Histol Histopathol (2015) 30: 1283-1294

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

A twist tale of cancer metastasis and tumor angiogenesis

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Summary. Twist1 is an evolutionally conserved transcription factor. Originally identified in Drosophila as a key regulator for mesoderm development, it was later implicated in many human diseases, including Saethre-Chotzen syndrome and cancer. Twist1's involvement in cancer has been well recognized. Driven by hypoxia-induced factor-1 (HIF-1), Twist1 has been considered as a proto-oncogene and its overexpression has been observed in a wide variety of human cancers. High expression level of Twist1 is closely related to tumor aggressiveness and metastatic potential. In cancer cells, Twist1 has been shown to function as a key regulator of epithelial-mesenchymal transition (EMT), a critical process for metastasis initiation. Twist1 has also been implicated in maintaining cancer stemness for self-renewal and chemoresistance. This review first summarizes the roles of Twist1 in embryo development and Saethre-Chotzen syndrome followed by a discussion of Twist1's critical functions in cancer. In particular, the review focuses on the recent discovery of Twist1's capability to promote endothelial transdifferentiation of cancer cells beyond EMT.

Key words: Twist1, EMT, cancer stem cell, angiogenesis

Introduction

Originally identified in *Drosophila*, *Twist1* was one of the essential zygotic genes involved in early mesoderm development and dorsal-ventral patterning in the embryo (Simpson, 1983; Thisse et al., 1987; Furlong et al., 2001; Castanon and Baylies, 2002). Its critical roles in mesodermal development have been well illustrated by genetic studies in the 1980s. The name "*Twist*" was based on the observations that, in the absence of the *Twist1* gene, *Drosophila* embryos failed to produced mesoderm for normal gastrulation and eventually died at the end of embryogenesis with a 'twisted' phenotype (Simpson, 1983). The *Twist1* gene was later shown to encode a transcription factor containing a basic helix-loop-helix (bHLH) domain (Thisse et al., 1988; Castanon and Baylies, 2002). bHLH transcription factors represent an important protein family involved in the regulation of organogenesis (Murre et al., 1989; Jan and Jan, 1993). Twist1 exercises its developmental functions by governing cell movement and tissue reorganization, and has a master regulator role for blastula gastrulation, mesoderm differentiation, and somatic muscle patterning and specification during early embryogenesis (Furlong et al., 2001).

As an essential transcription factor in embryonic development, Twist1 is evolutionally conserved from invertebrates to human (Gitelman, 2007). Mutations in the *Twist1* gene have been known to cause Saethre-Chotzen syndrome in human (SCS, or Acrocephalo-syndactyly type III) (el Ghouzzi et al., 1997; Howard et al., 1997; Krebs et al., 1997). SCS is a rare autosomal dominant inheritance disorder (prevalence of 1 in 25,000 to 50,000 people) and individuals with SCS typically show facial asymmetry, ptosis, small ears and craniosynostoses (premature closure of the cranial sutures) (Reardon and Winter, 1994). Experiments in mice with heterozygous *Twist1* ablation (*Twist1*$^{+/-}$) demonstrated craniofacial and structural abnormalities similar to those in SCS patients (Chen and Behringer, 1995; Bourgeois et al., 1998; Soo et al., 2002; Bialek et al., 2004).

In addition to its essential roles in the development of multiple organs and systems, a number of recent

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DOI: 10.14670/HH-11-638

studies have indicated the involvement of Twist1 in cancer aggressiveness and metastasis (Yang et al., 2004; Kwok et al., 2005; Puisieux et al., 2006). Twist1 has been identified as a proto-oncogene (Maestro et al., 1999), and its over-expression has been observed in many types of human cancer, including breast cancer (Yang et al., 2004), prostate cancer (Kwok et al., 2005; Yuen et al., 2007b), hepatocellular carcinoma (Lee et al., 2006; Yang et al., 2009b), pancreatic cancer (Satoh et al., 2008), esophageal squamous cell carcinoma (Yuen et al., 2007a; Sasaki et al., 2009), bladder cancer (Zhang et al., 2007; Wallerand et al., 2010), gastric cancer (Luo et al., 2008; Feng et al., 2009), and head and neck cancer (Yang et al., 2008, 2010; Wu et al., 2011). Twist1 plays several critical roles in cancer initiation, progression and metastasis. In particular, it has been shown that Twist1 overrides oncogene-induced cell senescence and apoptosis (Maestro et al., 1999; Valsesia-Wittmann et al., 2004; Ansieau et al., 2008), promotes epithelial-mesenchymal transition (EMT) (Yang et al., 2004; Firulli and Conway, 2008; Fu et al., 2011), increases resistance to chemotherapy (Cheng et al., 2007; Li et al., 2009), enhances cancer stemness (cancer stem cell, CSC) (Mani et al., 2008; Vesuna et al., 2009; Battula et al., 2010), and facilitates cancer cell invasion and metastasis (Yang et al., 2004; Puisieux et al., 2006; Fu et al., 2011).

This review will first discuss the roles of Twist1 in human conditions such as organogenesis and its involvement in human SCS. We then focus specifically on Twist1's roles in tumor biology. In particular, we discuss the intertwined oncogenic functions of Twist1 in two categories: 1. Promoting epithelial-mesenchymal transition (EMT) and cancer cell stemness during tumor initiation, and 2. Supporting tumor progression and angiogenesis by inducing endothelial differentiation of tumor cells.

Twist1's transcription factor functions and its involvement in human SCS

Twist1 is a transcription factor that contains a structurally conserved bHLH domain (Thisse et al., 1987). Human Twist1 has two nuclear localization signal (NLS) sequences, 37RKRR40 and 73KRGKK77. Nevertheless, Twist1 without functional NLS can still translocate into the nucleus via its partner's NLS in the heterodimer (Singh and Gramolini, 2009). The N-terminus of Twist1 is known to interact with p300, cAMP-response element binding protein (CREB), CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF). In addition, Twist1 inhibits the acetyltransferase activities of these histone remodeling enzymes (Hamamori et al., 1999). The C-terminus of Twist1 is known to interact with the DNA-binding domain of Runx2, a necessary transcription factor for osteoblast differentiation, in order to inhibit its function (Bialek et al., 2004). During skeletogenesis, Twist1 transiently suppresses Runx2's function, and relief of such inhibition is required for further development (Bialek et al., 2004). Thus, Twist1 not only directly regulates its target genes, but also indirectly regulates other gene expression through specific interaction with other transcriptional regulators.

The fact that a single point mutation in the bHLH domain can result in SCS emphasizes the domain's functional importance (el Ghouzzi et al., 1997; Howard et al., 1997). Like other bHLH proteins in the family, Twist1's bHLH domain comprises of a stretch of basic amino acids followed by an α-helix (I), an inter-helical loop and another α-helix (II) (Murre et al., 1989; Jan and Jan, 1993). The basic region, 109Q–T121, of human Twist1 protein is mostly responsible for DNA binding. The Twist1 with an R118C mutation in this region showed lower Twist1 DNA-binding capability, and resulted in SCS (el Ghouzzi et al., 1997). To exert its transcription function, Twist1 first forms a homo- or heterodimer with itself or another bHLH partner protein respectively via interaction between their bHLH domains (Castanon et al., 2001). The dimer then binds to a hexanucleotide DNA sequence (CATATG) known as the Nde1 E-box. Many genes essential for organogenesis contain E-boxes as regulatory elements (Jan and Jan, 1993).

During organogenesis, Twist1 is believed to exert its regulatory functions in part by regulating the FGF, BMP and to some extent the TGFβ signaling (Connerney et al., 2006). Different from most other bHLH proteins, Twist1 can distinctly regulate the expression of different target genes by forming functional homodimers (T/T) or heterodimers with ubiquitously expressed E proteins (T/E) (Castanon and Baylies, 2002). In addition to forming heterodimers with the E protein E12 or E47 for DNA binding, Twist1 has been shown to have an inhibitory role by interacting with MyoD, another key bHLH transcription factor that controls muscle differentiation (Hamamori et al., 1997). The Twist1/MyoD heterodimer results in inhibition of MEF2 transcription factor, and thus suppresses muscle differentiation (Spicer et al., 1996; Hamamori et al., 1997).

Since the T/T homodimers and T/E heterodimers distinctively regulate different gene sets, Twist1's binding partner is also tightly controlled. For example, it is known that, during cranial suture formation, the relative levels between T/T and T/E dimer can be regulated by the Id proteins, a unique class of helix-loop-helix proteins lacking the basic domain for DNA binding (Connerney et al., 2006). Id proteins compete with Twist1 for binding with the E proteins and thus prevent them from forming functional heterodimers with Twist1 (Massari and Murre, 2000). In other words, Id proteins directly control the balance between the T/T homodimer and T/E heterodimer. Id proteins promote formation of the T/T homodimers. In the absence of Id proteins, Twist1 prefers to form the T/E heterodimenr with E proteins (Connerney et al., 2006).

During cranial suture formation in normal mice,

Twist1 is expressed both in the osteogenic front and in the mid-suture, whereas Id1 is only expressed in the osteogenic front. With the presence of both Twist1 and Id1, the osteogenic front prefers Twist1 homodimer formation which induces the expression of the *FGFR2* gene for osteogenesis. In contrast, in the mid-suture mesenchyme, the Twist1 protein preferably forms heterodimers with the E proteins. The T/E heterodimer then induces expression of thrombospondin-1 (TSP-1), a protein known to inhibit osteoblast differentiation (Canfield et al., 1996). Thus, as the *Twist1*$^{+/-}$ mouse has lower Twist1 presence in the cranial suture, Twist1 mostly functions as a homodimer that results in an expanded pattern of T/T-regulated gene expression promoting osteogenesis (Connerney et al., 2008).

In addition, Twist1 and Id1 can regulate bone morphogenetic protein (BMP) signaling, a pathway predominantly active in the osteogenic fronts of cranial sutures. In particular, the Twist1 T/E dimers have recently been shown to inhibit BMP signaling (Hayashi et al., 2007). In the *Twist1*$^{+/-}$ mouse, the cranial suture has a higher level of the T/T homodimer than the T/E heterodimer, resulting in an expansion of BMP signaling. Interestingly, the BMP signaling is known to induce Id expression (Rice et al., 2000), which would further promote the T/T homodimer formation and thus intensify the BMP signaling. This positive feedback loop is likely responsible for the premature closing of the cranial sutures (craniosynostosis) in patients with SCS.

Twist1's oncogenic roles in EMT and cancer cell stemness

The involvement of Twist1 in human cancer has been well established by the observation that many types of human cancer overexpress Twist1 (Yang et al., 2004, 2006). Increased Twist1 expression is closely associated with a more aggressive disease and a poorer prognosis for the patient (Yang et al., 2004, 2006). The underlying molecular mechanism by which Twist1 exerts its oncogenic functions was further demonstrated by the fact that *Twist1* expression is directly triggered by hypoxia inducible factor-1 (HIF-1), a critical transcription factor involved in many aspects of cancer biology (Yang and Wu, 2008; Yang et al., 2008). In addition, the transactivation function of Twist1 has been implicated in epithelial-mesenchymal transition (EMT), an important process by which epithelial cells are converted to mesenchymal cells during embryonic development (Yang and Weinberg, 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009). In addition, EMT is believed to be a critical part of metastasis initiation since most human cancers are carcinomas of epithelial origins (80-90%). During EMT, the cell loses epithelial characteristics such as apical-basal polarity and cell-to-cell adhesion, but gains migratory mobility that is characteristically more related to a mesenchymal phenotype (Yang and Weinberg, 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009). As a result, EMT plays a role in tumor malignancy, invasiveness and metastasis (Yang and Weinberg, 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009). The role of EMT in organ fibrosis was also established (Yang and Weinberg, 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009).

At the molecular level, the epithelial markers decrease (e.g., E-cadherin, plakoglobin and desmoplakin), while the mesenchymal markers increase (e.g., vimentin, fibronectin and N-cadherin) on the cell surface during the EMT process (Yang and Weinberg, 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009). Several transcription factors have been identified as the "EMT regulators" for their ability to regulate EMT. These proteins include: Snail (also known as SNAI1), Slug (also known as SNAI2), Zeb1 (also known as TCF8 and δEF1), SIP1 (also known as Zeb2 and ZFXH1B), E47 (also known as TCF3), and Twist1 (Cano et al., 2000; Grooteclaes and Frisch, 2000; Comijn et al., 2001; Perez-Moreno et al., 2001; Hajra et al., 2002; Yang et al., 2004). Despite the importance of EMT in many biological aspects, including embryonic development, cancer metastasis and organ fibrosis; its underlying mechanisms for these important biological phenomena still remain to be explored.

Cancer stem cell (CSC) is a relatively new concept proposed to explain the functional heterogeneity of cancer cells and their difference in proliferation and differentiation capabilities (Reya et al., 2001; Gupta et al., 2009). Tumor cells with stem-like properties (or "stemness") possess self-renewal ability for tumor-initiation (Reya et al., 2001; Gupta et al., 2009; O'Brien et al., 2010). In tumor, tissue hypoxia plays a critical role in maintaining cancer cell self-renewal (Keith and Simon, 2007). The CSCs usually represent a small percentage of cells residing in a tumor mass (Reya et al., 2001). Nevertheless, since CSCs have the abilities for self-renewal and differentiating into secondary tumors, it is believed that CSCs are responsible for cancer cells' resistance to conventional chemo/radiation therapy (Reya et al., 2001; Gupta et al., 2009). In laboratory, several methods have been developed to monitor the 'stemness' population within a tumor mass, including surface marker staining, *in vitro* sphere formation, and *in vivo* tumor-initiating ability assay (Reya et al., 2001; O'Brien et al., 2010). Of note, the concept of CSC bears a strong resemblance to early embryo development and organogenesis which are known to be mediated by the EMT.

Recent experimental evidence suggests that the EMT process indeed contributes to cancer cell's stem-like properties (Mani et al., 2008; Morel et al., 2008; Hollier et al., 2009; Creighton et al., 2010; Fuxe et al., 2010; Kong et al., 2010). This observation provides the missing links between metastasis and cancer stemness in tumors undergoing EMT. For example, in head and neck squamous cell carcinoma (HNSCC), a subpopulation of highly tumorigenic cells has been identified with stem-like properties. This subpopulation of cells also overexpressed the polycomb complex protein Bmi1

(Prince et al., 2007), a key regulator necessary for self-renewing cell division of adult hematopoietic and neural stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2005). Bmi1 is a member of the polycomb group (PcG) proteins (Park et al., 2004). Functioning as chromatin modifiers, PcG proteins are involved in maintaining embryonic and adult stem cells and are known to promote cancer formation as well (Valk-Lingbeek et al., 2004; Gil et al., 2005; Buszczak and Spradling, 2006; Sparmann and van Lohuizen, 2006; Rajasekhar and Begemann, 2007; Pietersen et al., 2008; Konuma et al., 2010). Polycomb group proteins are multimeric transcriptional repressor complexes capable of silencing regulator genes to preserve stemness during development. Two main polycomb-repressive complexes, PRC1 and PRC2, have been characterized (Valk-Lingbeek et al., 2004; Gil et al., 2005; Buszczak and Spradling, 2006; Sparmann and van Lohuizen, 2006; Rajasekhar and Begemann, 2007; Pietersen et al., 2008; Konuma et al., 2010).

Bmi1 was first shown as an oncogene that collaborates with c-Myc to promote lymphomagenesis. Bmi1 is a core component of the PRC1 (Park et al., 2004). Bmi1 regulates cell proliferation and senescence by inhibiting the *INK4A* locus (Jacobs et al., 1999a,b). Bmi1 was also shown to be essential for maintaining both normal and leukemic hematopoietic stem cells. In that function, Bmi1 regulates the lineage specification and multipotency of hematopoietic stem cells (Lessard and Sauvageau, 2003; Park et al., 2003; Iwama et al., 2004). More importantly, Bmi1 can repress the *INK4A-ARF* locus to maintain the self-renewal properties of many cell types, including mammary epithelium, neuronal, pancreatic (including β-cell), and intestinal cells (Molofsky et al., 2003; Bruggeman et al., 2005; Ashton et al., 2007; Pietersen et al., 2008; Sangiorgi and Capecchi, 2008, 2009; Dhawan et al., 2009; Fasano et al., 2009).

Interestingly, repression of *INK4A-ARF* by Bmi1 is dependent on the polycomb repressive complex 2 (PRC2) (Pereira et al., 2010). In particular, such repression requires the enhancer of zeste homologue 2 protein (EZH2) in the PRC2. EZH2 is one of the components of the PRC2 complex, and EZH2 has histone H3 methyltransferase activity. After the EZH2-containing PRC2 binds to the promoter of target gene, EZH2 methylates the lysine 27 of histone H3 (H3K27) in the chromatin (Czermin et al., 2002; Muller et al., 2002). The trimethylated H3K27 (H3K27me3) is then recognized by the PRC1complex (Min et al., 2003). In this mechanism, both Bmi1-containing PRC1 and EZH2-containing PRC2 bind to the same promoter region of the target gene to maintain its repression. In order to achieve and maintain cell stemness, it is essential for both PRC complexes to bind and repress the *INK4A/ARF* locus (Gil and Peters, 2006; Bracken et al., 2007).

Since cancer stemness is maintained by hypoxia-inducible factors (HIFs), the HIF-inducible Twist1 may be directly involved in regulating expression of stemness genes, promoting synergetic EMT, and self-renewal capability (Keith and Simon, 2007; Yang and Wu, 2008; Yang et al., 2008). In a process of screening the stemness gene expression profiles that were triggered by various EMT regulators (e.g. Twist1, Snai1 and Slug), a tight correlation was established between Twist1 and Bmi1 (Yang et al., 2010). Other biochemical data, such as EMSA (electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation), showed that Twist1 directly binds to the Bmi1 promoter and activates Bmi1 expression in transiently transfected cells (Yang et al., 2010). A Co-immunoprecipitation assay also showed the direct interaction between Twist1 and Bmi1. In addition, head and neck cancer cells overexpressing HIF-1α, Twist1 or *Bmi1* demonstrated stem-like properties and EMT biomarkers (Yang et al., 2010). The link between Twist1 and Bmi1 for cancer EMT and stemness was further validated using siRNA-mediated knockdowns (Yang et al., 2010). Knocking down *Bmi1* expression in the Twist1-overexpressing cells resulted in reversion of EMT and abolishing stem-like properties. Likewise, siRNA-mediated knockdown of Twist1 in Bmi1-overexpressing cells prevented EMT and negated any stem-like properties. These results indicate that Twist1 and Bmi1 are mutually dependent in order to convey EMT and stemness to cancer cells (Yang et al., 2010).

Their interdependency for target gene regulation was tested using the qChIP (quantitative ChIP) assays on the promoters of *E-cadherin* and *p16INK4A*, whose gene expressions are known to be repressed during EMT (Yang et al., 2010). By performing a mutagenesis analysis, three E-box binding sites were located in the *E-cadherin* promoter region responsible for Twist1-induced repression (Yang et al., 2010). Using a luciferase gene reporter system, it was determined that full repression of the *E-cadherin* promoter requires the presence of both Twist1 and Bmi1 (Yang et al., 2010). Further, the co-occupancy of Twist1 and Bmi1 in the *E-cadherin* promoter was demonstrated by an electrophoretic mobility shift assay (EMSA), and later confirmed by supershifting with the anti-Twist1 or anti-Bmi1-specific antibody (Yang et al., 2010). Using a similar qChIP assay, the essential role of EZH2 in repressing E-cadherin expression was also established (Yang et al., 2010), a finding consistent with a previous report (Cao et al., 2008).

These data demonstrate simultaneous repression of E-cadherin and *p16INK4A* expression by Twist1 (an EMT regulator) and Bmi1/EZH2 (components of the polycomb group proteins). More importantly, these data provide the mechanistic link between the EMT and cancer stemness (Yang et al., 2010). Systematic profiling of several head and neck cancer cells' transcriptomes revealed that cells overexpressing either Twist1 or Bmi1 had their transcriptomes drifting toward the mesenchymal stem cell signatures and away from the epithelial signatures (Yang et al., 2010), a finding consistent with the notion that cancer stem cells tend to

display mesenchymal characteristics (Creighton et al., 2009). In addition to HNSCC, the involvement of Bmi1 in cancer stemness is further validated by the finding that Bmi1 is critical in maintaining prostate cancer stem cells (Lukacs et al., 2010).

These pre-clinical observations also have clinical relevance. By testing the expression levels of these genes in the HNSCC patient biopsy samples, Twist1 and Bmi1 have been used as prognostic tools for different human cancers (Prince et al., 2007; Cao et al., 2008; Yang et al., 2008, 2010), albeit their interdependence has never been fully explored. Consistent with the *in vitro* mechanistic findings on tumor cell lines, HNSCC patients with single-expression of either Twist1 or Bmi1 have a better prognosis, while those with double-expression have the worst prognosis. These clinical observations in HNSCC patients strongly support the notion that the Twist1-Bmi1 axis enhances cancer aggressiveness and metastasis by 1. Repressing tumor suppressor genes such as *E-cadherin* and *p16INK4A* (Yang et al., 2010), 2. Promoting EMT and cancer stemness (Yang and Wu, 2008). It remains to be explored if the Twist1-Bmi1 axis also contributes to the tumorigenesis process in other types of human cancer.

Tumor angiogenesis: Twist1 and the Notch signaling pathway

Beside Twist1's critical roles in embryogenesis, metastasis initiation, and cancer stemness, recent evidence indicated that Twist1's oncogenic functions may extend beyond EMT and cancer stemness. Twist1 has been shown to directly regulate de novo vasculature formation in tumor (Wu et al., 2011). Blood vessels are formed via different mechanisms during embryo development and in adults. During embryo development, de novo formation of vasculature is achieved through vasculogenesis, a process that derives endothelial cells from mesoderm cell precursors (Risau and Flamme, 1995). In adults, formation of new vasculature relies on angiogenesis, a process involving formation of new vessels from adjacent preexisting host vasculature.

Tumor angiogenesis is a hallmark of cancer. In fact, it is a critical feature for a tumor to survive, grow and eventual metastasize (Hanahan and Weinberg, 2011). Unlike angiogenesis in normal tissues, it is generally believed that the newly formed vascular endothelium in tumor can be derived from a variety of cellular origins. The endothelial cells that constitute tumor neovasculature can be generated from recruiting proliferating endothelial cells (ECs) from the nearby pre-existing vessels. Several signaling molecules have been identified for their capability to turn on the 'angiogenic switch' of these cells (Le Bras et al., 2010; Weis and Cheresh, 2011). In addition, much evidence suggests that the adult bone marrow (BM) is a source of cells that contributes significantly to postnatal angiogenesis (Carmeliet and Jain, 2000; Kopp et al., 2006). Several types of BM-derived hematopoietic cells have been identified to contribute to tumor angiogenesis, including tumor-associated macrophages (TAMs) (Lin et al., 2001; Pollard, 2004), Tie2-expressing monocytes (TEMs) (De Palma et al., 2005), VEGF receptor 1 (VEGFR1)-positive myeloid progenitors (Lyden et al., 2001), recruited BM-derived circulating cells (Grunewald et al., 2006), PDGFR$^+$ pericyte progenitors (Song et al., 2005), vascular leukocytes (Conejo-Garcia et al., 2005), and infiltrating neutrophils (Nozawa et al., 2006). In spite of these cells' significance in tumor angiogenesis, it still largely remains unknown about the precise contribution of each individual lineage.

Recent evidence also indicates that the cancer cells themselves can be an alternative cell source for generating neovasculatural endothelial cells. For example, endothelial stem-like cells with cancer-specific genomic alterations have been identified in different tumor types (Streubel et al., 2004; Pezzolo et al., 2007). Also, transdifferentiation of glioblastoma stem-like cells into endothelial cells has been demonstrated (Ricci-Vitiani et al., 2010; Wang et al., 2010; Soda et al., 2011). In this case, the cancer would require a prior epithelial-mesenchymal transition (EMT) before further differentiation into endothelial cells, and Twist1 may be an essential player in this process. Oncogenic overexpression of Twist1 in cancer cells may convey stemness to the cells (via the Bmi1-EZH2 axis) for subsequent differentiation into cells with endothelial properties for neovasculature formation. In order to support the development of tumor-derived endothelial cells, the master EMT regulator Twist1 protein should collaborate with other master regulatory signaling pathways involved in vasculature development.

Twist1 has been shown to directly activate Jagged1 expression for Notch signaling (Yen et al., 2010). Jagged1 is one of the key Notch ligands fundamental for cardiovascular development. In mice, Jagged1 deficiency is embryonic lethal and results in severe vascular defects (Xue et al., 1999). In humans, Jagged1 mutations have been linked to congenital heart defects in patients with Alagille syndrome (AGS) (High and Epstein, 2008). Since Jagged1 is expressed in the vasculature and many other tissues as well, AGS is a pleiotropic disorder with involvement of heart, liver, skeleton, eyes, and facial structures. In addition, Jagged1/Notch signaling also has an important role in tumor angiogenesis. In the head and neck squamous cell carcinoma cells (HNSCC), upregulation of Jagged1 expression has a positive influence on tumor angiogenesis, and its expression levels correlate with the levels of vasculature in tumors (Zeng et al., 2005). These results suggest that contact-dependent Notch ligand signals provided by tumor cells may be important in endothelial cell differentiation.

As a master transcriptional regulator for mesoderm development, Twist1 is capable of inducing EMT and promoting cancer metastasis (Furlong et al., 2001; Yang and Wu, 2008; Yang et al., 2008; Thiery et al., 2009). Twist1 expression is also upregulated in many types of

human cancer. In addition, Twist1 has been shown to establish a cross talk with the Notch signaling though direct activation of the Notch ligand Jagged1 (Yen et al., 2010). The Notch signaling pathway not only regulates vascular morphogenesis but also communicates with existing endothelial cells to promote tumor angiogenesis (Zeng et al., 2005; Dufraine et al., 2008; Bridges et al., 2011). More importantly, Our recent results indicate that the Twist1-Jagged1/Notch signaling promotes transdifferentiation of cancer cells into endothelium and contributes to de novo tumor angiogenesis (Chen et al., 2014). For example, the Twist1-Jagged1/Notch signaling activates expression of KLF4. KLF4 is a core component of the pluripotency transcription network that has been used to reprogram somatic cells into induced pluripotent stem cells (Yamanaka and Blau, 2010). KLF4 has been implicated in vasculogenesis during embryo development as a regulator of the endothelial barrier (Yet et al., 1998; Suzuki et al., 2005). Recent studies also demonstrated that these pluripotency transcription factors regulate endoderm specification (Teo et al., 2011). Nevertheless, the precise roles of KLF4 in the induction of endothelial differentiation remain to be explored.

Twist1 collaborates with Jagged1/Notch-KLF4 for tumor transdifferentiation into endothlium

Recently, much experimental evidence supported the roles of Twist1 in promoting tumor endothelial transdifferentiation (Chen et al., 2014). It has been shown that Twist1 is capable of inducing endothelial differentiation of tumor cells beyond EMT (Chen et al., 2014). In head and neck cancer cells, Twist1 overexpression triggered the expression of endothelial surface marker CD31 and CD144 on the cell surface, and interestingly, co-expression of both markers correlated with the highest Twist1 expression status in these cells (Chen et al., 2014). Further investigations indicated that the induced expression of CD31 and CD144 was not linked to chemokine receptor 4 (CXCR4), a known trigger for CD31 and CD41 expression (Chen et al., 2014). Other endothelial gene markers were upregulated by Twist1 in these HNSCC cells, including von Willebrand factor (vWF), TEK tyrosine kinase (TIE2), CD105, intercellular adhesion molecule 1 (ICAM1), ephrin B2 (EFNB2), and neuropilin 1 (NRP1) (Chen et al., 2014). In addition to promoting endothelial expression characteristics, Twist1 conveys endothelial functionalities to cancer cells, as demonstrated by increased tube formation, and the uptake of DiI-AcLDL fluorescent lipoprotein in Twist1-overexpressing HNSCC cells (Chen et al., 2014). The endothelial characteristics correlated with Twist1's expression levels in cells, since knocking down Twist1 expression repressed expression of endothelial marker genes and reduced vascular functions in culture and in tumor xenografts as well (Chen et al., 2014).

Histological evidence also pointed to the fact that Twist1 is capable of inducing tumor endothelial transdifferentiation (Chen et al., 2014). In tumor xenografts induced by HNSCC cells expressing Twist1 and the green fluorescence protein (GFP), fluorescent microscopic imaging showed that Twist1 contributed to the formation of tumor vessels double positive of GFP and CD144, indicating the tumor origin of these endothelial-like cells. Furthermore, tumors with Twist1 overexpression demonstrated higher hemoglobin levels compared with control tumors. These findings also have clinical relevance, since the majority of HNSCC cancer patients with Twist1-overexpressing (91.6%) showed tumor-derived vasculature, characterized by cells double positive of $Twist1^+/CD31^+$ or $Twist1^+/CD144^+$ (Chen et al., 2014).

Jagged is a Notch ligand known for its involvement in tumor angiogenesis (Dufraine et al., 2008; Bridges et al., 2011). The direct connection between Twist1 and Jagged1 in HNSCC was first made during a microarray approach to identify putative Twist1 downstream targets (Chen et al., 2014). It has been shown that Twist1 positively influences Jagged1 expression and its downstream targets, such as Hes1, Hey1 and Hey2 (Dufraine et al., 2008), and knocking down Twist1 reduced their expression levels (Chen et al., 2014). Twist1 regulates Jagged1 activity through direct binding to its promoter, as demonstrated by chromatin immunoprecipitation (ChIP) assays (Chen et al., 2014). Jagged1 promoter contains a Twist1-binding element responsible for direct interaction and activation (Chen et al., 2014). More importantly, Jagged1 functions as Twist1's downstream regulator for the expression of several endothelial marker genes, including CD31, CD144, vWF, CD105, ICAM1, EFNB2, NRP1 and EPHB4 (Chen et al., 2014). Knocking down Jagged1 abolished Twist1-induced expression of these endothelial marker genes and thus reduced their tube-formation ability, DiI-AcLDL uptake in culture (Chen et al., 2014). Consistent with the *in vitro* observations, Jagged1 knockdown also decreased the hemoglobin content and the $GFP^+/CD144^+$ and $CD31^+/Twist1^+$ double-positive populations in tumor xenografts (Chen et al., 2014). These *in vitro* and *in vivo* findings underline the essential role of Jagged1 in Twist1-mediated endothelial transition.

Transdifferentiation of tumor stem-like cells into endothelial cells may involve stemness-related transcription factors (Ricci-Vitiani et al., 2010; Wang et al., 2010; Soda et al., 2011). In particular, pluripotency factors (such as OCT4, SOX2, NANOG, KLF4, GFI1, WNT and BMI1) (Yamanaka and Blau, 2010) have been implicated in regulating endoderm specification (Teo et al., 2011). KLF4 is identified as the downstream factor of Twist1-Jagged1 activation. Knocking down Jagged1 reduced KLF4 expression in Twist1-overexpressing HNSCC cells (Chen et al., 2014). Functional activation of KLF4 by Jagged1 was later confirmed with a KLF4 promoter-driven reporter construct containing either a wild-type or a mutated Notch intracellular domain

(NICD)/CSL (CBF1, Suppressor of Hairless, Lag-1)-responsive site (Chen et al., 2014). NICD/CSL directly binds to the KLF4 promoter as demonstrated by ChIP analyses, and more importantly, such interaction was abolished in the absence of functional Jagged1 (Chen et al., 2014). Consistent with the laboratory findings, Immunohistochemistry (IHC) analyses on clinical HNSCC patient specimens also indicate KLF4's involvement in the Twist1-Jagged signaling pathway, and there was a significant correlation between Twist1 and Jagged1/KLF4 expressions as well as between Jagged1 and KLF4 expressions (Chen et al., 2014). Together, these results identified the transcription factor KLF4 as the downstream contributor, responsible for expression of various endothelial and vascular marker genes as a result of activation of the Twist1-Jagged1/Notch axis.

Functional differences of Twist1-mediated Bmi1 and KLF4 activation

As previously discussed, Twist1 has two main arms of functions, one is for maintaining EMT/stemness via the polycomb Bmi1 regulator (Yang et al., 2010), and the other is for cancer-endothelial transdifferentiation via the pluripotency factor KLF4 (Chen et al., 2014). Bmi1's contribution to Twist1-induced endothelial differentiation was tested and directly compared with KLF4's. Several observations have been made using the KLF4-, Bmi1- or KLF4/Bmi1-knockdown clones of Twist1-overexpressing cells (Chen et al., 2014). For example, flow cytometry and immunofluorescence staining revealed that either KLF4 or Bmi1 single knockdown decreased the $CD31^+/CD144^+$ double–positive population in Twist1-overexpressing OECM1 cells (Chen et al., 2014). Twist1-mediated enhancement in tube-formation ability was also abolished by either single knockdown. Endothelial functionalities, such as DiI-AcLDL uptake and hemoglobin levels in Twist1-overexpressing xenografts, were also negated in tumors with either single knockdown. Tissue sections of tumor xenograft showed the abrogation of co-expression of Twist1 and CD31 in these cells with either single knockdown. These data indicate Bmi1 may contribute some levels of endothelial contribution in Twist1-overexpressing cells (Chen et al., 2014).

Nevertheless, these two proteins do have functional differences. For example, elevated expression levels of various endothelial markers in response to Twist1-overexpression, including vWF, ICAM1, CD105, EFNB2, NRP1 and EPHB4, were only abolished in the KLF4 knockdown but not in the Bmi1 knockdown (Chen et al., 2014). qChIP assays using an anti-KLF4 antibody showed that KLF4 bound to the proximal promoters of CD31, CD144, vWF, CD105, ICAM1, EFNB2 and EPHB4 genes, and KLF4 is responsible for activating their expression in response to Twist1/Jagged1 (Chen et al., 2014). In contrast, parallel qChIP experiments using an anti-Bmi1 antibody showed that Bmi1 only bound to the promoters of CD31 and CD144 genes, and the promoter regions of CD31 and CD144 genes for Bmi1 binding were different from the regions bound by KLF4. These results indicate that Bmi1 alone is sufficient for activating various vascular markers, a process exclusively regulated by KLF4 (Chen et al., 2014).

Since both Bmi1 and KLF4 are shown to be capable of promoting cell stemness (Yamanaka and Blau, 2010; Yang et al., 2010; Lu et al., 2013), KLF4's contribution to Twist1-induced EMT (Wu et al., 2012) and stem cell-like property was also tested and compared directly with Bmi1's. Interestingly, knockdown of KLF4 was not able to reverse the EMT marker gene expressions induced by Twist1, whereas knockdown of Bmi1 did (Chen et al., 2014). Nevertheless, KLF4 knockdown still decreased the *in vitro* migration and invasion activity induced by Twist1, and thus suggests that KLF4 regulates metastatic activity independent of the EMT marker gene expressions. Similar findings were obtained in *in vivo*

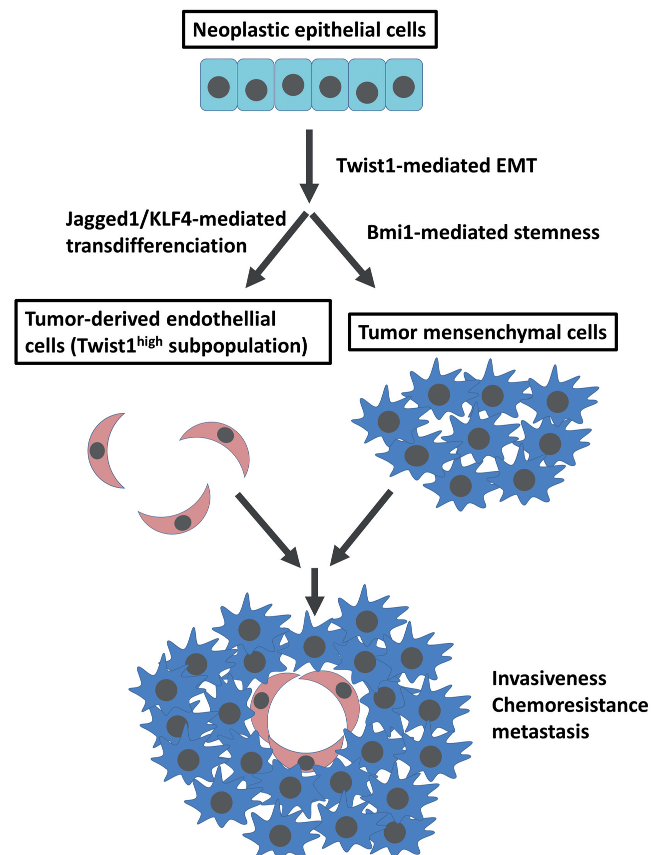

Fig. 1. A model to summarize the two crucial signaling pathways (Bmi1 and Jagged1-KLF4 axises) triggered by Twist1 to promote EMT, cancer stemness, and endothelial transdifferentiation to facilitate the metastatic process.

metastasis assays (Chen et al., 2014).

KLF4's contribution to Twist1-induced stemness was also determined in comparison with Bmi1 (Chen et al., 2014). KLF4 knockdown did not abolish the percentage of CD44 positive population induced by Twist1, whereas knockdown of Bmi1 completely negated the CD44 positive population induced by Twist1. Of note, either single knockdown of KLF4 or Bmi1 indeed negatively affected the size and/or numbers of tumor sphere formation as well as *in vivo* tumor-initiating ability, suggesting that both Bmi1 and KLF4 may contribute to tumor-initiating ability induced by Twist1 (Chen et al., 2014). In light of this observation, gene expression analysis revealed that KLF4 was responsible for the expression of *Wnt5A* and *CCND2* stemness genes that may be responsible for the observed KLF4 contribution to the tumor-initiating ability (Lee et al., 2009; Yang et al., 2009a; Yeh et al., 2011). In that capacity, KLF4 directly binds to the promoter regions of *Wnt5A* gene, as determined by a qChIP assay (Chen et al., 2014). These findings suggest that KLF4 is not as critical as Bmi1 in terms of promoting EMT and cell stemness in the presence of active Twist1 (Chen et al., 2014). It remains to be explored if these two proteins regulate other aspects of EMT/cell stemness using different mechanisms.

Conclusion

Twist1 is originally identified as a master regulator of mesoderm development in *Drosophila* (Simpson, 1983; Thisse et al., 1987; Furlong et al., 2001; Castanon and Baylies, 2002). Since its discovery, we have expanded our knowledge of this fascinating transcription regulator through understanding its critical roles in several human developmental and pathological conditions, including Saethre-Chotzen syndrome (el Ghouzzi et al., 1997; Howard et al., 1997; Krebs et al., 1997), cancer EMT (Yang and Weinberg, 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009), stemness (Yang et al., 2008) and endothelial transdifferentiation (Chen et al., 2014). As an important gene expression regulator, Twist1's expression levels have profound biological effects. In human, mutations in Twist1 cause developmental defects and Saethre-Chotzen syndrome (el Ghouzzi et al., 1997; Howard et al., 1997; Krebs et al., 1997), while Twist1 overexpression in cancer is associated with the disease's aggressiveness and metastasis (Yang et al., 2004, 2006). For cancer biology, we discussed the recent findings and molecular mechanisms in which Twist1 experts it oncogenic functions. Twist1 activation provides cancer cells several advantages. At the individual cellular level, Twist1-mediated EMT enhances cancer cell's ability to move and invade. The Twist1-imediated stemness and stem-cell like property give cancer cells the capability of self-renewal and, more importantly, resistance to conventional anti-cancer chemotherapy. At the multicellular tumor level, Twist1 can extend EMT one step further, to the epithelial-mesenchymal- endothelial transition (EMET) (Yang and Wu, 2008; Yang et al., 2008, 2010; Chen et al., 2014). In this concept, Twist1-overexpressing tumor cells can transdifferentiate into endothelial cells and directly contribute to the angiogenesis process (Chen et al., 2014). This model is in drastic contrast to the traditional angiogenesis process typically contributed by endothelial progenitors in adjacent pre-existing vessels or by bone marrow-derived progenitor cells in the circulation. This induction of endothelial differentiation may promote not only tumor progression but also cancer metastasis, since knockdown of either Jagged1 or KLF4 significantly decreases the *in vitro* or *in vivo* metastatic activity induced by Twist1. In summary, a model (Fig. 1) emerges for Twist1's critical role in tumor biology: 1. As the tumor grows, HIF-1α triggers the activation of Twist1 for EMT (Yang and Wu, 2008; Yang et al., 2008). 2. The Twist1-Bmi1 axis provides survival advantages such as stem-cell like properties (Yang et al., 2010). 3. A small population of stem-like tumor cells with high Twist1 expression can go through the Twist1-Jagged1/Notch-KLF4 axis for *de novo* vessel formation (Chen et al., 2014). Our understanding on this unique aspect of tumor biology could shed light on developing an effective anti-cancer therapy for Twist1 positive tumors.

Acknowledgements. This work was supported in part to K.J.W. by Ministry of Science and Technology Summit grant [MOST 103-2745-B-039-001-ASP]; National Science Council Frontier grant [NSC102-2321-B-010-001]; center of excellence for cancer research at Taipei Veterans General Hospital [MOHW104-TDU-B-211-124-001]; and National Health Research Institutes [NHRI-EX104-10230SI].

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Acceped June 18, 2015