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Nanoparticle delivery of inhibitory signal transducer and activator of transcription 3 G-quartet oligonucleotides blocks tumor growth in *HMGA1* transgenic model of T-cell leukemia

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While survival from childhood acute lymphoblastic leukemia (ALL) has improved significantly over the past three decades, pediatric patients with T-cell ALL (T-ALL) remain at high risk for relapse and poor outcomes compared to children with B-cell precursor ALL [1–3]. Outcomes for adults with T-ALL are even worse, with long-term survival rates of only 30–40% in patients less than 60 years, which falls to 10% in patients over 60 years [1–3]. Thus, studies are urgently needed to identify key molecular pathways that could be targeted in therapy. Similar to other malignancies, oncogene activation by mutation or overexpression is common in T-ALL. For example, overexpression of the *TAL-1* oncogene occurs in most cases of pediatric T-ALL [2–4]. We discovered that the *high mobility group A1* (*HMGA1*) oncogene is also overexpressed in T-ALL [1]. Activating mutations in other oncogenes have been identified, including *LMO2*, *TLX1/HOX1*, *TLX3/HOX11L2* and *HOXA*, which appear to specify distinct T-ALL subtypes [1–3]. Other recurrent mutations in T-ALL include chromosomal loss of the *CDKN2A/B* tumor suppressor loci or activating mutations in the *NOTCH1* pathway that occur in most subtypes of T-ALL [1–3]. *NKX2-1* and *MEF2C* are putative oncogenes found in T-ALL subtypes that lack known oncogenic

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rearrangements [4]. Activating mutations in genes regulating cytokine receptor and RAS signaling were discovered in about two-thirds of all cases of an aggressive subclass of T-ALL, designated early T-cell precursor ALL [5]. This study also uncovered inactivating mutations in genes involved in normal hematopoietic development as well as histone modifying lesions. Intriguingly, the spectrum of lesions was similar to that observed in myeloid tumors and includes genes expressed in normal hematopoietic stem cells. An investigation of transcriptional networks in T-ALL identified RUNX1 as a tumor suppressor induced by TLX1 or TLX3 [6]. Together, these studies suggest that T-ALL is associated with diverse molecular underpinnings, which could contribute to the high rates of relapse with current therapies.

In addition to T-ALL, the *HMGA1* oncogene is over-expressed in hematologic malignancies and solid tumors, including precursor B-ALL, refractory acute myeloid leukemia, and cancers of the breast, lung, colon, pancreas, uterus, brain, bladder and skin [1,7–12]. In fact, high expression of *HMGA1* in leukemic blasts correlates with relapse in childhood B-ALL [7]. *HMGA1* also induces leukemic transformation in cultured cells and causes aggressive leukemia in transgenic mice [1,7]. In a recent report, we found that *HMGA1* cooperates with loss of function of the *Cdkn2a* (*INK4A/ARF*) tumor suppressor locus in a mouse model of T-ALL [1]. *HMGA1* is also overexpressed in diverse, poorly differentiated solid tumors [7,12]. The *HMGA1* gene encodes the HMGA1a and HMGA1b chromatin remodeling proteins, which function in modulating gene expression by altering chromatin structure [7]. HMGA1 proteins are the most abundant, non-histone, chromatin binding proteins found in cancer cells, and high expression portends a poor prognosis in diverse tumor types [7]. *HMGA1* is also highly expressed during embryogenesis and in embryonic stem cells, hematopoietic stem cells, and cancer stem cells, including leukemic stem cells [7–12]. A recent landmark study revealed that HMGA1 is essential for reprogramming somatic cells into induced pluripotent stem cells by the four Yamanaka factors [10]. In addition, blocking *HMGA1* expression or function interferes with multiple cancer phenotypes, including uncontrolled proliferation, anchorage-independent cell growth, migration, invasion, xenograft tumorigenesis and tumor progression in murine models [7,11–12]. *HMGA1* is also required for cancer stem cell properties, such as three-dimensional sphere formation and limiting dilution tumorigenesis [11]. The transcriptional networks regulated by HMGA1 are emerging, and include genes involved in embryonic or adult stem cells, inflammation, signal transduction, cellular motility and hematopoiesis [7,10–12]. We previously reported that HMGA1 induces expression of the gene encoding the signal transducer and activator of transcription 3 (STAT3), and recent studies underscore a central role for STAT3 in inflammation, malignant transformation, tumor progression and a stem-like state [7,12–14]. *STAT3* overexpression is also a prominent finding in hematologic malignancies [7,12]. Our prior studies demonstrate that blocking STAT3 function leads to apoptosis *ex vivo* in T-ALL cells from our *HMGA1* transgenic model [12]. In addition, blocking HMGA1 or STAT3 function prevents colony formation in Burkitt leukemia cells [12]. Together, these studies suggest that targeting the HMGA1–STAT3 pathway could be an effective therapeutic approach in lymphoid tumors and other malignancies.

To determine whether blocking STAT3 function has anti-tumor activity *in vivo* in T-ALL induced by HMGA1, we treated *HMGA1* transgenic mice that are deficient in the *Cdkn2a* tumor suppressor locus (*HMGA1a-Cdkn2a*^{-/-}) with G-quartet oligodeoxynucleotides (GQ-ODNs) that specifically inhibit STAT3 binding to DNA [13,14]. GQ-ODNs were originally identified at the 3' end of telomeres in vertebrates where they inhibit telomerase activity [13,14]. GQ-ODNs form intra- and inter-molecular four-stranded structures, or G-quartets, and block protein–DNA binding. GQ-ODNs (T40214) that inhibit STAT3 DNA binding were identified and found to have potent anti-tumor effects in experimental models of solid tumors, although they had not been tested in hematologic malignancies [13,14]. We therefore tested STAT3 GQ-ODN nanoparticles comprised of a 20 base-pair duplex DNA conjugated with polyethyleneimine (PEI) as previously described [13,14]. Prior to treatment, we confirmed that the STAT3 GQ-ODNs block STAT3 binding in gel shift experiments [Figure 1(A)]. As shown, the STAT3 GQ-ODN (T40214) prevents formation of the STAT3–DNA binding complex. In contrast, the control GQ-ODN had no effect on the STAT3–DNA complex. Next, we treated *HMGA1a-Cdkn2a* mice with the STAT3 GQ-ODN nanoparticles or control ODN nanoparticles. Because *HMGA1a-Cdkn2a* mice develop aggressive T-ALL by 20 weeks of age and succumb to their disease by 24–28 weeks, we began treatment at 20–21 weeks of age and sacrificed mice after 2 weeks of therapy (22–23 weeks of age) to assess tumor burden. Based on the GQ-ODN half-life of 48–72 h, mice were treated twice weekly with GQ-ODN nanoparticles delivered intraperitoneally following conjugation with PEI. To assess tumor burden following treatment, we compared relative splenic weights (spleen weight/total body weight) in mice treated with STAT3 GQ-ODN or control GQ-ODN nanoparticles, because previous studies from our group found that relative spleen weight is the best surrogate for tumor burden in these mice [1]. We found a marked decrease in tumor burden in the mice treated with STAT3 GQ-ODN [Figures 1(B) and 1(C)]. As shown, the spleens from STAT3 GQ-ODN-treated mice were smaller and lacked the grossly visible tumor nodules observed in the mice treated with control nanoparticles. Histopathologic analysis showed that the STAT3 GQ-ODN-treated mice had more normal splenic architecture and significantly smaller tumor burdens as compared to mice treated with control GQ-ODN [Figure 1(D)]. These studies indicate that blocking STAT3 with GQ-ODNs could be effective in hematologic malignancies with *HMGA1* overexpression and high levels of activated STAT3, such as T-ALL. Our results further underscore the key role of STAT3 in leukemogenesis induced by HMGA1.

In summary, we report for the first time that nanoparticle delivery of STAT3 GQ-ODNs has anti-leukemia effects in an *HMGA1* transgenic model of aggressive T-ALL. Recent studies demonstrate a central role for *HMGA1* in poorly differentiated or refractory cancers, embryonic stem cells and cellular reprogramming to an induced pluripotent stem cell [10,11]. Similarly, STAT3 signaling has been linked to inflammatory pathways, tumor progression and stem cell properties [7]. In fact, prior functional studies in cultured cell and murine models of solid tumors indicate that both *HMGA1* and *STAT3* are required for cancer stem cell properties, including growth as three-dimensional spheres and tumor-initiator or cancer stem cells [7]. Together, these data suggest that HMGA1 drives leukemic transformation and refractory disease by inducing *STAT3* and stem-like transcriptional networks. Further studies will be needed to determine whether the *HMGA1*–*STAT3* network

is required for leukemic stem cell properties. In preliminary studies, we found enrichment of *HMGA* genes in leukemic stem cells from primary acute myeloid leukemia samples (Resar, unpublished data). In this brief report, we provide compelling evidence that blocking STAT3 function could be effective in T-cell lymphoid tumors driven, at least in part, by aberrant expression of *HMGA1*. Although further studies are needed, these findings indicate that the *HMGA1*–*STAT3* pathway plays an important role in T-ALL in this model and could serve as a rational therapeutic target. Future studies are also needed to explore the role of STAT3 GQ-ODNs in other models of hematologic malignancies characterized by up-regulation or activation of *HMGA1* and *STAT3*. Given the central role for HMGA1 as a master regulator in diverse cancers, targeting this oncogene directly should also have profound anti-tumor effects [11].

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Figure 1. STAT3 inhibitory GQ-ODNs block STAT3 DNA-binding activity and exhibit anti-tumor properties in a murine model of aggressive T-cell leukemia. (A) STAT3 inhibitory GQ-ODNs block STAT3 DNA-binding activity in *vSrc*-transformed NIH3T3 cells. Gel shift experiments were performed as previously reported [15]. In the first lane, the STAT3 dimer complex is shown. This complex is not disrupted by the STAT1 antibody (lane 2 labeled NIH3T3-*vSrc* + STAT1 antibody), although there is a supershifted band when the STAT3 antibody is added (lane 3 labeled NIH3T3-*vSrc* + STAT3 antibody). The control GQ-ODN

does not disrupt the STAT3 complex (lane 4), while the STAT3 GQ-ODN disrupts the STAT3 complex (lane 5). (B) Decreased splenic tumor burden was found in STAT3 GQ-ODN-treated mice. Relative splenic weight (spleen weight divided by total body weight) was used as a surrogate for tumor burden and significantly decreased in the mice treated with STAT3 inhibitory GQ-ODN ($p = 0.04$ by one-sided Student's t -test). Bars show mean $\pm$ standard error of the mean. An equal number of male and female mice were treated in each arm ($n = 10$ males and 10 females or 20 mice/treatment arm). (C) Decreased tumor nodules were apparent at necropsy. Control spleen (left) was taken from a mouse treated with control GQ-ODN and has large lymphoid tumor nodules, while smaller spleen (right) was removed from a mouse treated with STAT3 GQ-ODN. (D) Decreased tumor infiltration in spleens of mice treated with STAT3 GQ-ODN (bottom panels) as compared to control GQ-ODN (top panels). Tissues were sectioned and stained with hematoxylin and eosin as previously described [1]. Note effacement of the white pulp (WP) and decrease in red pulp (RP) in spleen treated with control GQ-ODN as compared to spleen treated with STAT3 GQ-ODN. Left panels $\times 100$ magnification (bar = 50 μM), right panels $\times 400$ magnification (bar = 10 μM).