Epithelial–mesenchymal transition and cancer stemness: the Twist1–Bmi1 connection

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Synopsis
EMT (epithelial–mesenchymal transition), a major mechanism of cancer metastasis, is a process that generates cells with stem-like properties. These stem-like cells in tumours are described as cancer stem cells. The link between EMT and cancer stemness is well documented without detailed mechanistic proof. Bmi1 belongs to the PRC1 (polycomb repressive complex 1) maintaining self-renewal and stemness together with EZH2 (enhancer of zeste homologue 2), which is a component of PRC2. Bmi1 is frequently overexpressed in different types of human cancers. Recent demonstration of an EMT regulator, Twist1, directly regulating the expression of Bmi1 provides a mechanistic explanation of the relationship between EMT and cancer stemness. The functional interdependence between Twist1 and Bmi1 provides a fresh insight into the common mechanism mediating EMT and cancer stemness. This observation is also confirmed using head and neck cancer patient samples. These results provide a critical mechanism of Twist1-induced EMT and cancer stemness in cancer cells through chromatin remodelling. The role of hypoxia and microRNAs in regulating EMT and cancer stemness is also discussed.

Key words: Bmi1, cancer, epithelial–mesenchymal transition (EMT), hypoxia, microRNA, stemness, Twist1

INTRODUCTION

EMT (epithelial–mesenchymal transition) is a process by which epithelial cells are converted into mesenchymal cells through loss of cell polarity, decrease in cell-to-cell adhesion and gain of migration ability [1,2]. EMT is regarded as the critical event during embryonic development, tumour metastasis and organ fibrosis [1–5]. Phenotypic changes of EMT include the repression of epithelial markers [e.g. E-cadherin (endothelial cadherin), plakoglobin and desmoplakin] and up-regulation of mesenchymal markers (e.g. vimentin, fibronectin and N-cadherin) [1–5]. The EMT regulators are transcription factors that include Twist1, Snail (also known as SNAI1), Slug (also known as SNAI2), Zeb1 (also known as TCF8 (T-cell factor 8) and δEF1), SIP1 (also known as Zeb2 and ZFXH1B) and E47 (also known as TCF3) [6–11]. Different EMT regulators were shown to induce EMT through repression of CDH1 (encoding E-cadherin) [6–11]. Although the role of EMT in embryonic development, cancer metastasis and organ fibrosis is well delineated, whether EMT plays a significant role in other aspects of cell biology is largely unknown.

Cancer stemness is a concept recently proposed to describe a small percentage of cells with stem-like properties residing in a tumour [12]. The concept is used to explain cancer cells’ resistance to conventional chemo/radiation therapy [12,13]. Tumour cells with stem-like properties possess a self-renewal ability and are termed CSCs (cancer stem cells) [12,13]. Different assays (e.g. staining of surface markers, in vitro sphere formation, in vivo tumour-initiating ability, etc.) were used to monitor the ‘stemness’ population within a tumour mass [12,14]. However, the molecular mechanisms to generate CSCs remain largely unknown. Several lines of evidence suggest that the process of EMT also generates cells with stem-like properties [15–20]. These results provide a critical connection between the induction of metastasis and the acquisition of cancer stemness in cancer cells undergoing EMT. Here, we review the relationship between EMT and cancer stemness, the regulation of Bmi1 by Twist1 and its significance in cancer stemness, and the role of hypoxia and miRs (microRNAs) in regulating Bmi1 and

Abbreviations used: ARF, alternative reading frame; CSC, cancer stem cell; CDH1, gene encoding E-cadherin; ChIP, chromatin immunoprecipitation; E-cadherin, endothelial cadherin; EMT, epithelial–mesenchymal transition; EMSA, electrophoretic mobility-shift assay; EZH2, enhancer of zeste homologue 2; HDAC, histone deacetylase; HIF-1, hypoxia-inducible factor-1; HNSCC, head and neck squamous cell carcinoma; INK4A, inhibitor of cyclin-dependent kinase 4a; IPS, inducible pluripotent stem cell; MET, mesenchymal–epithelial transition; miR, microRNA; P0, polycomb group; PRC, polycomb repressive complex; TCF, T-cell factor; TGF-β1, transforming growth factor-β1.

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stemness. We hope to provide insight into the process of EMT and cancer stemness mediated by the network of pathways.

EMT AND CANCER STEMNESS

The EMT process in tumour cells usually results in cells becoming more invasive, metastasize to distant organs and become drug-resistant, leading to subsequent demise of cancer patients [1,2]. Tumour progression and aggressiveness induced by EMT is well documented [1–3,5]. However, the mechanisms delineating the connection between EMT and tumour progression are not well defined. Several lines of evidence suggest that the process of EMT generates cells with stem-like properties [15–20], which are usually described as CSCs [12,13]. The CSCs usually represent a small percentage of cells residing in a tumour mass that are treatment-resistant. These CSCs have the ability to self-renew and generate secondary tumours. This property is described as a ‘tumour-initiating ability’ [12–14]. This observation could support the tumour progression model induced by EMT since CSCs may have characteristics different from the original tumour cells or the tumour cells sensitive to chemo/radiation therapy [12–14].

POLYCOMB GROUP PROTEINS, BMI1 AND STEMNESS

PcG (polycomb group) proteins are chromatin modifiers involved in cancer formation and maintaining embryonic and adult stem cells [21,22]. Stem cell chromatin constantly activates proliferation genes and represses differentiation genes [23]. PcG proteins include multimeric transcriptional repressor complexes that play a crucial role in stem cell maintenance and lineage specification [21,22,24]. The multimeric transcriptional repressor complexes include PRC1 (polycomb-repressive complex 1) and PRC2 [21,22,24]. Each complex contains multiple proteins tightly bound together. PcG proteins usually occupy the promoters of developmental regulators, and silencing of these genes in a PcG-dependent manner confers stemness [21,22,24–27].

Bmi1 is a member of PRC1 that is essential in maintaining chromatin silencing [21,28]. Bmi1 was first identified as an oncogene that collaborated with c-Myc to promote lymphomagenesis and regulated cell proliferation and senescence through inhibiting the INK4A (inhibitor of cyclin-dependent kinase 4a) locus [29,30]. Bmi1 was subsequently shown to be required for maintaining normal and leukaemic haematopoietic stem cells [31–33]. Bmi1 was later shown to be involved in the self-renewal of neuronal, mammary epithelium, pancreatic (including β-cell) and intestinal cells through repressing the INK4A/ARF (alternative reading frame) locus [34–41]. Bmi1 is also essential in the lineage specification and multipotency of haematopoietic stem and progenitor cells [42]. In a mouse glioma model, Bmi1 controls tumour development in an INK4A/ARF-independent manner [43]. Repression of INK4A/ARF by Bmi1 depends on PRC2 [44]. EZH2 (enhancer of zeste homologue 2), a member of PRC2 with histone H3 methyltransferase activity, methylates Lys27 of histone H3 (H3K27) after PRC2 binds to the promoters of target genes [45,46]. PRC1 then recognizes trimethylated H3K27 (H3K27me3) to maintain the repression of target genes together with PRC2 [47]. Repression of the INK4A/ARF locus is essential for PRC complexes to maintain stemness [48,49].

REGULATION OF BMI1 BY TWIST1 IS REQUIRED FOR BOTH EMT AND CANCER STEMNESS: FUNCTIONAL INTERDEPENDENCE BETWEEN TWIST1 AND BMI1

Twist1, a bHLH (basic helix–loop–helix) transcription factor, was first recognized for its role in the mesoderm development in Drosophila [50]. Twist1 is a master regulator of gastrulation, mesoderm differentiation and somatic muscles patterning and specification, governing cell movement and tissue reorganization during early embryogenesis [51,52]. Recent evidence demonstrated the important role of Twist1 in cancer metastasis as shown by induction of EMT by Twist1, overexpression of Twist1 in human cancers and the association of Twist1 with a more aggressive phenotype and a worse outcome [8,53]. Different signalling pathways were shown to regulate the expression of Twist1 [3]. We previously demonstrated direct regulation of Twist1 by HIF-1 (hypoxia-inducible factor-1) promotes metastasis [54]. In addition, a previous study identified a subpopulation of cells in HNSCC (head and neck squamous cell carcinoma) with stem-like properties, which was highly tumorigenic and expressed Bmi1 [55]. Owing to the critical role of hypoxia in maintaining self-renewal [56], we hypothesized that the EMT regulators activated by hypoxia could induce the expressions of stemness genes, resulting in promotion of EMT and tumour-initiating ability. Through screening possible activation of various stemness genes by different EMT regulators (Twist1, Snai1 and Slug), we observed a tight correlation between Twist1 and Bmi1. We subsequently demonstrated the direct activation of Bmi1 expression by Twist1 using transient transfection, EMSA (electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation) assays. Overexpression of HIF-1α, Twist1 or Bmi1 confers stem-like properties and induces EMT in head and neck cancer cell lines. Bmi1 is critical for Twist1-induced stem-like properties and EMT because siRNA-mediated knockdown of Bmi1 in Twist1-overexpressing cells abolishes stem-like properties and reverses EMT. In addition, Twist1 is also critical for Bmi1-induced stem-like properties and EMT because siRNA (small interfering RNA)-mediated knockdown of Twist1 in Bmi1-overexpressing cells abolishes both EMT and stem-like properties. The functional interdependence of Twist1 and Bmi1 to mediate
EMT and Cancer Stemness

**Figure 1** A model of functional interdependence between EMT regulators and PRC1–PRC2 complexes to co-operatively repress the expression of CDH1 and p16INK4A genes, leading to EMT and cancer stemness phenotypes

A link between EMT and cancer stemness is explained by this model. Stem-like properties and EMT was further demonstrated by qChIP (quantitative ChIP) assays to test the binding of these two proteins on both CDH1 and p16INK4A promoters when either Bmi1 or Twist1 is knocked down. Direct repression of CDH1 by Twist1 has not been shown previously [2]. Our results map three E-box sites located in the CDH1 promoter responsible for Twist1-induced repression. Mutation of the E-box-binding sites in the CDH1 promoter followed by transient transfection with Twist1 and/or Bmi1 expression vectors in reporter gene assays show full repression of the CDH1 promoter requires the presence of both Twist1 and Bmi1. Co-occupancy of the CDH1 promoter by Twist1 and Bmi1 is shown using EMSA followed by supershifting with either the anti-Twist1- or anti-Bmi1-specific antibody. The essential role of EZH2 has also been demonstrated using the assays mentioned above, which is consistent with the previous report [57]. Interaction between Twist1 and Bmi1 was shown using co-immunoprecipitation assays. Our own results present the first molecular demonstration that Twist1 (an EMT regulator) and Bmi1/EZH2 (components of the PcG proteins) are required simultaneously to repress both CDH1 and p16INK4A expression, providing one of the first mechanistic explanations of the link between EMT and cancer stemness [58]. A model is shown to depict this link (Figure 1). To confirm this observation derived from cell line experiments, HNSCC patient samples were used. The prognostic impact of Twist1 and Bmi1 has been demonstrated in different cancers [3,54,59–62], but their interdependence has never been explored. The co-operative role between Twist1 and Bmi1 in HNSCC is delineated since only co-overexpression of both proteins correlates with repression of CDH1 and p16INK4A and the worst prognosis of HNSCC patients. Patients expressing either Twist1 or Bmi1 alone have a better prognosis than those co-expressing both proteins. This observation further strengthens our discovery that Twist1 and Bmi1 interdependently promote EMT and cancer stemness, resulting in aggressive tumour behaviour and a poor outcome in HNSCC [58].

**Figure 2** A network of induction of EMT and cancer stemness by hypoxia

The pathways activated by HIF-1α or HIF-2α are outlined to depict the possible interconnection.

**HYPOXIA, miR AND THEIR RELATIONSHIP WITH THE TWIST1–BMI1 AXIS**

Tumour hypoxia is linked to tumour aggressiveness and correlates with worse survival for cancer patients [63–66]. HIF-1 directly or indirectly regulates the expression of different EMT regulators [1,3]. Accumulating evidence also suggests the important role of hypoxia in the proliferation and maintenance of stem cells and supports a possible link between dedifferentiated CSCs and the mesenchymal-like cells generated by hypoxia [67–71]. Our results show knockdown of either Twist1 or Bmi1 reverses EMT and attenuates stem-like properties induced by hypoxia [58]. These results provide a crucial link between hypoxia-induced EMT and cancer stemness. Previous evidence demonstrated HIF-1α activates the Notch pathway [72], inducing both EMT and stemness [73,74]. In addition, HIF-2α also activated the expression of Oct-4, a factor contributing to stemness [75]. Our results demonstrate that HIF-1α promotes EMT and stemness in cancer cells through the Twist1–Bmi1 axis [58]. A model depicting the interrelationship between these pathways is shown in Figure 2. Whether there is cross-talk between these pathways remains to be explored. Recent results from the iPSC (inducible pluripotent stem cell) experiments showed that Oct-4 (together with Sox2) represses Snail expression and induces MET (mesenchymal-to-epithelial transition) in the process of iPSC formation [76]. Other signalling pathways such as repression of TGF-β1 (transforming growth factor-β1) and TGF-β receptor 2 by c-Myc, induction of E-cadherin by Klf4 (Krüppel-like factor 4), and BMP (bone morphogenetic protein)-dependent induction of miR-205 and miR-200 family...
CONCLUSIONS AND PERSPECTIVES

Emerging evidence highlights the role of chromatin modification (e.g. promoter hypermethylation) in CDH1 repression [90]. Chromatin modifiers such as HDAC1 (histone deacetylase 1)/HDAC2, AJUBA/PRMT5 (protein arginine methyltransferase 5) or PRC2 were used by Snail to repress CDH1 expression [91–93]. Although p16INK4A is well documented to be regulated by Bmi1, our results further define the role of an EMT regulator (Twist1 in the HNSCC system) in the repression of p16INK4A. It is quite possible transcription regulator would act with chromatin modification complexes (PRC1 and PRC2 in the HNSCC system) to induce gene repression and cause EMT and cancer stemness.

Bmi1 acts as a critical fail-safe system to maintain cancer stemness through counteracting premature senescence induced by INK4A/ARF-dependent pathways [24,28,30]. Twist1 and Twist2 could also override oncogene-induced premature senescence in cancer cells by abrogating the activity of p16INK4A and p21CIP1 [94]. Together with our results, Bmi1 participates in multiple aspects of Twist1-mediated functions in cancer cells, including EMT induction and escape from fail-safe programmes induced by oncogenes. Bmi1 could also maintain stemness through the induction of telomerase activity and inhibition of TGF-β signalling [95,96]. Recent result also showed Bmi1 regulates EMT through repressing PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumour suppressor gene [97]. Whether these pathways cross-talk with each other will require further experimental proof.

In conclusion, the observation of the relationship between EMT and cancer stemness is well documented. The demonstration of the Twist1–Bmi1 axis further provides a mechanistic explanation of the link between EMT and cancer stemness. Although the Twist1–Bmi1 axis may not be the only pathway, the regulation of Bmi1 or other stemness genes through different mechanisms require further exploration and should be the subject of immediate attention. The functional interdependence between EMT regulators and PRC1–PRC2 complexes to mediate EMT and cancer stemness certainly provide a fresh insight into the molecular mechanism. Other EMT regulators should be tested as to whether they also co-operate with PRC1–PRC2 complexes to induce EMT and cancer stemness. The observation obtained from cell line experiments should be extrapolated into the analysis of patient samples as shown in the Twist–Bmi1 result [58]. The information obtained from patient sample analysis will be valuable for the prognostic prediction and treatment of metastatic cancers.

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