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High *MN1* expression increases the *in vitro* clonogenic activity of primary mouse B-cells

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Abstract

The *MN1* (Meningioma 1) gene is overexpressed in certain subtypes of acute myeloid leukemia (AML) and high levels of *MN1* expression in mouse bone marrow cells results in myeloid leukemia. We showed that compared with control bone marrow (BM) *MN1* expression was increased (2-fold or more) in 29 out of 73 (40%) pediatric B-cell acute lymphoblastic leukemia (B-ALL) patient BM. Additional analysis of *MN1* expression in sub-groups within our cohort carrying different chromosome translocations showed that carriers of the good prognostic marker *t*(12;21)(TEL–AML1) ($n = 27$) expressed significantly more *MN1* than both healthy controls ($n = 9$) ($P = 0.02$) and the group carrying the *t*(9;22)(BCR–ABL) ($n = 9$) ($P = 0.001$). In addition, *AML1* expression was also upregulated in 31 out of 45 (68%) B-ALL patient BM compared with control and there was a significant correlation between *MN1* and *AML1* expression ($r = 0.3552$, $P = 0.0167$). Retroviral *MN1* overexpression increased the colony forming activity of mouse Pro-B/Pre-B cells *in vitro*. Our results suggest that deregulated *MN1* expression contributes to the pathogenesis of pediatric B-ALL. Further investigation into the clinical and biological significance of elevated *MN1* expression in TEL–AML1^{positive} leukemia might provide insight into additional molecular mechanisms contributing to B-ALL and may lead to improved treatment options for patients.

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Conflict of interest statement
The authors declare no conflict of interest.

Contributions
A.K., G.G., and M.N. contributed to the design and analysis of this study. M.N. and M.D.Y. performed the experiments. M.C., S.T., R.K.G., performed RT-qPCR analysis of transduced mouse pro B-cells. E.Ö. performed statistical analysis. S.S.E., M.A., U.Ö., Ç.G., S.A., S.K., G.Ö., M.A., provided reagents. A.K., G.G., and M.N. wrote this manuscript, and all authors agreed on the final version.

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Keywords

MN1; ALL; TEL–AML1; AML1

1. Introduction

The involvement of the *MN1* gene in leukemia was initially characterized in AML and myelodysplastic syndrome (MDS) as part of the *MN1–TEL* fusion gene, which is generated by a *t*(12;22)(p13.2;q12.1) [1]. Subsequent studies showed that *MN1* expression is upregulated in subgroups of AML patients over-expressing the EVI-1 gene or carrying inv(16)(CBFB–MYH11) and *t*(8;21)(AML1–ETO) translocations [2]. CBFB–MYH11 and AML1–ETO each disrupt a different subunit of the core-binding factor (CBF) transcription factor complex [3,4]. Others and we have previously shown that ectopic expression of *MN1* in mouse bone marrow cells results in myeloid leukemia [2,5]. Additionally, overexpression of *MN1* in both mouse and human hematopoietic stem/progenitor cells causes abnormal proliferation and arrest of myeloid differentiation [6] whereas suppression of *MN1* expression in leukemia cell lines prevents their proliferation [7]. Despite this growing body of evidence describing *MN1*'s involvement in AML development, it is unknown if *MN1* also plays a role in the pathogenesis of lymphoblastic leukemia. Possible involvement of *MN1* was suggested by a recent study describing increased *MN1* expression in early T-cell acute lymphoblastic leukemia (ETP-ALL) as compared with T-cell acute lymphoblastic leukemia (T-ALL) [8].

Here, we analyzed the expression level of *MN1* in pediatric B-ALL patients carrying either the *TEL–AML1*, the *MLL–AF4*, or the *BCR–ABL* fusion genes, or patients who are negative for these translocations (TNB-ALL). In addition, we analyzed the expression of *AML1* (*RUNX1*), which is one of the subunits of CBF, in TEL–AML1^{positive} and TNB-ALL patient BM samples for which we had sufficient RNA. Finally, we determined the effect of the ectopic *MN1* expression on the *in vitro* clonogenic capability of primary mouse pro-B and pre-B cells.

2. Materials and method

2.1. Patient samples

Bone marrow (BM) samples of 73 pediatric B-ALL patients obtained at diagnosis (48 males, 25 females) and 9 healthy BM samples (5 males, 4 females) from pediatric BM transplantation donors were included in our study. Age distribution of patients and healthy controls was between 4 months-15 years and 6 months-15 years, respectively. One of the controls was 23 years old and because the RT-qPCR result of this control sample was similar to the other control samples, it was included in the control group. All patients were diagnosed based on FAB criteria and they were positive for one of three translocation products (TEL–AML1, BCR–ABL, MLL–AF4) or they were negative for these three translocations (TNB-ALL). The study was approved by the institutional review board (Istanbul University, Istanbul Medical Faculty, 2012/1045–1131) and informed consent was

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obtained from parents, guardians, or patients (based on the age of patients and donors) in accordance with the Decleration of Helsinki.

2.2. RT-qPCR

RNA extraction and cDNA synthesis of human BM samples and cell lines were performed as described [9] using the RNeasy total RNA extraction kit (Qiagen, Germany) and a cDNA synthesis kit (Roche Diagnostics, Germany). Real time PCR (qPCR) was performed using a LightCycler Instrument (Roche) and TaqMan primer/probe sets for *MN1* (Applied Biosystems, assay ID: Hs00159202_m1), *AML1* (Applied Biosystems assay ID: Hs02800695_m1) and the *HPRT* reference gene (Applied Biosystems, assay ID: 4326321E). Normalized *MN1* and *AML1* expression values (*MN1/HPRT*) were calculated using the standard curve and/or $2^{\ \ \ Ct}$ method. In addition, dividing the normalized values of the patient samples by the calibrator (average of the normalized *MN1* expression value of the 9 healthy controls) allowed us to express the differences as a fold change. RT-qPCR for the detection and normalization of *MN1* in the human cell lines was performed as described above using a 7500 Real-Time PCR system (Applied Biosystems).

Total RNA (2–5 μg) of primary murine B-cells was extracted with TRIzol Reagent (Invitrogen) and treated with DNase I (Invitrogen). First strand cDNA was synthesized using Oligo-dT priming and the SuperScript III First Strand Synthesis System (Invitrogen). RT-qPCR analysis was performed using TaqMan Gene Expression Master Mix (Applied Biosystems) and the TaqMan primer probe sets for *Mn1* (Applied Biosystems, assay ID: Mm03038457_m1) and the *Hprt* reference gene: probe (5′-CGAGCAAGTCTTTCAGTCCTGTCCA-3′), forward primer (5′-ATTATGCCGAGGATTTGGAA-3′), and reverse primer (5′-CCCATCTCCTTCATGACATCT-3′).

2.3. Retrovirus preparation

Retrovirus encoding MSCV-IRES-GFP (MIG) or MSCV-MN1-IRES-GFP (MIG-MN1) was prepared as described previously [2]. Briefly, the virus was produced in 293T cells by transient transfection of MIG or MIG-MN1 together with retroviral packaging plasmids [2]. Forty eight hours after transfection the virus was harvested and immediately used for transduction of lineage-negative (Lin^-) mouse bone marrow cells.

2.4. Mouse B-cell isolation and viral transduction

Mouse BM cells were harvested from femurs and tibiae of 8- to 10-week-old C57BL/6 mice ($n = 4$ to 5). The mice were sacrificed by CO_2 inhalation and the BM cells were flushed out into ice-cold PBS with 1% FBS using a 10 ml syringe with a 25-gauge needle. Red blood cells were lysed in Gey's solution (150 mM NH4Cl, 1 mM KHCO3, and 0.1 mM EDTA pH7.4). The cells were incubated with biotin-labeled antibodies (anti-Mac1, -Gr1, -Ter119, and -CD5, BD Biosciences) to deplete lineage-positive cells by using streptavidin-conjugated magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen). The lineage-depleted BM cells were cultured in IMDM with 20% heat-inactivated FBS, 55 μM β-mercaptoethanol, 200 mM GlutaMAX (Gibco), 10 mM HEPES (Gibco), 1× MEM Non-Essential Amino Acids (Gibco), and 1× penicillin and streptomycin (Gibco) supplemented

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with 10 ng/ml mSCF (PeproTech), 10 ng/ml mIL-7 (Invitrogen), and 10 ng/ml mFlt3 ligand (PeproTech) for 48 h prior to virus transduction. The cells were transferred to Retronectin-coated plates (Takara) and incubated with 0.22 μm filtered-retrovirus supernatant for two days in the presence of 8 μg/ml polybrene (Sigma-Aldridge). One day after the last transduction, the virus-transduced cells were labeled with anti-B220-eFluor605, -IgM-PE-Cy7, -IgD-APC, and -CD43-PE antibodies (BD Biosciences or eBioscience) and directly sorted into MethoCult M3630, (StemCell Technologies) using a BD FACSAria III Cell Sorter (BD Biosciences). For RT-qPCR of endogenous mouse *Mn1* and exogenous human *MN1* in transduced mouse bone marrow cells, we followed the same preparation procedure as above with the distinction that after transduction of Lin^- cells with MN1 or control GFP retrovirus the cells were plated on S-17 feeder cells [10] in the presence of rhIL-7 (50 μg/ml) to induce B-cell differentiation. After 2 weeks of culture both the MN1 and vector transduced cells were sorted for expression of $GFP^+/B220^+$ and expanded in rhIL-7 culture medium for an additional 2 weeks. RNA was extracted (Trizol) and 350 ng of each served as template for first strand cDNA synthesis using the SuperScript III First Strand Synthesis System (Invitrogen) and random hexamer priming. The level of human *MN1* and mouse *Mn1* versus endogenous mouse *Hprt* expression was determined using cyber green RT-qPCR analysis using the following probe sets:

MN1 forward-GAAGGCCAAACCCCAGAAGC

MN1 reverse-GATGCTGAGGCCTTGTTTGC

Mn1 forward-TGGTGGAGATGAGGACAAGA

Mn1 reverse-CTTGGGGTCACCATCTGTG

Hprt forward-CCC ATC TCC TTC ATG ACATCT

Hprt reverse-ATT ATG CCG AGG ATTTGG AA

2.5. Colony-forming unit (CFU) assay

MIG or MIG-MN1 transduced murine pro B-cells ($GFP^+B220^+CD43^+IgM^-$) were plated in methylcellulose-based media supplemented with rhIL-7 (MethoCult M3630, StemCell Technologies) at a density of 10,000 cells per dish (MC1). Colonies were counted seven days later, pooled, and replated into a secondary methylcellulose culture (MC2). MC1 colonies in each dish were photographed and the immunophenotype (GFP, B220, IgM, CD43) of the harvested B-cells from MC1 cultures was analyzed by FACS.

2.6. Statistical analysis

Pearson correlation, Mann–Whitney or Kruskal–Wallis analyses was performed using GraphPad Prism, version 4.0c for Mac or Prism, version 5.0 for Windows (GraphPad Software; www.graphpad.com).

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3. Results

3.1. MN1 expression is increased in TEL–AML1^{positive} patients

Using RT-qPCR we determined the *MN1* expression in BM samples of 73 pediatric B-ALL patients at diagnosis consisting of patients positive for one of three recurrent chromosome translocations [*t*(12;21)(TEL–AML1) ($n = 27$), *t*(9;22)(BCR–ABLp190) ($n = 9$), *t*(4;11) (MLL–AF4) ($n = 7$)] and patients negative for these translocations (TNB-ALL) ($n = 30$). Our results showed that compared with the average *MN1* expression in 9 healthy control samples, 40% of ALL patient samples showed 2-fold up regulated *MN1* expression (12/30 of TNB-ALL; 12/27 of TEL–AML1; 1/9 of BCR–ABL; 4/7 of MLL–AF4) and 21% of patient samples showed 2-fold down regulated *MN1* expression (2/27 of TEL–AML1; 5/9 BCR–ABL; 2/7 MLL–AF4; and 7/30 TNB-ALL). Statistical analysis revealed that only the TEL–AML1^{positive} patient samples expressed significantly higher levels of *MN1* than healthy control samples ($P = 0.02$) (Fig. 1A). In addition, the mean expression was higher in the TNB-ALL group due to relatively higher *MN1* expression in 3 patient samples as shown in Fig. 1A and B. By ordering the samples in each group based on the *MN1* expression level (from lowest to highest), there was a notable trend of increased *MN1* expression in TEL–AML1^{positive} patient samples as compared with TNB-ALL, with 22 out of 27 TEL–AML1^{positive} samples expressing a higher level of *MN1* (Fig. 1B). Consistent with these results, RT-qPCR analysis of *MN1* expression in different human leukemia cell lines also detected increased *MN1* expression both in REH (TEL–AML1^{positive}) and ME-4 (inv(16)^{positive}) cells, the former showing the highest expression (Fig. 1C).

Additional comparison between the different translocation groups indicated that BCR–ABL^{positive} patient samples exhibited significantly decreased *MN1* expression when compared with the TNB-ALL ($P = 0.04$) or TEL–AML1^{positive} group (Fig. 1A). When all 73 patients were grouped based on age or white blood cell count (WBC), the difference in the *MN1* expression was not significant (Table 1).

Given that AML1 is targeted by TEL–AML1 fusion, we next analyzed the expression of wildtype *AML1* [11] in the same TEL–AML1^{positive} ($n = 17$) and TNB-ALL ($n = 28$) samples, for which there was still sufficient RNA on hand. RT-qPCR analysis indicated that *AML1* expression was significantly higher in TEL–AML1^{positive} patients compared with that in both control ($n = 9$) and TNB-ALL samples (Fig. 1D). *MN1* and *AML1* expression correlated significantly in the total number of analyzed B-ALL ($n = 45$) samples or in the TNB-ALL group ($r = 0.3552$, $P = 0.0167$ and $r = 0.5779$, $P = 0.0013$, respectively) (Fig. 1E), whereas the correlation in the TEL–AML1^{positive} group was not significant ($r = 0.2955$, $P = 0.2495$). This was probably due to the small number of patient samples (Fig. 1E, right panel) analyzed.

3.2. Primary mouse pro-B cells show higher Mn1 expression compared to pre-B and immature B-cells

The observation that *MN1* expression was relatively high in B-ALL patients prompted us to analyze the expression level of endogenous *Mn1* at different developmental stages of primary mouse B-cells. RT-qPCR analysis of FACS-sorted mouse pro-B

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($B220^+IgM^-CD43^+$), pre-B ($B220^+IgM^-CD43^-$), immature-B ($B220^+IgM^+IgD^-$) and mature-B ($B220^+IgM^+IgD^+$) cells (Fig. 2A) showed that *Mn1* expression was highest in the pro-B cells whereas it was not detectable in pre-B and immature-B cells (Fig. 2B). These results suggest that the *Mn1* expression level changed during the development of mouse B-cells.

3.3. Ectopic MN1 expression increases colony-forming activity of Mouse Pro-B cells

Based on our results showing that mouse pro-B cells express increased levels of endogenous *Mn1* (Fig. 2B) and *MN1* expression was upregulated in B-ALL patient samples (Fig. 1A and B), we next wished to determine the effect of ectopic *MN1* expression in primary mouse pro-B cells, a B cell differentiation state from which human B-ALL can arise [12]. Lineage-depleted BM cells were transduced with MIG or MIG-MN1 retrovirus (ectopic human *MN1* expression in transduced mouse pro-B cells was confirmed by RT-qPCR (Fig. 3A)), followed by FACS to directly seed sorted GFP-positive pro-B ($GFP^+B220^+IgM^-CD43^+$) cells into MethoCult medium and assess their CFU-pre-B colony forming activity (Fig. 3B). MN1-transduced pro B-cells (MIG-MN1) produced 6-fold more CFU-pre B cell colonies than non-transduced pro-B cells (Mock) or control pro-B cells (MIG) (Fig. 3C). In addition, the MIG-MN1 pre-B cell colonies were larger than MIG control colonies (Fig. 3D). FACS analysis of MIG and MIG-MN1 CFU-pre B colonies showed no difference in immunopheotype as both mainly consisted of $GFP^{positive}$ pro-B or pre-B cells (Fig. 3D). Replating cells from the first methocult (MC1) into secondary cultures (MC2) diminished the colony forming activity in both MIG and MIG-MN1 cultures. These results indicated that overexpression of human *MN1* in murine pro-B cells enhanced their proliferation and colony forming activity.

4. Discussion

Treatment protocols of leukemia patients are mainly based on clinical and molecular parameters, including the presence of recurrent chromosome translocations producing oncogenic fusion proteins. AML1–ETO, CBFB–MYH11 and TEL–AML1 fusions are associated with a good response to therapy and favorable prognosis [13,14], and all disrupt one of the two subunits of the CBF transcription factor complex AML1(RUNX1)/CBFB [3]. Based on previous findings showing that AML patients with AML1–ETO or CBFB–MYH11 express higher levels of *MN1* compared with control BM or other AML subgroups [2] we assessed if *MN1* expression was also deregulated in patients with TEL–AML1, a fusion protein associated with a good prognosis, which occurs in about 22% of pediatric B-ALL patients [13]. We show that expression of *MN1* is higher in certain subgroups of pediatric B-ALL patients, revealing a notable trend toward increased expression in the patients with TEL–AML1. Ectopic *MN1* expression increased the size and number of primary mouse pro-B/pre-B cell-colonies in methylcellulose assays, thereby functionally linking a high level of *MN1* to an increased colony forming activity of these cells, *in vitro*.

In addition to patients with TEL–AML1, we also analyzed *MN1* expression in a small cohort of ALL patients carrying BCR–ABL or MLL–AF4 fusions. Although the patients expressing the poor prognostic factor BCR–ABL displayed significantly lower *MN1*

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expression when compared with TNB-ALL or TEL–AML1^{positive} patients, the difference was not significant when compared with that in control samples.

Our results raise intriguing questions as to the mechanisms driving the increased *MN1* expression in leukemia. It was shown before by us and others that *MN1* expression is higher in human CD34^{+} cells [7,15] and decreases during myeloid differentiation in culture [6]. Here we also showed that mouse pro-B-cells express more *Mn1* (Fig. 2A) than more mature B-cells. Therefore, although increased *MN1* expression could be reflecting the immature characteristic of the B-ALL cells, it also raises the possibility that expression of the *MN1* could be under direct or indirect regulation by the CBF transcription factor complex, given that the gene rearrangements targeting this complex are associated with increased *MN1* in AML [2] and B-ALL (this study). Interestingly, expression of *AML1* was also upregulated in B-ALL, showing a significant correlation with *MN1* expression. Our results form the basis for future studies toward the delineation of the molecular mechanisms of normal and abnormal MN1 expression in B-ALL.

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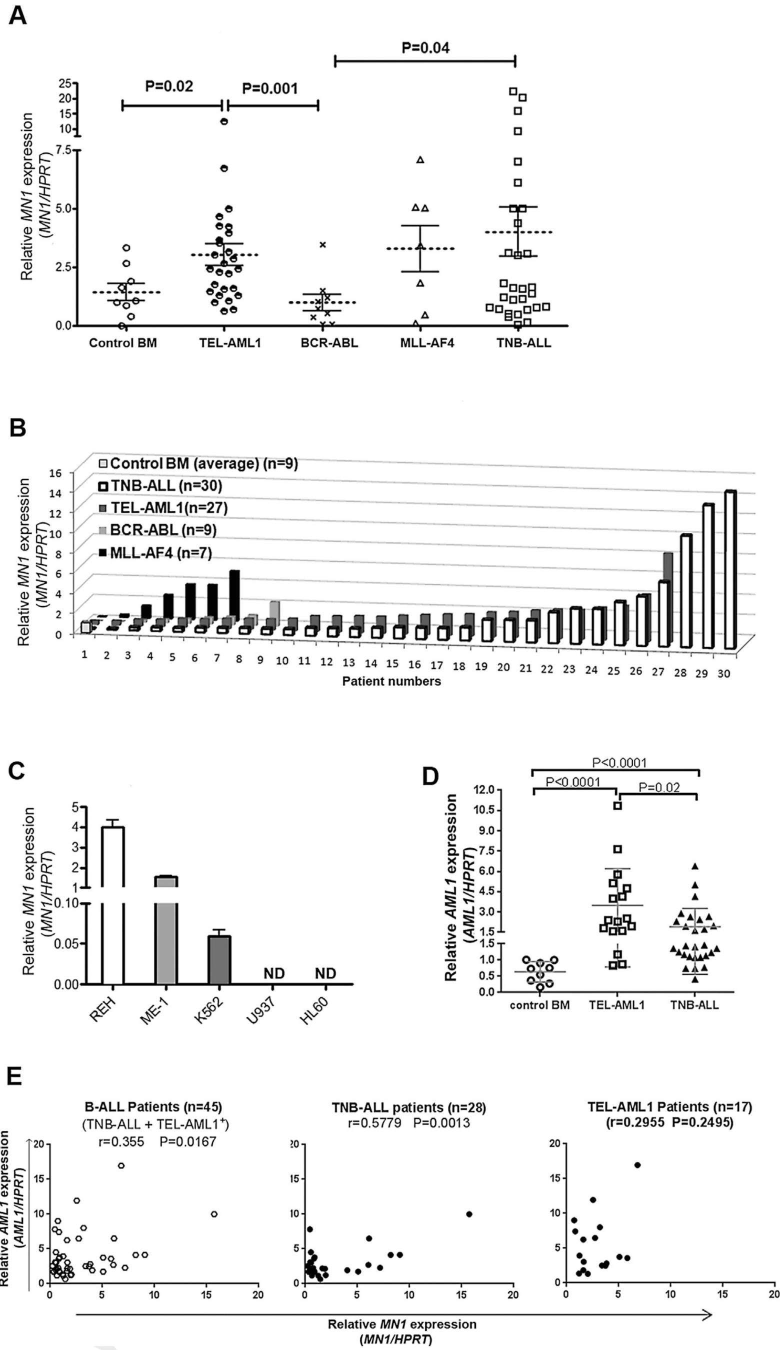

Fig. 1. MN1 expression in different subtypes of B-ALL

(A) RNA samples of patients were analyzed by RT-qPCR and normalized *MN1* expression levels (mean of triplicates). The respective P values of the *t*(9;22)(BCR–ABL), *t*(4;11) (MLL–AF4) and translocation negative (TNB-ALL) group were compared with the control (0.391, 0.71 and 0.189, respectively) and the *t*(12;21)(TEL–AML1) group (0.001, 0.834 and 0.457, respectively). Selected P values are indicated in the graph. (B) The results shown in panel A are depicted as a bar graph to more clearly show the difference in *MN1* expression in each patient. (C) RT-qPCR analysis of endogenous *MN1* expression in different human

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leukemia cell lines (mean of triplicates ± SEM) (ND = not determined). (D) RT-qPCR analysis of *AML1* expression (mean of triplicates). (E) Pearson correlation of *MN1* and *AML1* expression. Fold-expression values relative to the average of 9 controls are plotted in all analyzed B-ALL (left panel) or in TNB-ALL (middle panel) and TEL–AML1^{+} group (right panel).

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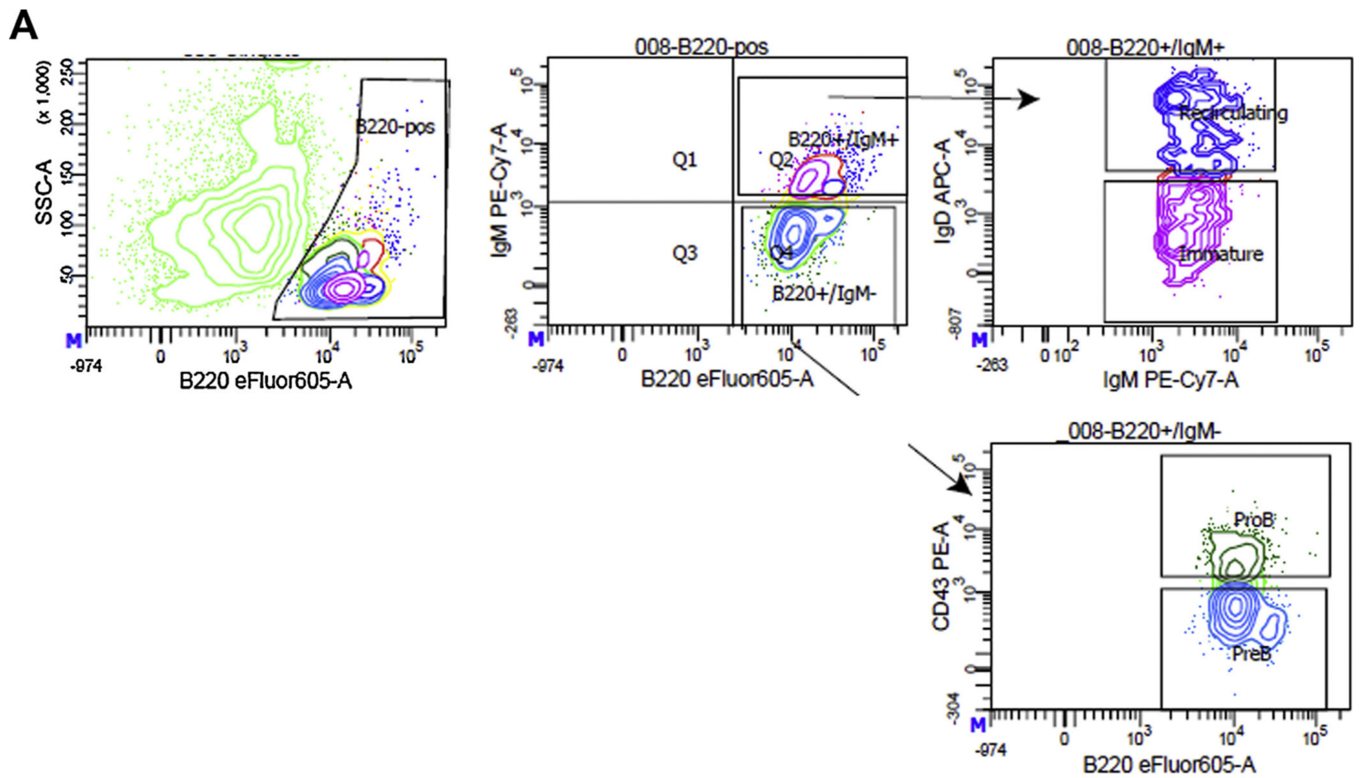

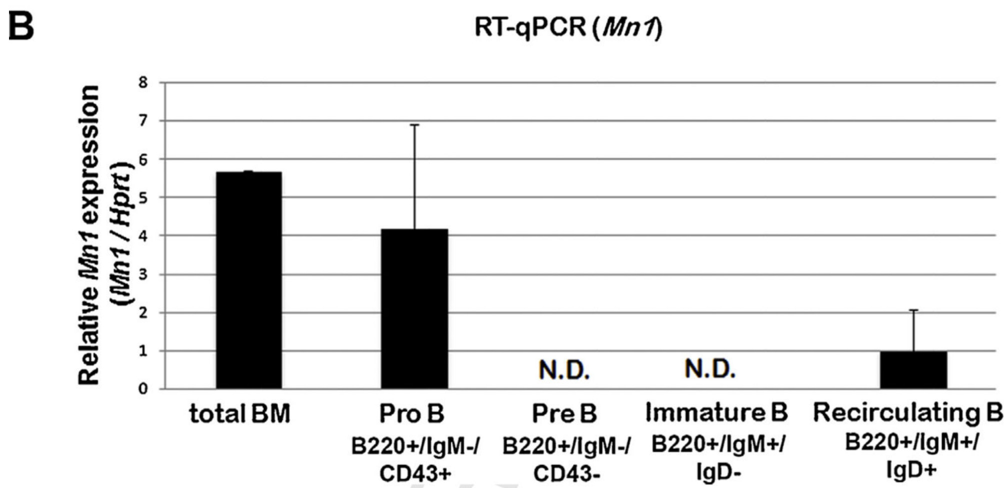

Fig. 2. Expression level of endogeneous Mn1 mRNA in various primary B-cells

(A) Data showing the isolation of distinct differentiation stages of wild-type B-lineage cells from murine BM using FACS. (B) RNA from the FACS-isolated cell fractions was examined for *Mn1* expression by RT-qPCR. The relative expression level of *Mn1* was normalized for *Hprt* expression and shown in the graph as a mean of triplicates ± SEM.

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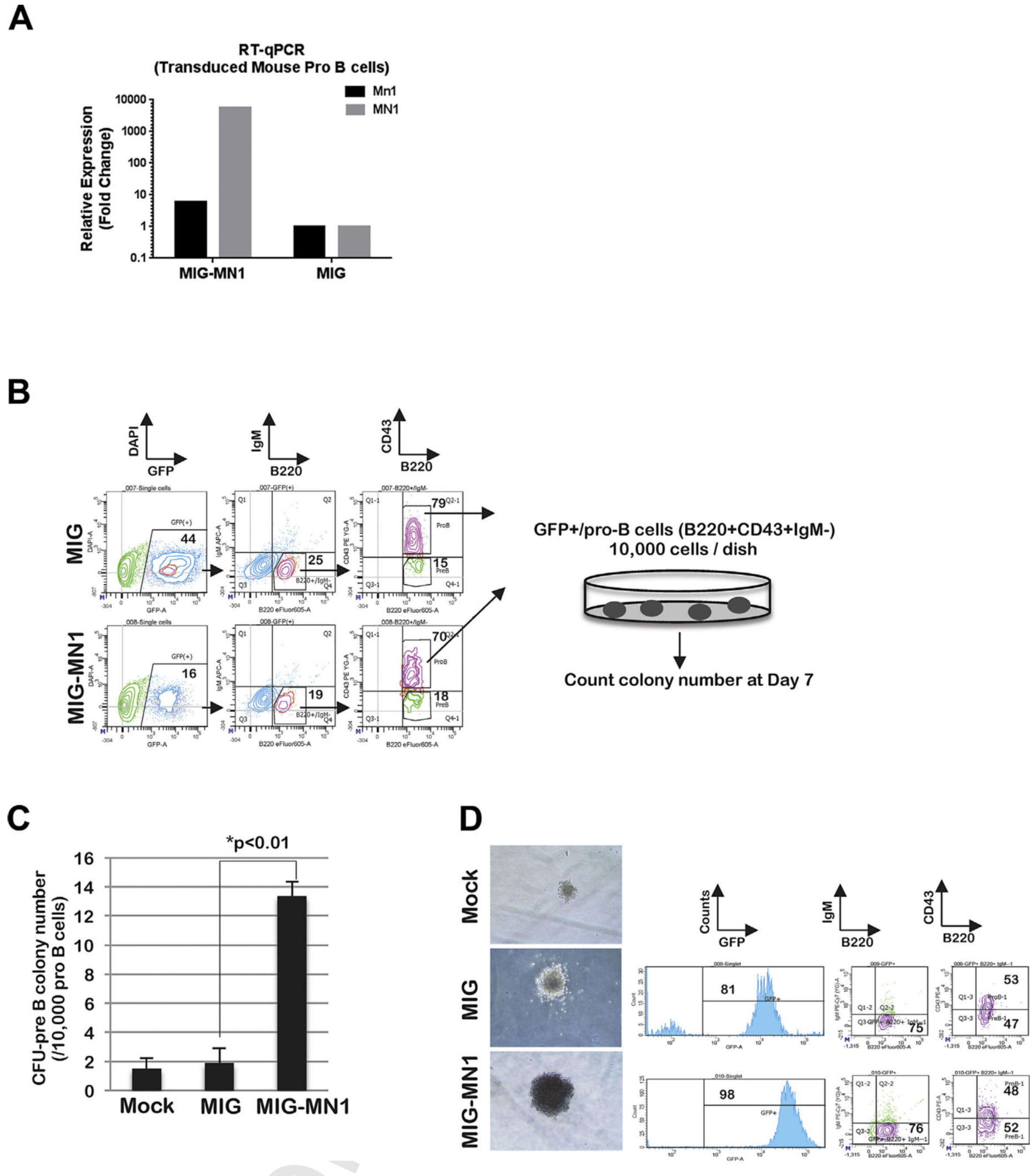

Fig. 3. Overexpression of MN1 enhances the colony forming activity of murine pro B-cells

(A) RT-qPCR analysis of ectopic human *MN1* and endogenous mouse *Mn1* expression in MIG-MN1 or MIG-transduced mouse pro-B cells. Relative expression level of *Mn1* or *MN1* was normalized for *Hprt* expression and shown as fold-change relative to MIG-transduced cells. (B) FACS isolation diagrams of GFP^{positive} pro B-cells (B220^{+}IgM^{-}CD43^{+}) from MIG and MIG-MN1 transduced BM (left) and an explanatory scheme of the subsequent methylcellulose (MethoCult) (MC1) assay (right). GFP^{positive} pro-B cells (10^6 per dish) were seeded and the number of CFU-pre-B colonies was counted 7 days later. (C) The

colony numbers obtained in panel B are depicted by a bar graph showing the results of three independent experiments (* $P < 0.01$). (D) Representative images of CFU-pro-B/pre-B colonies in the mock, MIG, or MIG-MN1 plates are shown in the left panel and FACS analysis of MIG and MIG-MN1 CFU-pro-B/pre-B colonies is shown in the panel on the right.

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Table 1

Median *MN1* expression in B-ALL patients.

	Number of patients (%)	MN1 expression (median)	*P* value
Age (year) (*n* = 73)			
1	2 (2.7)		
1–10	56 (76.7)	2.22	0.590#
10	15 (20.5)	1.38	
WBC (*n* = 67)			
1.000–10.000	18 (26.8)	1.44	0.468#
10.000–50.000	29 (43.2)	1.81	
50.000	20 (29.8)	2.36	
***t*(4;11)**			
Positive	7	3.44	0.658
Negative	30	1.57	
***t*(9;22)**			
Positive	9	0.73	0.042*
Negative	30	1.57	
***t*(12;21)**			
Positive	27	2.55	0.258
Negative	30	1.57	

P value: Kruskal–Wallis (#) or Mann–Whitney test.

*Statistically significant.