The emerging role of Twist proteins in hematopoietic cells and hematological malignancies

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Twist1 and Twist2 (Twist1–2) are two transcription factors, members of the basic helix-loop-helix family, that have been well established as master transcriptional regulators of embryogenesis and developmental programs of mesenchymal cell lineages. Their role in oncogenesis in epithelium-derived cancer and in epithelial-to-mesenchymal transition has also been thoroughly characterized. Recently, emerging evidence also suggests a key role for Twist1–2 in the function and development of hematopoietic cells, as well as in survival and development of numerous hematological malignancies. In this review, we summarize the latest data that depict the role of Twist1–2 in monocytes, T cells and B lymphocyte activation, and in associated hematological malignancies.

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INTRODUCTION

Twist1–2 main functions

Twist1 and Twist2 (Twist1–2) are two distinct tissue-restricted transcription factor members of the basic helix-loop-helix (bHLH) class B family that display high sequence similarity with each other. They play a critical role in embryogenesis, particularly in the inhibition of mesenchymal cell development. In humans, mutations in twist1 cause the Saethre–Chotzen syndrome, an autosomal dominant inheritance disease principally characterized by craniosynostosis (premature closure of clavicular suture).¹,² Heterozygous twist1+/− mice show craniofacial and limb abnormalities, whereas twist1−/− mice die early during embryogenesis. Twist2 is expressed after Twist1 in mesodermal tissues during embryogenesis. Twist2 inhibits terminal differentiation of mesoderm-derived cells, such as myocytes, osteoblasts and adipocytes.³,⁴ In humans, mutations in twist2 are associated with the Setleis Syndrome, an inherited developmental disorder characterized by bilateral temporal marks and other facial features.⁵ Twist2−/− mice exhibit normal embryogenesis but die 2–3 days after birth of generalized cachexia caused by high levels of proinflammatory cytokines.⁶

A complex regulation

The expression of Twist1–2 is triggered by diverse signaling pathways linked to inflammatory-related cytokines via nuclear factor (NF)-κB and signal transducer and activator of transcription 3 (STAT3),¹⁶ and to stress conditions via WNT/β-catenin and hypoxia-inducible factor-1.¹²,¹³ As described throughout this review, their expression is tightly regulated, context dependent and multifactorial.

To confuse the situation, once expressed, Twist1–2 may function either as transcriptional activators or repressors, through both direct and indirect mechanisms.¹⁴–¹⁶ They act through direct mechanisms when they bind to conserved DNA E-box sequences (5′-NCANNTGN-3′) present in the regulatory region of many lineage-specific genes¹⁷ and function as transcription factors (Figure 1); through indirect mechanisms when they activate/repress gene expression via recruitment of co-activators/repressors at the E-boxes they are bound to, or when they associate with other transcription factors such as MEF2, RUNX1, RUNX2, CEBP-α and NF-κB to modulate gene transcription.⁵,⁸,¹⁴,¹⁶,¹⁹ (Figure 1).

Mechanisms that influence transcription regulation by Twist1–2 include (1) their spatial and temporal regulation, (2) their post-translational modifications (acetylation and phosphorylation), (3) their dimer choice, (4) their affinity with potential dimers and (5) protein–protein interactions (Figure 1).

The most characterized post-translational modification of Twist1 is its phosphorylation on serine 68 (S68) by mitogen-activated protein kinases such as p38, c-Jun N-terminal kinase and extracellular signal-regulated kinase-1/2.²⁰,²¹ S68 phosphorylation inhibits ubiquitylation-mediated degradation of Twist1 and thus regulates its half-life and availability. PKA (protein kinase A), PKC, PKB/Akt and PP2 can all modulate Twist1 phosphorylation levels at several other amino acid positions.²²–²⁴

Twist1–2 can also influence gene transcription through epigenetic modulation of the promoter region. Indeed, Twist1–2 were reported to interact with and recruit histone acetyl transferases/histone deacetylases to the promoter regulatory region that results in transcription repression through histone modifications.⁴,¹⁴,¹⁵ (Figure 1).

Twist1–2 may form homodimers and heterodimers with class A bHLH and E protein transcription factors or E2A that contain both E12 and E47-Tcf3 and are implicated in immune cell development.²⁶ Twist1–2 partner choice depends mostly on its accessibility and on Twist1–2 phosphorylation acetylation...
A major role for Twist1–2 in regulating inflammation

The perinatal death due to high levels of proinflammatory cytokines of mice with a twist2−/− genotype or carrying twist1 and twist2 haploinsufficiency hints at a key role for Twist1–2 in the control of proinflammatory response, and illustrates the complexity of the transcriptional regulation mechanisms by and of Twist1–2. On one hand, they both regulate proinflammatory cytokine expression by interfering with NF-κB-dependent gene transcription. They interact physically with NF-κB (as demonstrated for Twist1 in COS cells), and bind to E-boxes in the regulatory regions of NF-κB-regulated cytokines, such as tumor necrosis factor-α (TNFα) and interleukin (IL)-1β, as shown in murine macrophages6 (Figure 1). Furthermore, Twist2 is able to inhibit IL12 and interferon-γ (IFNγ) expression and to activate the production of the anti-inflammatory cytokine IL-10,31 whereas upon phosphorylation on S542 by PKB/Akt, Twist1 is able to enhance anti-inflammatory TGFβ receptor signaling.21

On the other hand, Twist1–2 expression itself is activated by NF-κB, suggesting the existence of a negative feedback loop where NF-κB pro- and anti-apoptotic pathways are activated by cytokines (such as TNFα), leading to downstream activation of other cytokines and of Twist1–2. In turn, Twist1–2 interact with p65/RelA subunit of NF-κB, causing the repression of NF-κB-mediated transactivation of cytokines.9 Their activation and interaction by and with NF-κB is conserved across species. This negative feedback loop sets Twist1–2 as central modulators of the NF-κB proinflammatory pathway.

Finally, their role in the proinflammatory response incidentally implicates Twist1–2 in the modulation of the immune system response. Indeed, recent studies show their considerable role in immune cell function. Moreover, dysregulation of Twist1 or Twist2 are implicated in the pathogenesis of various hematological malignancies.31–33

FUNCTIONS OF TWIST1–2 IN HEMATOPOIETIC LINEAGES

Myeloid lineage

In the hematopoietic system, the expression of Twist1 is largely observed in CD34+ hematopoietic stem cells34–36 whereas Twist2 is mostly expressed in the myeloid lineage.31 Twist2 is a major negative modulator of both the development of myeloid cells and their proinflammatory responses. It is expressed in granulocyte–macrophage progenitors, and inhibits their proliferation and differentiation into macrophages, neutrophils and basophils,
through direct interaction and inhibition of RUNX1 and C/EBPα transcription factors. 31 In mature myeloid cells, Twist2 negatively regulates the proinflammatory responses by inhibiting the expression of proinflammatory cytokines such as IL12, IFNγ, IL1, TNFα, IL6, macrocyte chemoattractant protein-1 and macrophage inflammatory protein-1α through inactivation of C/EBPα and NF-κB, while activating anti-inflammatory IL10 expression 31; (Table 1).

In murine mature macrophages, Twist1–2 are also implicated in an anti-inflammatory feedback loop triggered by IFNα to counteract production of TNFα. 31 Indeed, IFN type I strongly induced (1) mRNA and protein expression of Twist1–2 through induction of the receptor tyrosine kinase Axl, (2) responsiveness to Axl ligands and (3) concomitant reduction of TNFα expression. Sharif et al. 33 showed evidence that suppression of TNFα expression was mediated in part by Twist1–2 binding to E-boxes in the TNFα promoter region. Twist1–2 also modulate the function of several other transcription factors expressed in macrophages such as E-proteins, myocyte enhancer factor 2 (MEF2) and sterol regulatory element-binding protein-1 (SREBP1), positioning them as central in the regulation of macrophage priming and anti-inflammatory response. 4,5,18,37

Although its precise role is still unknown, Twist1 is also expressed in follicular dendritic cells that are specialized mesenchymal-derived stromal cells found in the germinal centers of lymphoid tissues that trap and present immune complexes to B cells, enabling their maturation. 37

Lymphoid lineage

T lymphocytes. The T lymphocyte family encompasses CD8⁺ T lymphocytes that are cytolytic effector cells, CD4⁺ helper T cells whose role is auxiliary to other immune cells, and CD4⁻ regulatory T cells. T cells are further differentiated into naive, effector memory (EM), central memory (CM) and terminally differentiated effector memory phenotypes depending on their exposure to and activation by their antigen (reviewed in Appay et al.48). CD4⁺ helper T lymphocytes are also divided in different subsets that display different functions. The best characterized includes T helper 1 (Tₜ₁) and T helper 2 (Tₜ₂), and other subsets such as T helper 17 (Tₜ₁₇), follicular helper T cells (Tₜ₅), and the more recently described T helper 9 (Tₜ₉) (reviewed in Wan et al.49).

There are ever-increasing data aiming to decipher the role of Twist1–2 in the T-cell response. These studies seem to suggest that, in this context, Twist1–2 display different and nonredundant functions.

**Twist1 and T lymphocytes.** In 2008, Niesner et al. 40 were the first to identify Twist1 as a key modulator of Tₜ₁ lymphocyte proinflammatory response in mice. They observed that Twist1 expression was predominant in differentiated Tₜ₁ effector memory cells. Accordingly, Twist1 expression was transiently induced following T cell receptor stimulation through NFAT (nuclear factor of activated T cells) and NF-κB, then enhanced and constantly detected upon repeated T cell receptor stimulations. Its expression was also activated by IL12 via STAT4. Following activation of naive Tₜ₁ cells, NFAT, NF-κB and STAT4 had to be bound to their respective binding sites in the promoter region of twist1 to induce its transcription, whereas in activated Tₜ₁ cells, NFAT and NF-κB binding to the twist1 promoter region was sufficient to trigger its expression. Ex vivo, Twist1 expression was mostly observed in memory CD4⁺ T cells and was especially high in chronically activated CD4⁺ T cells. Furthermore, the authors described that ectopic overexpression of Twist1 in 6-day-old Tₜ₁ cells drastically reduced their pathogenic contribution in inflammation, whereas Twist1 silencing enhanced their potential to induce chronic inflammation. These results imply a key role for Twist1 in limiting inflammation in Tₜ₁ cells. In T cells, as in macrophages, Twist1-mediated repression of NF-κB-activated pathways seems to act through binding to E-boxes in regulatory regions of NF-κB target genes. In particular, Twist1 appeared to dampen inflammation triggered by Tₜ₁ memory cells in

| Table 1. Functions of Twist1 and Twist2 in hematopoietic cells |
|-----------------------|-----------------------|
| **Myeloid lineage:** | **Twist1** |
| - Key regulators of priming and of the anti-inflammatory response of macrophages | - Expressed in myeloid progenitors, inhibition of proliferation and differentiation into macrophages, neutrophils, basophils through inhibition of RUNX1 and C/EBPα. 31 |
| Mediators of an anti-inflammatory feedback loop triggered in response to IFNα to counteract TNFα production | - Expressed in mature myeloid cells. |
| Modulation of E-proteins, MEF2 and SREBP1 | - Expressed in mature myeloid cells. |
| - Expressed in follicular dendritic cells (unknown function). 37 | - Inhibition of proliferation and differentiation into macrophages, neutrophils, basophils through inhibition of RUNX1 and C/EBPα. 31 |
| **T lymphocytes** | **Twist2** |
| - Expressed mainly in Tₜ₁ lymphocytes | - Expressed in thymocytes and mature T lymphocytes |
| Induction by TCR stimulation through NFAT and NF-κB, by IL12 via STAT4 | Prevention of galectin-1 mediated apoptosis after NF-κB activation during negative selection. 19,32 |
| Prevention of chronic inflammation 40,42,43 | |
| Inhibition of the proinflammatory response. 40 | |
| Repression of NF-κB activated pathways, TNFα, IL2, SOCS1, SOCS2, IL1R decoy and Jak2 40 | |
| Inhibition of IFN-γ through modulation of T-bet, STAT4 and RUNX3 41 | |
| - Inhibition of Tₜ₁₇ polarization and T follicular helper development 45 | |
| Induced by STAT3 to control IL6 signaling | |

| B lymphocytes | **Twist2** |
|-----------------|-----------------|
| Expressed in B cells (unknown function). 45 | |

Abbreviations: C/EBPα, CCAAT/enhancer binding protein; IFN, interferon; IL, interleukin; MCP, macrocyte chemoattractant protein; MEF2, myocyte enhancer factor 2; MIP, macrophage inflammatory protein; NA, not available; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NFAT, nuclear factor of activated T cells; RUNX1, runt-related transcription factor-1; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; SREBP1, sterol regulatory element-binding protein-1; TCR, T cell receptor; Tₜ₁, T helper; TNF, tumor necrosis factor.
mice experiencing delayed-type hypersensitivity and antigen-induced arthritis through the specific targeting of TNFα, IL2, IFNγ, SOCS1, SOCS2, IL1R decoy and Jak2. Interestingly, and in contrast to the well-known properties of Twist1 in the induction of the epithelial-to-mesenchymal transition (EMT),41 Twist1 expression did not interfere with the homing capacity of T<sub>eff</sub>1 cells to the inflamed tissue. The authors further validated their results in humans, showing that expression of Twist1 was low in naïve CD4<sup>+</sup> T cells, enhanced in activated effector memory CD4<sup>+</sup> T cells and even higher in terminally differentiated effector memory CD4<sup>+</sup> T cells.40 Twist1-expressing CD4<sup>+</sup> T cells exhibited the phenotype of activated T<sub>eff</sub>1 effector memory cells (CD45RA<sup>−</sup> CCR7<sup>−</sup> CD27<sup>−</sup> and CD57<sup>+</sup> ) and continuously expressed in CD4<sup>+</sup> T cells recovered from inflamed tissues of patients with chronic inflammation (that is, rheumatic diseases, Crohn’s disease and ulcerative colitis), despite therapeutic treatment. However, they did not definitely validate whether Twist1 was a hallmark of T<sub>eff</sub>1 rather than of T<sub>eff</sub>2 or of T<sub>eff</sub>17 polarization in humans (Table 1).

In summary, Twist1 expression in T cells considerably lowered their contribution to inflammation and tissue destruction in mice. Expression of Twist1 in T<sub>eff</sub>1 cells acts as an endogenous regulator limiting the proinflammatory potential of T<sub>eff</sub>1 cells in the continuous presence of antigen with repeated activation of NFAT and NF-κB. In humans, the specificity of Twist1 upregulation in CD4<sup>+</sup> T cells recovered under a chronic inflammation context lead the authors to propose that Twist1 could be used as a biomarker of chronic inflammation.

Pham et al.<sup>42</sup> further defined the role of Twist1 in T<sub>eff</sub>1 cell-mediated inflammation. They showed that Twist1 modulated inflammation, as measured by inhibition of IFNγ production, by decreasing the expression of T-bet, STAT4 and RUNX3 and their binding to the IFNγ locus. Of note, only RUNX3 overexpression was able to compensate for Twist1-mediated inhibition of IFNγ, and this was achieved through direct physical interaction with Twist1. The authors did not observe direct binding of Twist1 to the promoter regions of IFNγ, TNFα and IL12Rβ2. Interestingly, they showed that Twist1 expression was not restricted to T<sub>eff</sub>1 cells, but could also be measured in T<sub>eff</sub>2 cells, although at a lower level.

In a subsequent study, the same group identified Twist1 as a key factor that hindered T<sub>eff</sub>17 polarization.43 They showed that Twist1 was a key component of a STAT3-induced feedback loop that controls IL6 signaling through inhibition of IL6Rα to block T<sub>eff</sub>17 and TF<sub>γ</sub><sub>δ</sub> cell development differentiation. Phenotypically, mice with a T cell-specific deletion of Twist1 also exhibited early-onset experimental autoimmune encephalomyelitis, and increased antigen-specific antibody responses, demonstrating a significant role for Twist1 in limiting both cellular and humoral immunity.

**Twist2 and T lymphocytes.** The function of Twist2 in T lymphocytes and immature thymocytes seems to focus mainly around the prevention of galectin-1-induced apoptosis, a process normally occurring after NF-κB activation during the negative selection in the thymus. This has been explored in in vitro experiments on mouse embryonic fibroblasts.48 N-Myc expression is amplified in neuroblastomas accompanied by a systematic overexpression of Twist1. Prevention of apoptosis by Twist1 in this context could be partly attributable to impaired p14ARF activity, once again through hindering of the p14ARF–MDM2–p53 axis.49 In fact, Twist2 are able to repress the p53 proapoptotic response at each step that may lead to its activation, through: (1) inhibition of transcriptional co-activators of p53, such as acetyltransferase p300,2(2) prevention of p53 phosphorylation and (3) preclusion of p53 DNA-binding activity.49–51

Twist1 also offsets the apoptosis response induced by other pathways in addition to the DNA damage response. For example, Twist1 is able to block the c-Myc-induced apoptotic response through modulation of the p14ARF–MDM2–p53 pathway in in vitro experiments on mouse embryonic fibroblasts.48 N-Myc expression is amplified in neuroblastomas accompanied by a systematic overexpression of Twist1. Prevention of apoptosis by Twist1 in this context could be partly attributable to impaired p14ARF activity, once again through hindering of the p14ARF–MDM2–p53 axis.51 As a direct consequence of their capacity to override oncogene-induced senescence and apoptosis, Twist1–2 cooperate in vitro and in vivo with mitogenic oncoproteins for malignant transformation.<sup>46,49–51</sup> (Figure 2).

In addition, Twist1–2 were recently shown to be involved in the direct regulation of microRNAs (such as miR-199a-214 and miR-10b involved in breast cancer invasiveness)52 and of genes linked to cancer progression.<sup>53,54</sup> Namely, Twist1 is able to bind to the promoter of the proto-oncogene AKT2 to upregulate its

**TWIST1–2 AS EMERGING RISK FACTORS IN HEMATOLOGICAL MALIGNANCIES**

The overexpression of Twist1 has been described as a poor prognostic factor in numerous epithelium-derived malignancies such as breast cancer, prostate cancer, colorectal cancer, bladder cancer, melanoma, hepatocellular carcinoma and neck carcinoma (reviewed in Puisieux et al<sup>55</sup>). Twist1–2 deregulations have also been more recently detected in hematological malignancies. Their precise roles are ill-defined in this latter context, and probable mechanisms of their oncogenic properties must be extrapolated from results obtained in solid tumors that we briefly summarize below.

The central role of Twist1–2 in embryogenesis and mesodermal development and their targeting of multiple genes coding for cell-fate proteins inevitably links them to cancer and oncogenesis. In brief, Twist1 has been implicated in cancer initiation by counteracting both senescence and apoptosis programs and in cancer progression by increasing resistance to treatment.

In cancer cells where Twist1 or Twist2 expression is increased, it has been demonstrated that they neutralize senescence and cell death through inhibition of both p53 and RB tumor suppressor pathways.<sup>47</sup> (Figure 2). Interestingly, Twist1–2 E-boxes are found in the regulatory region of the cyclin-dependent kinase inhibitors p21, p15 (INK4B) and p16 that can induce cell cycle arrest upon DNA damage. Both Twist1–2 repress senescence by inhibiting the transcription of p53 target p21 and of p16.<sup>47</sup> Furthermore, they inhibit the expression of p14ARF and perturb the p14ARF–MDM2–p53 axis.<sup>48</sup> In fact, Twist1–2 are able to repress the p53 proapoptotic response at each step that may lead to its activation, through: (1) inhibition of transcriptional co-activators of p53, such as acetyltransferase p300, (2) prevention of p53 phosphorylation and (3) preclusion of p53 DNA-binding activity.49–51

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In addition, Twist1–2 were recently shown to be involved in the direct regulation of microRNAs (such as miR-199a-214 and miR-10b involved in breast cancer invasiveness)52 and of genes linked to cancer progression.53,54 Namely, Twist1 is able to bind to the promoter of the proto-oncogene AKT2 to upregulate its
expression, and to activate the transcription factor Y-box-binding protein 1 (YB-1) associated with cellular proliferation. Overexpression of YB-1 and AKT2 have been observed in several malignancies and linked to poor prognosis.\textsuperscript{53,54} In breast cancers, Twist1 oncogenic functions that promote EMT are controlled by its phosphorylation mediated by PKB/Akt, leading to modulation of TGF\(\beta\)2 transcription and enhanced TGF\(\beta\) receptor signaling.\textsuperscript{21}

Finally, Twist1–2 have also been implicated in metastasis formation through EMT, thereby facilitating cancer cell invasion.\textsuperscript{51,59} Twist1 induction of metastasis through EMT plays a central role in epithelium-based cancers but may not be the most relevant pathological effect in hematological malignancies, because they derive from cells with intrinsic motility capacity.

Twist 1–2 in malignancies derived from the myeloid lineage

\textit{Myelodysplastic syndromes (MDS)}. MDS are clonal disorders of hematopoietic stem/precursor cells characterized by abnormal maturation and differentiation, an increasing resistance to proapoptotic signals and a high risk for MDS patients to progress to acute myeloid leukemia (AML).

Upregulation of TNF\(\gamma\)2 is typically observed in MDS bone marrow (BM) and is associated with a decreased expression of Twist1 in stromal cells.\textsuperscript{50} In contrast, Twist1 expression is augmented in the CD34\textsuperscript{+} hematopoietic stem cell compartment from MDS BMs.\textsuperscript{34} This increase in Twist1 expression appears to be induced in hematopoietic precursors by stroma contact and more frequent in

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\textbf{Figure 2.} Targets of Twist proteins in the DNA damage response pathway. In healthy conditions, DNA damage triggers the activation of ataxia telangiectasia mutated (ATM) and ATR kinases that usually results in activation of p53, of its transcriptional targets cyclin-dependent kinase inhibitor p21 (CIP/WAF1, CDKN1A) and/or Bax, and ultimately leads to senescence or apoptosis. DNA damage can also induce the cyclin-dependent kinase inhibitor p16 (INK4A) and the tumor suppressor retinoblastoma (Rb) that will further stabilize senescence cell cycle arrest. p14ARF might also come into play during the DNA damage response and is additionally induced by cellular stresses such as oncogene signaling and replication stress. p14ARF is an upstream regulator of p53 that acts through repression of p53-inhibitor MDM2. Myc transcription factor family members promote cell growth and proliferation, but might at the same time induce p53-dependent (through p14ARF) and -independent apoptosis. In cancer cells where Twist1 or Twist2 expression is increased, they neutralize senescence and cell death through inhibition of both p53 and Rb tumor-suppressor pathways. Twist1–2 repress senescence through binding to E-boxes in the regulatory region of p21 and p16. They might also inhibit the expression of p14ARF. Furthermore, Twist1 is able to block the Myc-induced apoptotic response through modulation of the p14ARF–MDM2–p53 pathway. Conversely, they increase cellular proliferation and trigger resistance to chemotherapy through the upregulation of AKT2 and YB-1 and the activation of the antiapoptotic NF-\(\kappa\)B response.
advanced-stage compared with low-grade MDS. A physical interaction between endogenous Twist1 and p53 has been detected and associated with resistance to apoptosis through inhibition of proapoptotic protein Bax expression. Furthermore, forced inhibition of Twist1 in myeloid cell lines and primary CD34+ MDS cells resulted in increased expression of p53 and Bax, and enhanced TNFα-mediated apoptosis (Table 2). Li et al. also observed an inverse correlation between the protein levels of Twist1 and p53 phosphorylated at serine 46. In 2013, the same group established that interactions between Twist1-dependent miR-10 family member and p53 played a central role in regulating TNFα-mediated apoptosis in MDS clonal cells. They showed that miR-10a/b facilitated apoptotic responses and decreased cell proliferation through direct modulation of the p53 promoter and of downstream targets of NF-κB after transcriptional activation by Twist1. Of note, Twist1 inhibition also increased apoptosis in cell lines displaying mutated p53, suggesting that Twist1 contributes to additional pathways in the apoptotic response to stroma in MDS that have yet to be defined.

**Acute myeloid leukemia.** AML is characterized by a rapid growth and an increased number of myeloid-derived blast cells that accumulate in the BM, paired with an arrest in their maturation. This process interferes with hematopoiesis and frequently results in hematopoietic insufficiency. N-Myc overexpression is frequently detected in AML. Similar to observations made in neuroblastoma, experiments in mice showed that Twist1 is upregulated in AML-expressing N-myc, therefore suggesting that Twist1-mediated disruption of the apoptotic response through the ARF/p53/MDM2 pathway could be involved in N-Myc-positive AML (Table 2). Furthermore, studies on the myeloid leukemic cell line U937 showed that Twist1 expression activated by the PKB/Akt/mTor (mammalian target of rapamycin) pathway through the neutrophin receptor TrkC (tropomyosin-related kinase C) molecular network (implicated in leukemogenesis) was involved in resistance to apoptosis, whereas when activated by Axl, Twist1 expression was implicated in resistance to treatment.

Finally, plasma levels of TNFα, IL6 and IL10 are increased in AML patients. IL6 level is inversely correlated to patient survival. IL6 signalization acts through STAT3/5 activation to promote survival in AML and, in theory, this may lead to the activation of Twist1–2 expression. Importantly, deregulation of Twist1–2 expression in AML patients remains to be demonstrated.

### Table 2. Functions of Twist1 and Twist2 in hematological malignancies

|                  | Twist1                                                                                           | Twist2                |
|------------------|--------------------------------------------------------------------------------------------------|-----------------------|
| **Myeloid malignancies** |                                                                                                  |                       |
| MDS              | Expression associated with disease stage                                                         |                       |
|                  | Inhibition of apoptosis                                                                          |                       |
|                  | Interaction with p53, and Bax inhibition                                                        |                       |
|                  | Regulation of TNFα-mediated apoptosis through miR-10                                           |                       |
|                  | Modulator of unknown p53-independent pathway of apoptosis                                        |                       |
|                  | - Upregulated in N-Myc AML                                                                      | - Hypermethylation of twist2 promoter region in some cases of childhood AML function unknown |
|                  | - Possible implication in resistance to apoptosis and treatment                                  | - No hypermethylation of the twist2 promoter104 |
| AML              | - Increased expression13,68,83                                                                   |                       |
|                  | - Associated with stage disease and treatment resistance.                                       |                       |
| CML              | - Increased expression13,68,83                                                                   | - No hypermethylation of the twist2 promoter104 |
|                  | - Associated with stage disease and treatment resistance.                                       |                       |
| **T-cell lymphoma** |                                                                                                  |                       |
| CTCL             | - Highly expressed                                                                              | - Highly expressed    |
|                  | Correlated with MF and S2 disease stage                                                         | - Antiapoptotic role81,82 |
|                  | - Probable inhibition of apoptosis through association with c-Myc and p53                      | Downregulation of CD7, galectin-1 receptor. |
| ALC              | - Highly expressed                                                                              |                       |
|                  | Upregulated via STAT3                                                                           |                       |
|                  | - Probable role in invasiveness and chemoresistance linked to phospho-Akt and Bmi-189         |                       |
| **B-cell lymphoma** |                                                                                                  |                       |
| DLBCL,MZBCL      | - Overexpressed in DLBCL,89 MZBCL and FL (unknown function) (Merindol et al., this study, Figure 3a). | - Not overexpressed (Merindol et al., this study, Figure 3b). |
| FL               | - Overexpressed                                                                                 |                       |
| **Leukemia**     |                                                                                                  |                       |
| ALL              | - No hypermethylation of twist2 promoter region frequent in childhood ALL2,105                  |                       |
|                  | - Associated with loss of Twist2 expression                                                      |                       |
|                  | - Loss associated with disease stage/relapse                                                     |                       |
|                  | - Tumor-suppressor properties                                                                    |                       |
|                  | - Loss of Twist2 linked to cell growth and to resistance to apoptosis and chemotherapeutic agents.|                       |
|                  | - Frequent promoter region methylation in mutated Ig VH                                        |                       |
|                  | - Associated with favorable prognosis.44                                                        |                       |

**Abbreviations:** ALC, anaplastic large cell lymphoma; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; Ig, immunoglobulin; CML, chronic myeloid leukemia; CTCL, cutaneous T-cell lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MDS, myelodysplastic syndrome; MF, mycosis fungoides; MZBCL, marginal zone B-cell lymphoma; NA, not available; STAT, signal transducer and activator of transcription; S2, Sézary syndrome; TNF, tumor necrosis factor; VH, variable heavy chain.
Chronic myelogenous or myeloid leukemia (CML). CML is a clonal stem cell disorder characterized by an increased number and unregulated growth of myeloid cells in the BM and accumulation of these cells in the blood. This disease is associated with a characteristic chromosomal translocation called the Philadelphia chromosome, consisting of a Bcr-Abl tyrosine kinase fusion.

In 2003, Tipping et al. detected the expression of Twist1 in KCL22 CML cell lines in a genome-wide microarray analysis. Twist1 expression was further amplified in KCL22 clones that were resistant to Imatinib, a tyrosine kinase inhibitor (TKI). In 2010, Casset et al. confirmed that Twist1 expression increased in CD34⁺ hematopoietic stem cells and CD34⁺ cells throughout CML disease progression. Its expression significantly decreased upon complete remission and increased again after relapse. Furthermore, they observed a 100-fold increase of Twist1 expression in TKI nonresponder compared with TKI responder samples (Table 2). The increase in Twist1 expression was specific to TKI-resistant patients in the CD34⁺ cells compartment. Silencing of Twist1 by RNA interference experiments in cell lines decreased cell number and viability in response to Imatinib, whereas overexpression of Twist1 led to improved Imatinib resistance of previously sensitive cell lines. Therefore, Twist1 seems to be implicated in resistance to TKI in cell lines and the authors suggest that Twist1 may be used as a novel predictive marker of TKI resistance in CML.

A recent study proposed a new role for Twist1 in CML. Xin et al. studied differences between the CML cell lines K562 and the multidrug-resistant K562/A02 derived by enrichment through treatment with doxorubicin. They showed that Twist1 was highly expressed in the multidrug-resistant cell lines derived from K562. Moreover, twist1 knockdown decreased the expression of stem cell markers such as Oct4, Sox2 and Nanog in this multidrug-resistant cell line, suggesting that, in addition to participating in chemoresistance, Twist1 is involved in maintaining tumour-initiating or cancer stemness properties.

Twist1–2 in lymphoid lineage-derived malignancies

T-cell lymphoma

Cutaneous T-cell lymphoma (CTCL). CTCLs are a group of lymphoproliferative disorders characterized by localization of neoplastic T cells in the skin. Classical forms of CTCLs are plaque-type mycosis fungoides (MF) and Sézary syndrome (Sz). The link between MF and Sz remains controversial. Sz was described by Albert Sézary, it can arise de novo or evolve from MF and is considered a rare leukemic variant. Sz is characterized by erythroderma, peripheral lymphadenopathy and the presence of malignant T cells that are CD4⁺/CD45RO⁺ and CD3⁺/CD26⁻ in the skin, lymph nodes and peripheral blood, chronically activated and skewed toward T H2 differentiation. These T cells are derived from the same T-cell clone. MF is a slowly progressive disease (87% 5-year survival), whereas Sz is more aggressive with a poor prognosis (11% 5-year survival). In both diseases, chemotherapy and BM transplantation are not curative and do not significantly improve survival.

Twist1. Sz and MF are defined by aberrations in four pathways: (1) evasion of activation-induced cell death, (2) T Cells-specific lymphocyte differentiation, (3)TGFβ receptor expression and (4) modulation of TNF receptor ligands (reviewed in Dulmage et al.). Molecularly, typical observations of these diseases include the constitutive activation of STAT3 and decreased expression of TGFβ receptor II, of downstream modulators such as SMAD3 and of its ligand TGFβ1, diminished detection of p15 and p16 and dysregulation of the c-Myc and of the transcription factor Twist1 in Sz (Table 2). Similar results were observed in a subset of patients with MF and with CD30-negative primary CTCL. Although they did not perform any mechanistic experiments, the authors proposed that Twist1 could block c-Myc-induced apoptosis by antagonizing the p53 pathway. In 2012, a study by Goswami et al. showed that Twist1 expression was indeed correlated with MF and Sz stages. Moreover, they observed an association between increased Twist1 and c-Myc expression and abnormal p53 expression. The precise role of Twist1 and of other Sz- and MF-specific upregulated proteins (that is, PLS3, KIR3DL2, NKP36) in CTCL remains to be characterized.

Twist2. Similar to conclusions from experiments in primary T lymphocytes, studies by SH Koh et al. suggested that Twist2, like Twist1, plays an antiapoptotic role in T-cell lymphoma but through a distinct pathway (Table 2). Indeed, they showed that Twist2 was overexpressed in mature T-cell lymphoma cells and specifically highly expressed in Sz cells. Ectopic expression of Twist2 was able to downregulate the expression of the galectin-1 receptor CD7 through induction of chromatin deacetylation. This led to a decrease in galectin-1-mediated apoptosis in activated T cells that promoted malignant T-cell accumulation and progression of T-cell lymphoma. Accordingly, CD7 expression is dramatically downregulated in Sz and Twist1-induced apoptosis by CD7 is inhibited.

Of note, downregulation of CD7 is also related to the progression of adult T-cell lymphoma/leukemia where galectin-3-induced apoptosis is inhibited, although Twist2 expression has not yet been detected in this context.

Anaplastic large cell lymphoma (ALCL). ALK⁺ ALCL represents a small subset of non-Hodgkin’s T-cell lymphoma characterized by a specific chromosomal translocation and the fusion of nucleophosmin (NPM) and anaplastic large cell lymphoma kinase (ALK) genes in the majority of cases. This leads to ubiquitous expression of ALK, whose expression is normally restricted to the brain and nervous system. NPM–ALK promotes oncosignals by activating cellular signaling proteins, including STAT3, MEK/ERK, mTOR and PI3K/Akt (reviewed in Amin et al. and Charlie et al.). The activation of these pathways has been linked to Twist1 expression in other malignancies as well as to increased cell proliferation and resistance to apoptosis.

NPM–ALK promotes tyrosine phosphorylation of STAT2 on Y705, essential for dimerization and activation of STAT3. Inhibition of STAT3 leads to apoptosis and cell cycle arrest in ALK⁻ ALCL cell lines and is required for its lymphomagenesis. In 2012, Zhang et al. reported that STAT3 directly upregulated Twist1 and that Twist1 was highly expressed in three ALK⁺ ALCL cell lines and in vivo in three ALK⁺ ALCL tumor-derived samples (Table 2). They performed functional assays that suggested that Twist1 contributes to the invasiveness of ALK⁺ ALCL. In two or three cell lines, Twist1 was linked to expression of phospho-Akt and Bmi-1 and downregulation of p66Shc (a member of Src homology and collagen homology family). Twist1 knockdown resulted in increased sensitivity to the chemotherapeutic agent ALK inhibitor Crizotinib in those two cell lines. These results need to be confirmed on a larger population of patients. Nevertheless, they suggest a key role for Twist1 as a regulator of apoptosis and resistance to treatment in this T-cell malignancy.

In addition, an in vitro study by Degerman et al. hints at a role for Twist1 during spontaneous oncogenesis of two Nijmegen breakage syndrome T-cell cultures established following mitogenic stimulation in the presence of IL-2. Twist1 expression is strongly increased in cell lines undergoing immortalization and appears to be of significance for senescence bypass in T cells.

B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL), marginal zone B-cell lymphoma and follicular lymphoma. DLBCL is the most common subtype of
non-Hodgkin’s lymphoma among adults with an overall survival at 5 years of 60%. Marginal zone B-cell lymphoma and follicular lymphoma are two other types of B-cell lymphoma, the latter being the second most common non-Hodgkin’s lymphoma.

DLBCL arises from normal B cells or from the transformation of other types of lymphoma and leukemia. The disease is characterized by an aggressive and rapidly growing tumor that can arise in any part of the body. DLBCL is heterogeneous and encompasses a diverse set of clinical presentations and diagnosis. With the help of gene expression profiling, DLBCL can be divided into germinal centre B-cell-like, activated B-cell-like and primary mediastinal B cell-like subgroups.10,11,92

The first suggestions of a possible Twist1 dysregulation came from genetic studies performed by Davis et al.93 showing that the NF-κB pathway was constitutively activated in activated B-cell-like and primary mediastinal B cell-like DLBCL. In addition, the expression of STAT3 and IL6 is frequently triggered in activated B-cell-like subtypes.94 c-Myc overexpression because of chromosomal rearrangement or translocation is observed in ~10% of DLBCL (regardless of the subtype) and associated with poor prognosis.95–98

In 2012, Lemma et al.99 detected the expression of Twist1 in the nucleus of the majority of DLBCL samples (83/99) by immunohistochemical staining. Twist1 expression correlated with Slug and Zeb1 expression, two other transcription factors associated with EMT97 (Table 2). Nevertheless, the expression of Twist1 could not be associated with any prognostic value. Interestingly, overexpression of YB-1, a known target of Twist1 implicated in cellular proliferation, has also been described and linked to poor prognosis and rituximab resistance in DLBCL samples.100,101

With the aim of investigating a possible role of Twist1–2 in B-cell lymphoma, we performed real-time Q-PCR on samples derived from Origene (Lymphoma cDNA Array II LYT102, OriGene Technologies, Inc., Rockville, MD, USA) (Figure 3). A description of each sample is found at www.origene.com/assets/documents/TissueScan/LYT102.xls. As a positive control, an analysis of Twist1 expression from a sample of a patient with MF was included. Reference values used as healthy controls were obtained from six samples of lymph nodes and spleen containing 100% normal cells, originally part of the mRNA samples present on the LYRT102 plate. We detected an increased expression of Twist1 in DLBCL samples and also in follicular and marginal zone B-cell lymphomas samples (although results were statistically significant only for follicular and marginal zone B-cell lymphoma compared with spleen and lymph node controls of normal appearance; Figure 3a). Twist2 expression was positively correlated with Twist1 expression. Although a high expression of Twist2 was detected in some patients in all three types of lymphoma, no significant overexpression of Twist2 could be measured compared with controls (Figure 3b).

In conclusion, Twist1 seems to be overexpressed in several types of B-cell lymphomas. Given its potential as a therapeutic target in other types of cancer, further investigations are needed to characterize its function and its regulation.

Leukemia. Although there are considerable data showing Twist1–2 oncogenic properties in T-cell lymphoma and in myeloid leukemia, publications on their implication in lymphoid leukemia remain sporadic and results remain to be confirmed.

Acute lymphoblastic leukemia (ALL). ALL is the most common type of leukemia in childhood and still accounts for 25% of all deaths from pediatric cancers. In adults, it is even more difficult to treat with a 5-year survival of 40%. It can derive from both B lymphocytes and T cells.

In several types of cancer such as leukemia, differences in biology and survival between patients may be due, in part, to inappropriate epigenetic regulation, such as silencing of tumor suppressor genes through DNA methylation.102,103

Preliminary evidence suggesting that Twist2 might play a role in ALL came from observations of specific dysregulated pathways of transcription factors that are known targets of Twist2, namely RUNX1 (fused with ETV6 in 25% of childhood ALL) and NF-κB.31 In 2012, Thathia et al.104 identified that hypermethylation of the twist2 promoter is frequent in both childhood (30 of 54 including 28 of 43 B-ALL and 2 of 7 T-ALL) and adult ALL (52 of 77 patients including 27 of 39 B-ALL and 13 of 15 T-ALL), whereas it was only moderately observed in childhood AML (4 of 28 cases) and rarely

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**Figure 3.** Twist1–2 mRNA levels in several types of non-Hodgkin’s B-cell lymphomas. The mRNA levels of twist1 and twist2 were measured by real-time PCR using the Applied Biosystems StepOnePlus Realtime PCR systems (Life Technologies, Carlsbad, CA, USA) for 35 amplification rounds in Fast runs using the Fast SYBR Green Master mix (FastStart Universal SYBR Green Master, Roche, Basel, Switzerland). CTRL (n = 6), healthy control samples; DLBCL (n = 16), diffuse large B-cell lymphoma samples; follicular (n = 10), follicular lymphoma samples; MCL (n = 4), mantle cell lymphoma samples; MF (n = 1), mycosis fungoides sample; MZBC (n = 5), marginal zone B-cell lymphoma samples, Gus (ß-Glucuronidase) was the endogenous control gene used to normalize DNA quantity. Relative expression was calculated as 2(-ΔΔCt). A t-test was performed between healthy and DLBCL samples derived from lymph nodes and P = 0.0357 between healthy and DLBCL samples derived from spleen, data not shown. As twist1 expression is known to be upregulated in MF, one sample derived from a patient with MF was included as a positive reference value in the figure. (b) twist2 relative expression. twist2 expression was measured in the same samples as in Figure 1a. No statistically significant difference could be observed between groups. Of note, high variability was observed in DLBCL- and MZBL-derived samples.
in CLL (5 of 110 cases). Noteworthy, Twist2 hypermethylation was not observed in CML and adult AML. Twist2’s upstream regulatory region hypermethylation was more frequent in ETV6-RUNX1-positive childhood ALL than in other childhood ALL cases (Table 2). Moreover, twist2 hypermethylation was especially frequent in relapsed adult ALL (20 of 22 cases), and the corresponding frequencies of samples exhibiting hypermethylated twist2 was higher following relapse than at the time of diagnosis.104

Hypermethylation resulted in loss of Twist2 expression in patient samples and ALL cell lines. Interestingly, restoration of Twist2 expression through transfection of Nalm6 and Reh ALL cell lines resulted in a dramatic inhibition of cell growth (aggravated by decamethasone treatment), associated with induction of apoptosis in ETV6-RUNX1−Reh cell lines.104 The authors propose that Twist2 inhibition of cell growth could be mediated in some cases through inactivation of RUNX1 because the latter has been shown to increase proliferation and enhance B-cell survival105; although no experiments were conducted to test this. Finally, re-expression of Twist2 leads to an increased sensitivity to the chemotherapeutic agents etoposide and daunorubicin. The authors hypothesize that chemotherapeutic sensitivity modulation by Twist2 is mediated through NF-κB inhibition, because increased expression of the latter is correlated with chemoresistance.

In summary, in opposition to its role in T-cell lymphoma and in myeloid leukemia, Twist2 appears to exhibit tumor-suppressor properties in ALL.

Chronic lymphocytic leukemia. CLL is the most common type of leukemia, displaying a great variability with regard to clinical course and survival. It is characterized by slow and uncontrolled proliferation with disrupted apoptosis of a clonal B cell that results in the accumulation of cells and disruption of normal immune function. Prognostic factors associated with worsened survival include expression of ZAP70 and CD38, chromosomal aberrations (reviewed in Stilgenbauer et al106) and chromosomal deletions,107,108 p53 mutations109 and absence of somatic mutations in genes coding for immunoglobulin variable heavy chain (lg Vh).110,111 In addition, a high frequency of patients with unmethylated Ig Vh ALL exhibit p53 dysfunction. DNA methylation is also implicated in the biology of CLL.112 In 2005, Rehali et al44 demonstrated that the Twist2 promoter was frequently methylated in patients exhibiting mutated Ig Vh subtypes of CLL (associated with a favorable prognosis) but not in patients that exhibited unmethylated Ig Vh subtypes (associated with a poor prognosis) (Table 2). Methylation resulted in twist2 silencing in mutated subtypes, whereas Twist2 was expressed in unmethylated subtypes. The authors suggest that the expression of Twist2 in unmethylated Ig Vh patients (with unmethylated twist2) could lead to disruption of the p53 pathway as observed in several other types of cancer, although they did not confirm this experimentally. In conclusion, parallel to what is seen in T-cell lymphoma but in opposition to observations in ALL, the expression of Twist2 in CLL could be associated with a poor prognosis.

CONCLUSION AND PERSPECTIVES: OTHER BHLH PROTEINS IN HEMATOLOGICAL MALIGNANCIES

Twist1-2-mediated gene expression modulation is intrinsically linked to the abundance of other BHLH members such as E2A and Id proteins. Interestingly, deregulation of expression of these proteins has been observed in various hematological malignancies. For instance, E2A20 mice display a high risk of developing T-cell tumors, as do E2AId120 mice.112 E47 knockdown experiments in T-cell lymphoma cell lines linked E47 deficiencies to the development of T-cell malignancy.113 Loss of expression of E2A, or its sequestration by Id2, has been linked to resistance to apoptosis in primary effusion lymphoma,114 to loss of B-cell phenotype and reprogramming of neoplastic B cells in Hodgkin’s lymphoma.115 Id1 is frequently overexpressed in various malignancies and its deregulated expression in mice has been associated with MDS development and immortalization of myeloid progenitors.116 In AML, high expression of Id1 is implicated in leukemic cell proliferation and has been associated with poor prognosis.117 Id4 silencing is systematic in CLL and MDS and plays a role in its pathogenesis.118,119 In contrast, Id4 seems to act as an oncogene in B cell-derived malignancies such as ALL.120,121 Unfortunately, Twist1–2 function was not investigated in the studies mentioned herein.

In conclusion, numerous questions remain to be addressed concerning the role of Twist1–2 in hematological malignancies. There is still a disturbing paucity of data on Twist2 implication in blood malignancies and on the role of Twist1–2 in non-T-cell lymphoma/leukemia. As potential therapeutic targets and as central regulators of apoptosis and senescence, large efforts should be undertaken to promptly clarify the role of Twist1–2 and their partners in these types of cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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