TPA alone [82]. It was found that v-Ha-ras levels in tumors from arsenic plus TPA were 3-fold higher than that from TPA alone; and the up-regulation of v-Ha-ras level occurred early in arsenic-exposed fetal skin [82]. Moreover, CD34, a marker for both KSCs and skin CSCs, and Rac1, a gene being capable of stimulating KSC self-renewal, were found to be greatly increased in tumors produced by arsenic plus TPA co-

exposure versus TPA treatment alone [82]. These findings strongly suggest that fetal arsenic exposure promotes skin carcinogenesis in association with distorted skin CSC signaling and population dynamics, implying stem cells as a key target of arsenic in the fetal basis of skin cancer in adulthood [82].

Follow-up studies from Waalkes' group using a human prostate stem cell/progenitor line WPE-stem derived from its parental, heterogeneous mature normal prostate epithelial cell line RWPE-1, Tokar EJ et al. found that WPE-stem cells express higher levels of anti-apoptotic genes, stress-related genes, and arsenic adaptation factors but lower levels of pro-apoptotic genes; and WPE-stem cells thus exhibit innate resistance to arsenic-induced cytolethality and apoptosis and hyper-adaptability to chronic arsenite exposure compared with the parental RWPE-1 cells [84]. These findings suggest that malignant transformation of RWPE-1 cells by arsenic involves the selection/enrichment and transformation of stem cells leading to an increase in the number of CSC-like cells [84,85]. Further studies from this same group showed that arsenic exposure (5 μM and 18-weeks for human WPE-stem cells; 0.5 μM and 28-weeks for rat kidney stem/ progenitor cell line RIMM-18) is able to transform epithelial stem/progenitor cells into a CSC-like phenotype, providing evidence showing that arsenic exposure may produce CSCs or CSC-like cells from directly transforming normal stem cell/progenitor cells [86,87]. Alternatively, based on the theory that stem cells reside in certain special niche and that the surrounding microenvironment has a significant impact on stem cell dynamics, studies from this same group also tested the idea whether normal stem cells (NSCs) could be malignantly transformed into CSCs by nearby arsenic-transformed malignant epithelial cells (MECs) without direct physical contact in the absence of arsenic [88]. It was found using a Transwell noncontact co-culture system that noncontact co-culture of MECs and NSCs rapidly (≤ 3 weeks) converts NSCs into CSC-like cells probably through the action of a high level of a tumor microenvironment remodeling factor interleukin-6 (IL-6) secreted by the MECs [88]. These findings have important implications for arsenic in vivo carcinogenicity suggesting that the soluble factors secreted by arsenic-transformed cells may transform nearby NSCs into CSCs or CSC-like cells promoting cancer initiation and progression. These findings also provided an alternative explanation for the observed CSC or CSC-like cell overabundance in arsenic-transformed cells and arsenic exposure-caused tumors [89,90].

Other studies further demonstrated the capability of arsenic exposure inducing CSC-like property and explored the underlying mechanisms, particularly the epigenetic mechanisms. To identify abnormally-expressed genes associated with arsenic-induced malignant transformation of human prostatic epithelial cells (RWPE-1), Benbrahim-Tallaa L et al. performed microarray screening and found that the oncogene K-Ras is highly expressed in arsenic-transformed cells [91]. Time-course analysis showed that increased K-Ras expression occurred before arsenic-induced cell malignant transformation, suggesting that up-regulation of K-Ras expression may play an important role in RWPE-1 cell transformation by arsenic [91]. It was subsequently determined that K-Ras expression is also significantly increased in arsenic-transformed prostate stem cells (As-CSC), suggesting a role of K-Ras in arsenic-induced CSC-like property [92]. Indeed, the important role of K-Ras in maintaining arsenic-transformed cell malignant features and CSC-like property was further demonstrated by the study showing that down-regulation of K-Ras expression in

arsenic-transformed prostate epithelial and stem cells impairs their malignant phenotypes [93]. Mechanistically, no mutations in K-Ras codons 12, 13, and 61 were found and no changes in CpG island methylation in K-Ras promoter region were detected in arsenic-transformed cells [91]. Instead, qRT-PCR profiling analysis of expression levels of 84 human miRNAs revealed that the levels of a number of miRNAs targeting K-Ras are significantly reduced in arsenic-transformed prostate epithelial cells and stem cells [92]. Given the important roles of oncogene K-Ras and tumor suppressive miRNAs in regulating cancer stemness, these findings provide a potential mechanistic explanation for arsenic exposure increasing K-Ras expression and induction of CSC-like property [92].

The expression levels of let-7 family of miRNAs found to be down-regulated in arsenic-transformed human prostate epithelial cells and stem cells was also significantly decreased in arsenic-transformed human keratinocytes [92,94]. Jiang R et al. reported that arsenic exposure (1 μM, 15-weeks) induces CSC-like property and malignant transformation of human keratinocytes [95]. Q-PCR analysis revealed that the expression levels of let-7 family of miRNAs are significantly reduced in arsenic-transformed human keratinocytes (HaCaT) [94]. Overexpressing let-7c, a member of let-7 family, in arsenic-transformed keratinocytes reduced their CSC-like property as evidenced by forming much less suspension spheres [94]. Mechanistically, it was found that arsenic exposure reduces let-7c miRNA expression probably through increasing let-7c promoter DNA methylation as treatment with 5-Aza-2'-deoxycytidine (5-AZA), an inhibitor of DNA methyltransferases, partially reverses the effect of arsenic treatment reducing the level of let-7c [94]. Interestingly, the levels of let-7 family miRNAs were also greatly decreased in arsenic-transformed human prostate epithelial cells and stem cells although the underlying mechanism was not determined [92]. Together, these findings suggest that epigenetic down-regulation of let-7 family of miRNAs may play an important role in maintaining CSC-like property in arsenic-transformed cells. It remains to be determined whether down-regulation of let-7 family miRNAs is critically involved in the process of arsenic exposure producing CSC-like cells.

Epithelial to mesenchymal transition (EMT) is an important developmental process featuring the loss of epithelial cell properties and acquisition of mesenchymal cell characteristics [96]. Numerous cancer biology studies revealed that EMT is critically involved in cancer metastasis [97,98]. Moreover, EMT has been linked to the generation of CSCs or CSC-like cells [99-101], implying that EMT could also play an important role in cancer initiation. Our arsenic carcinogenesis studies provided compelling evidence showing that chronic arsenic exposure induces EMT, which plays a causal role in arsenic-induced cell malignant transformation and tumorigenesis [102]. We found that chronic arsenic exposure (2.5 μM, 16-weeks) causes malignant transformation of p53 knockdown human bronchial epithelial cells ($p53^{low}$HBECs) but not p53 intact HBECs [102]. During the cell transformation process, arsenic-exposed $p53^{low}$HBECs gradually lost their epithelial cell features and acquired mesenchymal-like properties as evidenced by loss of expression of epithelial cell marker gene E-cadherin and epithelial cell morphology and acquisition of expression of mesenchymal cell marker gene vimentin and mesenchymal cell morphology, indicating the occurrence of EMT [102]. Moreover, arsenic-transformed cells were highly migratory and invasive [103-105]. Our mechanistic studies revealed that arsenic exposure down-regulate the expression level of EMT-suppressing miR-200 family and increases the expression level

of EMT-inducing transcription factors ZEB1 and ZEB2 (zinc-finger E-box-binding homeobox factor). Re-expressing miR-200b (a member of miR-200 family) in arsenic-transformed cells is capable of causing mesenchymal to epithelial transition (MET) and abolishing the malignant phenotypes such as soft agar colony formation and nude mouse tumorigenicity. Moreover, stably overexpressing miR-200b in parental $p53^{low}$HBECs was able to completely block arsenic exposure inducing EMT and cell malignant transformation, indicating that down-regulation of miR-200 plays a causal role in arsenic-induced EMT and cell malignant transformation [102]. Further mechanistic studies showed that chronic arsenic exposure down-regulates miR-200 family expression by increasing its promoter DNA methylation and induction of the expression of ZEB1, a negative regulator of miR-200 family [102].

Based on the findings from our cell transformation studies [102-105], we performed animal studies using p53 and ZEB1 heterozygous knockout mice ($p53^{+/-}$, $ZEB1^{+/-}$) to further determine the role of miR-200 down-regulation and ZEB1 up-regulation in arsenic carcinogenesis. Briefly, both $p53^{+/-}$ and $ZEB1^{+/-}$ mice were backcrossed to A/J mouse back ground for 10 generations and crossed with each other to produce p53 and ZEB1 double knockout A/J mice ($p53^{+/-}ZEB1^{+/-}$). Timed pregnant $p53^{+/-}$, $ZEB1^{+/-}$ and $p53^{+/-}ZEB1^{+/-}$ A/J mice were given regular drinking water (Control group) or drinking water containing arsenic (20 ppm, $NaAsO_2$) (Arsenic exposure group) starting from gestation Day 18 (Fig. 2). Maternal mice were allowed to give birth and arsenic exposure group were continuously given arsenic water. After genotyping and weaning at 3-week old of age, male offspring from Control group or Arsenic exposure group were divided into four groups based on their genotypes (wild type, $p53^{+/-}$, $ZEB1^{+/-}$ and $p53^{+/-}ZEB1^{+/-}$). The offspring from Control group maternal mice were given regular drinking water; the offspring from Arsenic exposure group maternal mice were continuously given the same arsenic water; and all mice were sacrificed at 34-week old of age to determine their lung tumor formation (Fig. 2). As shown in Table 1, no lung tumors were found from all mice in Control group given regular drinking water. Similarly, no lung tumors were found from wild type and $ZEB1^{+/-}$ mice in Arsenic exposure group. However, 40% (6 out of 15) of $p53^{+/-}$ mice in arsenic exposure group developed lung adenoma. In contrast, only 6.7% (1 out of 15) of $p53^{+/-}ZEB1^{+/-}$ mice in Arsenic exposure group developed lung adenoma, indicating that down-regulation of EMT-inducing transcription factor ZEB1 significantly reduces arsenic-induced lung tumorigenesis (Table 1). Given the recognized role of EMT in producing CSCs or CSC-like cells, the findings from our arsenic cell transformation and animal studies suggest that miR-200 down-regulation and ZEB1 up-regulation could play a critical role in arsenic lung tumorigenesis by promoting EMT and generating CSC-like cells or tumor initiating cells. Indeed, this statement is supported by the findings from subsequent other studies showing the down-regulation of miR-200 family in arsenic-transformed human urothelial cells [106] and the involvement of EMT in arsenic-induced CSC-like property [95,107].

Dysregulation of miRNA expression was also observed in arsenic-transformed human prostate stem/progenitor WPE-stem cells (As-CSCs) [92,108]. Ngalame N et al. found that several miRNAs were significantly up- or down-regulated in As-CSCs compared to the untransformed WPE-stem cells [92]. The most significantly down-regulated miRNA was miR-143 (4.8-fold), so the authors re-expressed miR-143 in As-CSCs and determined the

effect of miR-143 re-expression on the malignant phenotypes of As-CSCs [108]. It was found that miR-143 re-expression not only reduces As-CSC cell proliferation and apoptotic resistance, also dysregulated the expression of SC/CSC self-renewal genes including NOTCH-1, BMI-1, OCT4 and ABCG2, suggesting a potential role for miR-143 down-regulation in maintaining arsenic-induced CSC-like property or in arsenic-induced malignant transformation of prostate stem cells [108]. These findings along with others showing other miRNA abnormal expression during arsenic cell transformation process suggest that miRNA dysregulation (probably through abnormal promoter DNA methylation) is an important epigenetic mechanism in arsenic-induced CSC-like property and tumorigenesis.

In addition to miRNA dysregulation, other epigenetic mechanisms may also be critically involved in arsenic-induced CSC-like property. For example, Chang Q et al. reported that a continuous 6-month treatment with low dose arsenic (0.25 μM, $NaAsO_2$) is capable of reprogramming immortalized human bronchial epithelial cell (BEAS-2B) to CSC-like cells [109]. Mechanistically, it was found that continuous low dose arsenic exposure causes a sub-lethal stress to the cells, which triggers a sustained activation of JNK resulting in increased expression of an important stem cell factor c-Myc and production of CSC-like cells [109]. Interestingly, studies from this same group also found that arsenic treatment causes Akt-mediated phosphorylation of EZH2, a key epigenetic regulator, through the JNK-STAT3 signaling pathway [110]. Given the important role of JNK pathway in arsenic-induced CSC-like cells [109], it is likely that the epigenetic dysregulations resulting from JNK-STAT3-Akt-mediated EZH2 phosphorylation may also be critically involved in the production of CSC-like cells by arsenic exposure.

5.2 Cadmium

Cadmium is a common environmental pollutant released by various metal industrial sources and general population exposure to cadmium mainly through cadmium-contaminated air, food and water in addition to cigarette smoking [2]. Long term cadmium exposure causes lung cancer in humans; however, the mechanism of cadmium carcinogenesis has not been well understood. Cadmium is not a strong genotoxic carcinogen; non-genotoxic mechanisms such as epigenetic mechanism may play important roles in cadmium carcinogenesis [111-113].

To explore the mechanism of cadmium carcinogenesis by determining whether cadmium exposure induces CSC-like property, Qu W et al. exposed immortalized human pancreatic ductal epithelial (HPDE) cells to low dose cadmium (1 μM, $CdCl_2$) for 29 weeks and suspension culture spheroid formation assay was used to determine CSC-like cell production [114]. It was found that chronic cadmium-exposed HPDE cells express significantly higher levels of molecular markers for pancreatic stem cells or CSCs (CXCR4, OCT4, CD44) and produce 3-fold more suspension spheres than the control cells [114]. These findings provide some initial evidence suggesting that chronic cadmium exposure may produce CSC-like cells, which may play an important role in cadmium carcinogenesis [115].

To determine whether cadmium-transformed cells have a similar effect to that of arsenic-transformed cells converting normal stem cells to CSCs [88], Xu Y et al. performed a similar

non-contact co-culture study [116]. It was found that after 2-week non-contact co-culture with cadmium-transformed prostate epithelial cells, normal prostate stem/progenitor cells displayed cancer cell characteristics and morphological evidence of occurrence of EMT, implying the conversion from normal prostate stem/progenitor cells to CSCs [116]. Interestingly, similar non-contact co-culture with a strong genotoxic carcinogen N-methyl-N-nitrosourea (MNU)–transformed prostate epithelial cells was not able to convert normal prostate stem/progenitor cells to CSCs [116]. The mechanisms explaining the different capabilities of arsenic- and cadmium-transformed cells from that of MNU-transformed cells in converting normal prostate stem/progenitor cells to CSCs are not clear, however, an apparent difference among arsenic, cadmium and MNU is that both arsenic and cadmium are weakly genotoxic and mutagenic while MNU is strongly genotoxic. Whether arsenic and cadmium-caused epigenetic reprograming in arsenic- and cadmium-transformed cells plays a key role in the production of CSCs from nearby normal stem cells remain to be investigated.

Since accumulating evidence supports the idea that non-CSC cancer cells could be converted into CSCs under certain conditions [17,117,118], Ju H et al. recently determined whether cadmium treatment could produce CSCs from the bulk tumor cell population [119]. It was found that treatment of human breast and liver cancer cells with cadmium (0.1-1.0 μM) for 72 hours significantly increases the CSC-like cell population as evidenced by analysis of stem-cell markers [119]. Although no CSC-related functional studies were carried out, these findings still have some important implications suggesting that cadmium exposure may be able to promote cancer progression and recurrence by producing CSC-like cells from non-CSC cancer cells.

A recent animal model study provided evidence suggesting that in utero cadmium exposure is able to cause an expansion in the mammary stem/progenitor cell population as evidenced by increased numbers of mammary sphere-forming cells in the neonatal mammary gland and increases in branching, epithelial cells, and density in the prepubertal mammary gland of offspring from cadmium-exposed maternal rats [120]. The enrichment of stem cell/progenitor cell population may make more stem cells be available as the target of cadmium or other oncogenic hits thus increasing the risk of developing cancer. Since cadmium exposure has been shown to cause lung cancer in both humans and rodents, it will be interesting to see whether cadmium exposure is capable of increasing the number of lung epithelial stem cells or sensitizing other carcinogen-induced lung carcinogenesis.

Although above-summarized *in vitro* and *in vivo* studies provided preliminary evidence suggesting that stem cells/progenitor cells could be cadmium targets and cadmium exposure may be able to produce CSC-like cells, the underlying mechanisms remain largely unknown. Given the fact that cadmium is not a strong genotoxic carcinogen, it is likely that cadmium-caused epigenetic effects may play a significant role in cadmium-induced CSC-like property and carcinogenesis.

5.3 Nickel

Nickel is another non-genotoxic metal carcinogen released into environmental mostly by metal industrials and general population exposure to nickel mainly through inhalation of

nickel-contaminated air or smoking tobacco [3]. Long term exposure to nickel causes lung and nasal cancer, however, the mechanism of nickel carcinogenesis remain largely unknown. It is proposed that epigenetic mechanisms may play important roles in nickel carcinogenesis [112,121,122].

Nickel is the least studied metal carcinogen in terms of the capability of inducing CSC-like property [123]. Wang L et al. recently reported that chronic exposure of BEAS-2B cells to 100 μM of nickel chloride for 12 months induces cell malignant transformation [124]. By using suspension culture sphere formation assay and analyzing common stemness markers such as ALDH1A1, CD44, and Notch1, the authors concluded that the CSC-like cells are accumulated in nickel-transformed BEAS-2B cells. This conclusion was further supported by the strong tumor initiation ability of suspension sphere-forming cells from nickel transformed cells [124]. Mechanistically, it was determined that increased expression of superoxide dismutase 1 (SOD1) plays an important role in maintaining CSC-like property of nickel-transformed cells [124]. Interestingly, a very recent study showed that chronic nickel exposure is capable of inducing EMT and producing highly migratory and invasive cells through epigenetic activation of ZEB1 [125]. EMT was linked to the generation of CSCs; however, it remains to be determined whether nickel exposure-caused epigenetic changes play an important role in chronic nickel exposure-induced CSC-like property.

5.4 Chromium

Chromium (Cr) and its compounds are widely used in the manufacture of many consumer products; as a result, continuous industrial Cr emissions and improper waste disposal plus the presence of Cr in particulate matters from automobile emissions are the main sources of general population Cr exposure [3]. Cr exists in several valence and the most common forms of Cr found in occupational and general environment include Cr(0), Cr(III), and Cr(VI), however, only hexavalent chromium [Cr(VI)] has been recognized as a human carcinogen [126]. Many epidemiological studies have established a link between Cr(VI) exposure and increased risk of cancer and other diseases although the underlying mechanism has not been well understood [127,128].

Unlike other metal carcinogens (arsenic, cadmium and nickel), Cr(VI) is generally considered as a genotoxic carcinogen due to the fact that Cr(VI) undergoes a series of metabolic reductions inside cells to generate various reactive Cr metabolites and reactive oxygen species, which produce a variety of genotoxic effects [126,129,130]. However, studies on human lung tumors resulting from chronic Cr(VI) exposure showed that fewer mutations are found in lung cancers from patients who had been exposed to Cr(VI) than those who had not [131,132]. Instead, epigenetic changes such as increased DNA methylation levels in the promoter regions of several tumor suppressor genes were identified in lung tumors from workers exposed to chromate [133-135]. Laboratory studies in cell culture models provided additional evidence showing that Cr(VI) exposure is capable of causing epigenetic dysregulations as evidenced by increasing DNA methylation [136], altering histone posttranslational modification patterns [137-140], and changing the expression of miRNAs [8]. However, whether Cr(VI) exposure-caused epigenetic changes play important roles in Cr(VI) carcinogenesis remains largely unknown.

Our recent study found that chronic low dose Cr(VI) exposure (0.25 μM of $K_2Cr_2O_7$ for 20 to 40 weeks) induces BEAS-2B and 16-HBE cell malignant transformation and CSC-like property [140]. Moreover, chronic low dose Cr(VI) exposure caused epigenetic changes as evidenced by the significantly increased levels of histone H3 repressive methylation marks H3K9me2 and H3K27me3 in Cr(VI)-transformed cells. Mechanistically, Cr(VI) exposure increased the levels of H3K9me2 and H3K27me3 by up-regulating the expression of histone H3K9 and H3K27 methyltransferases (HMTases). Importantly, increased expression levels of these HMTases were also observed in Cr(VI) exposure-caused human lung cancer tissues [140]. Furthermore, by using shRNA stable knockdown and pharmacological inhibitors targeting these HMTases, it was determined that up-regulation of these HMTases contributes causally to Cr(VI)-induced CSC-like property and cell malignant transformation [140]. Since Cr(VI) is generally considered as a genotoxic carcinogen, we also determined whether Cr(VI)-caused epigenetic changes may affect its genotoxic effects. It was found that knockdown of HMTases significantly reduces a higher level of Cr(VI) treatment-induced DNA damage [140]. Together, the findings from this study suggest that Cr(VI) exposure-caused epigenetic changes play a critical role in Cr(VI)-induced CSC-like property; and one potential mechanism of Cr(VI)-caused epigenetic dysregulations promote CSC-like property is to enhance Cr(VI) genotoxicity through down-regulating the expression of key DNA repair genes.

Other study also revealed the existence of CSC-like cells in Cr(VI)-transformed cells as evidenced by forming suspension culture spheres and forming tumors in nude mice when a small number of these cells were injected [141]. Another study showed that chronic Cr(VI) exposure (1 μM of $Na_2Cr_2O_7 \cdot H_2O$ for 24 weeks) causes BEAS-2B cell malignant transformation and down-regulation of miR-143 [142]. Re-expression of miR-143 in Cr(VI)-transformed cells significantly reduced tumor angiogenesis and tumor growth, suggesting an important role of miR-143 down-regulation in maintaining malignant phenotypes of Cr(VI)-transformed cells [142]. Interestingly, Ngalame N et al. found that the expression level of miR-143 is also significantly decreased in arsenic-transformed prostate stem cells; and re-expressing miR-143 reduced arsenic-transformed cell CSC-like property [92,108]. Other studies reported that miR-143 expression is down-regulated in glioblastoma stem-like cells and breast cancer stem-like cells; and re-expressing miR-143 significantly impaired CSC-like properties of glioblastoma and breast cancer cells [143,144]. Together, these findings suggest that miR-143 down-regulation is critically involved in maintaining CSC-like property. Further studies on the role of miR-143 down-regulation in Cr(VI) exposure-induced CSC-like property is needed.

6. Conclusions and perspectives

Arsenic, cadmium, nickel and hexavalent chromium are common environmental pollutants and potent carcinogens causing multiple types of cancer in humans upon chronic exposure; however, the mechanisms of their carcinogenicity have not been clearly defined after many year active investigations. The evolution of CSC theory has opened exciting new avenues for metal carcinogenesis research. Since the initial reports suggesting that arsenic may target stem cells to induce cancer [78-80], it is now clear that metal carcinogen exposure is able to produce CSC-like cells representing a novel mechanism of metal carcinogenesis.

Mechanistically, epigenetic reprograming via abnormal DNA methylation, dysregulated histone posttranslational modifications or altered miRNA expression resulting from chronic metal carcinogen exposure may play crucial roles in producing CSC-like cells (Fig. 3). Although significant progresses have been made during the past decade, further studies are needed for better defining the mechanism of how metal carcinogen exposure generates CSC-like cells; and translating the knowledge for designing strategies to achieve early diagnosis, effective prevention and improved therapeutic outcomes for metal carcinogen exposure-caused cancer.

First, up to date studies almost exclusively were done in *in vitro* cell culture models. While cell culture models are convenient for identifying and characterizing metal exposure-produced CSC-like cells, they fail to reflect potential important roles of metal exposure on tissue microenvironment. It has been proposed that stem cells may reside in certain special niche and alterations of surrounding microenvironment may activate and transform normal stem cells to CSCs to initiate cancer [16,23]. It is imperative to develop appropriate animal models to study whether metal carcinogen exposure has a significant impact on target tissue microenvironment. Second, the majority of mechanistic studies determined the role of identified mechanisms involved in maintaining metal carcinogen-induced CSC-like phenotypes. Fewer studies attempted to investigate the mechanisms leading to the production of CSC-like cells. For example, while many studies described in parallels that metal carcinogen exposure causes epigenetic dysregulations and CSC-like property; how epigenetic dysregulations lead to production of CSC-like property remains largely unknown. Future studies need to determine how metal carcinogen exposure generates CSC-like cells establishing the causal relationship. Third, the statement of metal carcinogen exposure producing CSC-like cells is currently mostly based on the analysis of certain stem cell markers, cancer cell-like behaviors and the capability forming suspension spheres. Fewer studies have examined their tumorigenicity in nude mice using the limited dilution approach. Future studies need to identify specific markers for separating metal carcinogen-produced CSC-like cells for testing their tumorigenicity and ensure the target cells are indeed CSCs or CSC-like cells. And forth, tumor immunology studies revealed that reduced immunosurveillance plays important roles in cancer initiation and progression [145]. The effect of metal carcinogen exposure on immunosurveillance is unknown, it is critical to determine whether metal carcinogens cause epigenetic dysregulations in immune cells to reduce immunosurveillance promoting tumor initiation and progression.

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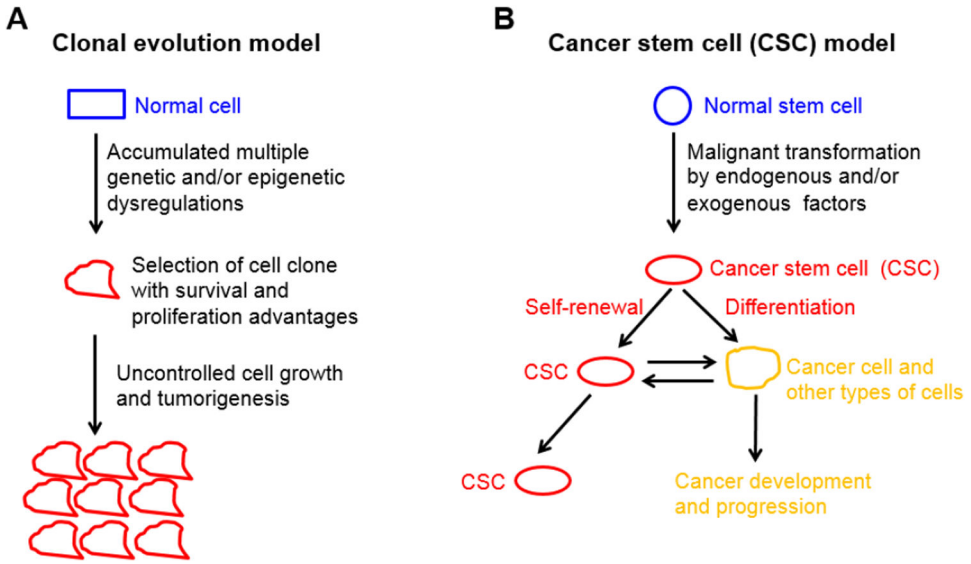

Fig. 1.

Models of carcinogenesis. **A**. Clonal evolution model: accumulated multiple genetic and/or epigenetic hits provide cells with survival and proliferation advantages leading to uncontrolled cell growth and tumorigenesis. **B.** Cancer stem cells (CSC) model: normal stem cells are malignantly transformed by endogenous and/or exogenous factors into CSCs, which differentiate into cancer cells and other types of cells resulting in cancer development and progression.

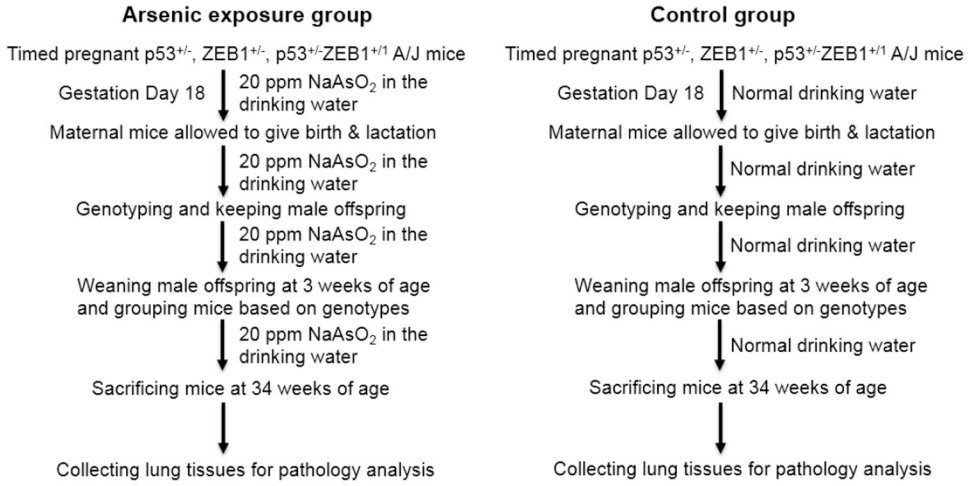

Fig. 2.

Schematic description of arsenic exposure through drinking water from gestation day 18 to 34-weel old of age in wild type, $p53^{+/-}$, $ZEB1^{+/-}$ or $p53^{+/-}ZEB1^{+/-}$ A/J male mice.

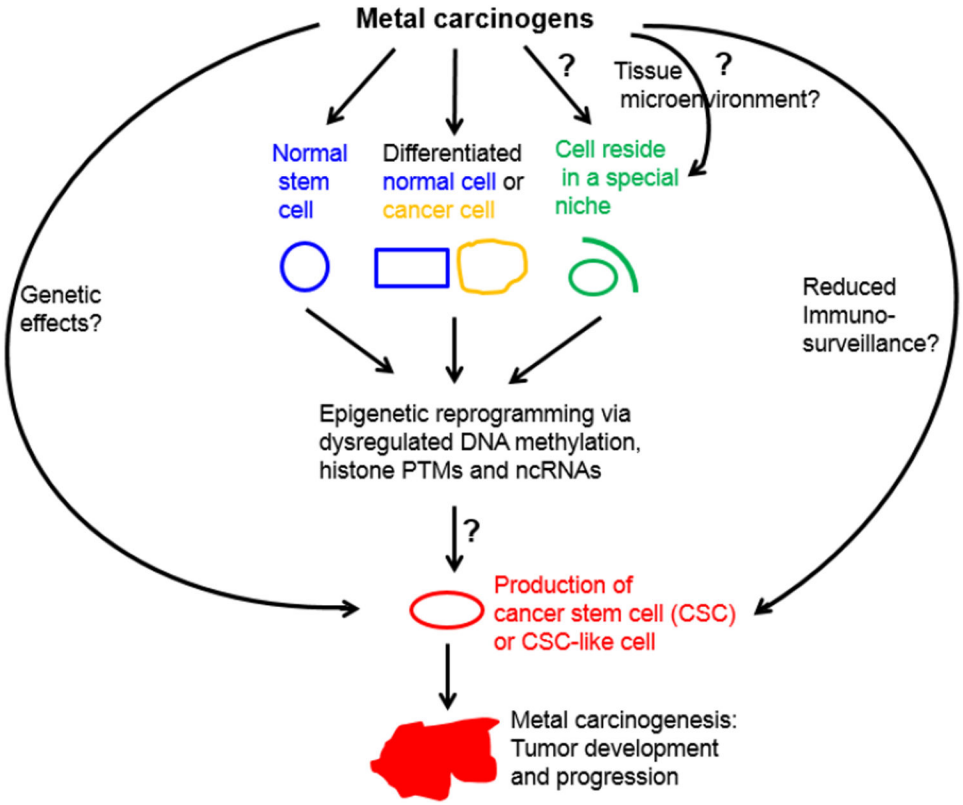

Fig. 3.

Proposed model of metal carcinogen exposure inducing CSC-like property as a novel mechanism of metal carcinogenesis. Metal carcinogens may target normal stem cell, differentiated normal cell or cancer cell to produce CSCs or CSC-like cells promoting carcinogenesis via epigenetic reprogramming. Metal carcinogens may also target a special population of cell reside in a special niche or niche environment to produce CSC/CSC-like cells. It is likely that metal carcinogen-caused genetic effects and immunosurveillance changes may contribute significantly to metal carcinogen-induced CSC-like property.

Table 1.

Down-regulation of ZEB1 significantly reduces arsenic-induced lung tumorigenesis

Mouse genotype & treatment groups	Number of mice per group	Number of mice with lung tumors	Percent of mice with lung tumors
A/J male mice with normal drinking water			
Wild type	15	0	0
$p53^{+/-}$	15	0	0
$ZEB1^{+/-}$	15	0	0
$p53^{+/-}ZEB1^{+/-}$	15	0	0
A/J male mice with 20 ppm arsenic in drinking water			
Wild type	15	0	0
$p53^{+/-}$	15	6	40
$ZEB1^{+/-}$	15	0	0
$p53^{+/-}ZEB1^{+/-}$	15	1	6.7^{*}

Abbreviations: ZEB1: zinc-finger E-box-binding homeobox factor 1.

$^{*}p<0.05$, compared to arsenic-exposed $p53^{+/-}$ mice, analyzed using a two-sided Chi-square test. The schedule of arsenic exposure through drinking water is described in Fig.2.