Transient depletion of RUNX1/RUNX1T1 by RNA interference delays tumour formation *in vivo*

To the Editor

The chromosomal translocation t(8;21)(q22;q22) occurs in about 10% of all cases of acute myeloid leukaemia (AML) and is one of the most frequent chromosomal abnormalities found in AML (1). This translocation generates the *RUNX1/RUNX1T1 (AML1/MTG8, AML1/ETO)* fusion gene, which by itself is not sufficient for full leukaemic transformation, but which supports human haematopoietic stem/progenitor cell self-renewal *in vivo* as well as leukaemic proliferation and clonogenicity *ex vivo* (2-4). Generation of *RUNX1/RUNX1T1* may not only be an initiating event in leukaemogenesis, but might also become a leukaemia-specific target for therapeutic approaches. However, its role in leukaemic persistence *in vivo* and, in particular, its significance for leukaemic stem cells has not been established yet.

One prerequisite for studying possible functions of *RUNX1/RUNX1T1* in maintaining leukaemia *in vivo* and the development of therapies targeting this genetic lesion is the availability of suitable animal model systems. Several transgenic and knock-in mouse models have been developed to study *RUNX1/RUNX1T1*-driven leukaemogenesis in murine haematopoietic cells (5). However, *in vivo* studies of *RUNX1/RUNX1T1* in the human leukaemic background are currently limited due to a lack of suitable xenotransplantation models, as t(8;21)-positive leukaemic cells including established cell lines do not or only rarely engraft in *Nod/Scid* mouse strains (6).

The *Rag2*$^{-/-}\gamma c^{-/-}$ mouse strain which lacks B, T and NK cells represents an interesting alternative to *Scid*-based mouse strains. For instance, intrahepatic injection of CD34^{+} human cord blood cells into newborn BALB/c *Rag2*$^{-/-}\gamma c^{-/-}$ mice leads to the development of an adaptive immune system (7). We tested this immunodeficient mouse strain in combination with the t(8;21)-positive AML cell line Kasumi-1 in order to establish a xenotransplantation model for t(8;21)-associated AML. Sublethally irradiated newborn mice received an intraperitoneal injection of 10^6 Kasumi-1 cells. Within 53 to 55 days, transplanted mice developed swollen abdomen or showed other signs of tumour formation. *Postmortem* examination revealed mainly solid tumours of 1 cm - 1.5 cm in diameter (Figure 1a) located intraperitoneally. Tumour histology and flow cytometry analyses revealed that the tumours consisted almost exclusively of human myeloid CD45^{+} CD34^{+} CD33^{+} cells, which is concordant with the immunophenotype of Kasumi-1 cells (Figure 1b, c, and data not shown). Notably, such extramedullary myeloid tumours (granulocytic sarcomas, chloromas) are found in some 20% of all t(8;21) AML cases (8). In less than half of the animals we observed infiltration of the spleen by leukaemic cells, but neither bone marrow nor liver infiltration (Figure 1c). Consistent with this, RUNX1/RUNX1T1 protein was strongly expressed in tumours, occasionally and weakly in spleen and never in liver (Figure 1d).

We recently analysed the significance of RUNX1/RUNX1T1 for leukaemic proliferation and clonogenicity using short interfering RNAs (siRNAs). A single siRNA treatment caused a transient reduction in fusion protein levels lasting 5 to 7 days. siRNA-mediated RUNX1/RUNX1T1 depletion restored myeloid differentiation capacity, inhibited proliferation and

severely impaired leukaemic clonogenicity *in vitro* (3, 4). However, these experiments did not address the *in vivo* significance of this leukaemic fusion protein in human leukaemic cells. To examine the consequences of a transient RUNX1/RUNX1T1 depletion on leukaemic engraftment and tumour formation, we used the *Rag2*$^{-/-}\gamma c^{-/-}$ transplantation system. We electroporated Kasumi-1 cells either in the absence of any siRNA ("Mock"), with siRNA targeting the *RUNX1/RUNX1T1* fusion site ("siAGF1") or with a mismatch control siRNA ("siAGF6") as described previously (3). siRNA-mediated *RUNX1/RUNX1T1* suppression was examined by western blotting and functionally analysed by colony formation assays (4). In comparison to mock and mismatch siRNA-treated cells, siAGF1-treated cells showed at least a threefold decrease in fusion protein and a sixfold decrease in colony forming cells *in vitro* (Figures 2a, b). One day after siRNA treatment, cells were either transplanted or stored in liquid nitrogen till transplantation. Transplantations were performed by intraperitoneally injecting 10^6 electroporated cells into sublethally irradiated newborn mice. Total group sizes were 12 animals for the mock group, 11 animals for the active siRNA group and 10 animals for the mismatch control siRNA group. Transplantation of mock- or mismatch siRNA-treated cells resulted in tumour formation in all transplanted animals with a median survival of 50 days (Figure 2c). In contrast, pre-treatment with the active RUNX1/RUNX1T1 siRNA siAGF1 resulted in an extended median survival of 73 days ($p < 0.02$). Notably, 3 animals of the active siRNA group examined 71 days post transplantation showed no signs of tumour formation in histological analyses. In a second set of experiments, 2.5×10^5 cells were transplanted 8 hours after siRNA electroporation into non-irradiated mice. In

this experiment, groups of 8 and 7 animals were transplanted with active and mismatch siRNA-pre-treated cells. This setting resulted in a median survival of 64 and 90 days for the mismatch siRNA and the active siRNA group, respectively. Again, the difference in median survival was statistically significant ($p < 0.02$) suggesting that siRNA-mediated transient reduction of RUNX1/RUNX1T1 causes a substantial decrease in cancer-initiating cells.

In summary, we show that already a transient siRNA-mediated depletion of RUNX1/RUNX1T1 causes a significant increase in median survival in a xenotransplantation model. These findings suggest that RUNX1/RUNX1T1 siRNAs compromises the engraftment and/or self-renewal capacities of t(8;21) leukaemia-initiating cells. Future studies will show, whether RNAi-mediated *RUNX1/RUNX1T1* suppression during and after leukaemic engraftment may stop or even reverse tumour formation, consequently paving the way for developing therapeutic approaches directly targeting this leukaemic fusion protein.

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Figure legends

Figure 1: Analysis of *Rag2*$^{-/-}\gamma c^{-/-}$ mice xenotransplanted with t(8;21) AML cells.

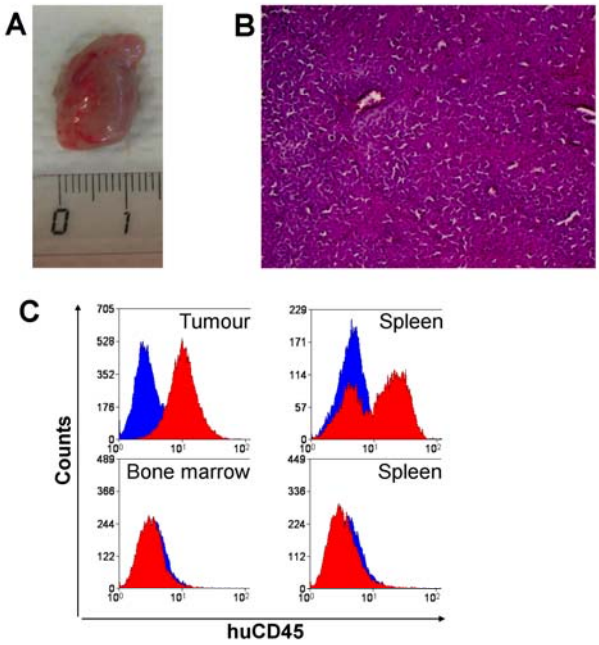
Newborn mice were transplanted with t(8;21)-positive Kasumi-1 cells. Animals had to be humanely killed within 50-70 d because of tumour burden. (A) Tumour excised from the abdominal cavity of an animal transplanted with 10^6 cells. (B) Tumour histology. The animal was transplanted with 2.5 x 10^5 cells pre-treated with the mismatch control siRNA siAGF6. The tumour was stained with Hematoxylin/Eosin. The original magnification was 20x. (C) Flow cytometry analysis of tumour-,spleen- and bone marrow-derived cells. Examples for infiltrated (top right) and non-infiltrated spleens (bottom right) are shown. The red curves show cells stained with α-human CD45 antibody, the blue curves show isotype controls.The top right panel shows an example for a spleen with leukaemic infiltration. (D) Immunoblot analysis for RUNX1/RUNX1T1 expression. Tissues are indicated at the top, detected proteins on the right and length marker sizes on the left. Mouse 1 and 2 were transplanted animals; control indicates a non-transplanted control animal. Kasumi-1 cells served as positive control. RUNX1/RUNX1T1 was detected with an anti-AML1 antibody as described (4). Tubulin (TUB) served as a

loading control. Note the RUNX1/RUNX1T1 signal in spleen lysate from animal 2 indicating weak leukaemic spleen infiltration.

Figure 2: siRNA-mediated *RUNX1/RUNX1T1* suppression decreases leukaemic clonogenicity and extends median survival.

Kasumi-1 cells were electroporated with the indicated siRNAs as described previously (4). Cells were examined for RUNX1/RUNX1T1 protein levels and colony formation *in vitro*, and transplanted into newborn *Rag2*$^{-/-}\gamma c^{-/-}$ mice. (A) Immunoblot for RUNX1/RUNX1T1 expression in siRNA-treated Kasumi-1 cells. siRNA treatments are indicated at the top, detected proteins on the right and length marker sizes on the left. RUNX1/RUNX1T1 and RUNX1 were detected with an anti-AML1 antibody as described (4). Tubulin (TUB) served as a loading control. (B) Colony formation of siRNA-treated Kasumi-1 cells. Colony formation assays were performed as described (4). CFC, colony forming cells. Error bars indicate standard deviations. (C) Survival curves of *Rag2*$^{-/-}\gamma c^{-/-}$ mice transplanted with 10^6 siRNA-treated Kasumi-1 cells. Pre-treatment with the RUNX1/RUNX1T1 siRNA siAGF1 extended median survival significantly compared to mock or control siRNA siAGF6 pre-treatment ($p < 0.02$ according to log-rank test). In each treatment arm, at least 10 mice were transplanted. (D) Survival curves of *Rag2*$^{-/-}\gamma c^{-/-}$ mice transplanted with 2.5×10^5 siRNA-treated Kasumi-1 cells. Pre-treatment with the RUNX1/RUNX1T1 siRNA siAGF1 extended median survival significantly compared to control siRNA siAGF6 pre-treatment ($p < 0.02$ according to log-rank test). In each treatment arm, at least 7 mice were transplanted.

Figure 1


A
B
C
Tumour
Spleen
Bone marrow
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Counts
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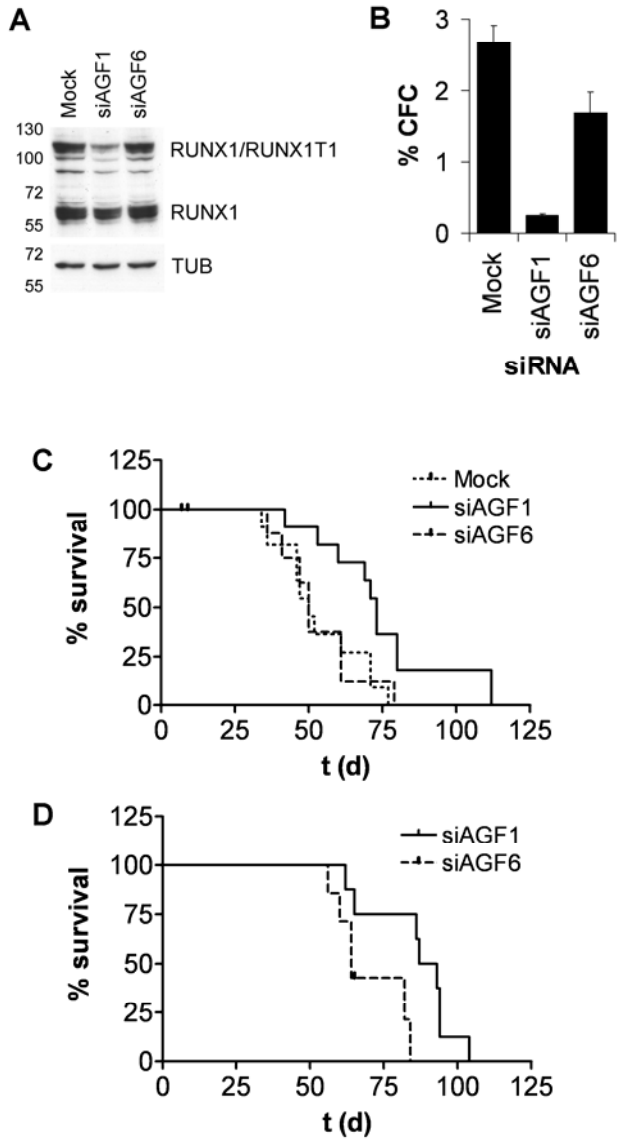
Kasumi-1
Control
Liver
Spleen
Mouse 1
Tumour
Liver
Spleen
Mouse 2
Tumour
Liver
Spleen
130
95
55
RUNX1/RUNX1T1
TUB

Figure 2


A
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siAGF1
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