

Bone marrow mesenchymal stem cells from infants with MLL-AF4⁺ acute leukemia harbor and express the MLL-AF4 fusion gene

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MLL-AF4 fusion is a hallmark genetic abnormality in infant B-acute lymphoblastic leukemia (B-ALL) known to arise in utero. The cellular origin of leukemic fusion genes during human development is difficult to ascertain. The bone marrow (BM) microenvironment plays an important role in the pathogenesis of several hematological malignancies. BM mesenchymal stem cells (BM-MSC) from 38 children diagnosed with cytogenetically different acute leukemias were screened for leukemic fusion genes. Fusion genes were absent in BM-MSCs of childhood leukemias carrying TEL-AML1, BCR-ABL, AML1-ETO, MLL-AF9, MLL-AF10, MLL-ENL or hyperdiploidy. However, MLL-AF4 was detected and expressed in BM-MSCs from all cases of MLL-AF4⁺ B-ALL. Unlike leukemic blasts, MLL-AF4⁺ BM-MSCs did not display monoclonal Ig gene rearrangements. Endogenous or ectopic expression of MLL-AF4 exerted no effect on MSC culture homeostasis. These findings suggest that MSCs may be in part tumor-related, highlighting an unrecognized role of the BM milieu on the pathogenesis of MLL-AF4⁺ B-ALL. MLL-AF4 itself is not sufficient for MSC transformation and the expression of MLL-AF4 in MSCs is compatible with a mesenchymal phenotype, suggesting a differential impact in the hematopoietic system and mesenchyme. The absence of monoclonal rearrangements in MLL-AF4⁺ BM-MSCs precludes the possibility of cellular plasticity or de-differentiation of B-ALL blasts and suggests that MLL-AF4 might arise in a population of prehematopoietic precursors.

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Abbreviations used: B-ALL, B-acute lymphoblastic leukemia; FISH, fluorescence in situ hybridization; HSC, hematopoietic stem cell; MSC, mesenchymal stem cell.

Pediatric leukemias possess unique biological features. They are characterized by the presence of tumor-specific chromosomal translocations that entail the generation of oncogenic fusion genes (Pui et al., 2008). These chromosome translocations contribute to the molecular pathogenesis of childhood leukemia, and many are well characterized, defining the different subtypes of childhood leukemia (Wiemels et al., 2009). There is compelling evidence that several of the common chromosome translocations (i.e., MLL-AF4, TEL-AML1, and AML1-ETO) that are seen in pediatric leukemia often originate prenatally in utero during embryonic/fetal

development (Ford et al., 1993; Greaves and Wiemels, 2003; Bueno et al., 2009).

The cellular origin of translocations within the stem cell hierarchy of the hematopoietic system is difficult to ascertain, particularly as the functional impact of the translocation and resulting clonal expansion can occur downstream of the origin of the translocation (Greaves and Wiemels, 2003). Stem cells are the main target

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for oncogenic events (McCulloch, 1983; Reya et al., 2001). Stem cells are essential for embryogenesis, and their vulnerability to cancer development might be seen as an evolutionary trade-off for their unique properties (Weissman, 2000). Furthermore, many cell signaling pathways and transcription factors essential for normal embryonic development are also master regulators involved in cancer onset and progression, supporting a strong link between embryonic/fetal development and cancer (Clark et al., 2007; Deshpande and Buske, 2007; Dreesen and Brivanlou, 2007; Bueno et al., 2007, 2009; Grigoryan et al., 2008; Jiang and Hui, 2008; Laird et al., 2008).

The cellular organization and relationships among precursors that initiate embryonic angiogenesis and hematopoiesis in the human have been characterized (Wang et al., 2004; Menendez et al., 2004). A bipotent primitive hemangioblast derived from human embryonic stem cells is uniquely responsible for endothelial and hematopoietic development (Wang et al., 2004; Menendez et al., 2004). The detection of the BCR/ABL oncogene and lymphoma-specific genetic aberrations in endothelial cells from chronic myelogenous lymphoma and B cell lymphoma patients suggests that endothelial cells may be part of the neoplastic clone (Gunsilius et al., 2000; Streubel et al., 2004; Fang et al., 2005), and that hemangioblasts rather than hematopoietic stem cells (HSCs) appear to be target cells for the first oncogenic hit, which could occur during the first steps of embryonic stem cell differentiation and/or in hemangioblasts persisting in adults (Prindull, 2005).

The existence during development of mesendodermal progenitors that are multipotent precursors common for the vasculature and for a variety of mesoderm-derived tissues has long been suggested (Waller et al., 1995; Minasi et al., 2002; Cossu and Bianco, 2003; Tada et al., 2005; Bakre et al., 2007). Furthermore, the BM hematopoietic microenvironment plays a role in the pathogenesis of a variety of hematological malignancies, including acute leukemia, multiple myeloma, lymphomas, or myelodysplastic syndrome (Streubel et al., 2004; Blau et al., 2007; Corre et al., 2007; Walkley et al., 2007; Lopez-Villar et al., 2009). Mesenchymal stem cells (MSCs) are key components of the BM milieu, and many efforts are being undertaken to assess their role in several hematopoietic tumors (García-Castro et al., 2008).

During in utero development, leukemic fusion genes may arise in a population of mesodermal prehematopoietic precursors that would give rise throughout development to a variety of mesoderm-derived tissues, including HSCs and MSCs. However, the question of whether BM-MSCs from childhood leukemia harbor leukemia-specific fusion genes has not been addressed.

Here, fusion genes were not detected in BM-MSCs from any subtype of childhood leukemia, but in infant MLL-AF4⁺ B-acute lymphoblastic leukemia (B-ALL) MLL-AF4 was detected and expressed in BM-MSCs from all patients studied. MLL-AF4⁺ BM-MSCs displayed no monoclonal Ig gene rearrangements. Expression of MLL-AF4 in normal BM-MSCs

is compatible with a mesenchymal phenotype. Our data suggest a differential impact of MLL-AF4 in the hematopoietic system and mesenchyme.

RESULTS

Establishment of BM-MSC cultures from children with different acute leukemias

MSC cultures were successfully established and expanded from the BM of 38 children diagnosed with distinct subtypes of childhood acute leukemia defined by specific chromosomal abnormalities. Table I depicts how leukemic patients were grouped according to specific cytogenetic abnormalities. BM-MSCs displayed typical fibroblastoid morphology (Fig. 1 A) and immunophenotype (Fig. 1 B). MSC cultures were consistently devoid of contaminating hematopoietic cells, being negative for CD45, CD34, HLA-DR, CD19, and CD14, but express common MSC markers, including CD90, CD73, CD105, CD166, and CD106 (Fig. 1 B). To further characterize MSCs from pediatric acute leukemias, adipogenic and osteoblastic differentiation assays were performed as suggested (Dominici et al., 2006; Fig. 1 C). Osteoblastic and adipogenic differentiation was achieved with a similar efficiency than those of normal BM-MSCs (Fig. S1). Thus, MSCs derived from a variety of pediatric leukemic BM samples seem to be phenotypically and functionally similar to those from healthy donors.

MLL-AF4 is present and expressed in BM-MSCs from infants with MLL-AF4⁺ B-ALL

We determined by fluorescence in situ hybridization (FISH), whether BM-MSCs from cytogenetically different subtypes of pediatric acute leukemias share the specific genetic aberrations present in the leukemic blasts. The corresponding leukemic fusion gene could never be detected in BM-MSCs from childhood acute leukemias carrying TEL-AML1, BCR-ABL, AML1-ETO, MLL-AF9, MLL-AF10, or MLL-ENL fusions or hyperdiploidy (Table I and Fig. 2 A). In contrast, MLL-AF4 was detected in $6.8 \pm 1.7\%$ of BM-MSCs from all the cases of MLL-AF4⁺ infant B-ALL (Table I and Fig. 2 A).

We next examined whether MLL-AF4⁺ BM-MSCs express the MLL-AF4 transcript. MLL-AF4 was expressed in MLL-AF4-carrying BM-MSCs and in the leukemic blasts as assessed by real-time RT-PCR (Fig. 2 B), indicating that among the cytogenetically distinct pediatric acute leukemias, the leukemia-specific fusion gene is only present and expressed in the stroma microenvironment from infants with the aggressive MLL-AF4⁺ pro-B-ALL.

V(D)JH monoclonal rearrangements were present in MLL-AF4⁺ leukemic blast cells, but absent in BM-MSCs from infants with MLL-AF4⁺ B-ALL

Monoclonal Ig gene rearrangements were performed to exclude contamination of the MSC cultures by leukemic cells and to rule out de-differentiation of ALL blasts into MSCs. Using PCR, gene scanning, and sequencing, we characterized

Table I. Presence of leukemic fusion genes (and hyperdiploidy) in BM-MSCs from a cohort of infants/children with cytogenetically distinct acute leukemia

Patient ID	Diagnosis	Cytogenetics (fusion gene)	Age (months)	Fusion gene in MSC ^a	Positive cases in each diagnostic group
1	Pre-B-ALL	t(12;21) TEL-AML1	30	NO	0/12 (0%)
2	Pre-B-ALL	t(12;21) TEL-AML1	24	NO	
3	Pre-B-ALL	t(12;21) TEL-AML1	36	NO	
4	Pre-B-ALL	t(12;21) TEL-AML1	42	NO	
5	Pre-B-ALL	t(12;21) TEL-AML1	70	NO	
6	Pre-B-ALL	t(12;21) TEL-AML1	84	NO	
7	Pre-B-ALL	t(12;21) TEL-AML1	42	NO	
8	Pre-B-ALL	t(12;21) TEL-AML1	96	NO	
9	Pre-B-ALL	t(12;21) TEL-AML1	60	NO	
10	Pre-B-ALL	t(12;21) TEL-AML1	48	NO	
11	Pre-B-ALL	t(12;21) TEL-AML1	30	NO	
12	Pre-B-ALL	t(12;21) TEL-AML1	54	NO	
13	M2-AML	t(8;21) AML1-ETO	144	NO	0/1 (0%)
14	Pre-B-ALL	t(9;22) BCR-ABL	72	NO	0/5 (0%)
15	Pre-B-ALL	t(9;22) BCR-ABL	48	NO	
16	Pre-B-ALL	t(9;22) BCR-ABL	60	NO	
17	Pre-B-ALL	t(9;22) BCR-ABL	72	NO	
18	Pre-B-ALL	t(9;22) BCR-ABL	72	NO	
19	T-ALL	Hyperdiploid	30	NO	0/10 (0%)
20	Pre-B-ALL	Hyperdiploid	48	NO	
21	Pre-B-ALL	Hyperdiploid	24	NO	
22	Pre-B-ALL	Hyperdiploid	36	NO	
23	Pre-B-ALL	Hyperdiploid	36	NO	
24	Pre-B-ALL	Hyperdiploid	48	NO	
25	Pre-B-ALL	Hyperdiploid	142	NO	
26	Pre-B-ALL	Hyperdiploid	24	NO	
27	Pre-B-ALL	Hyperdiploid	60	NO	
28	Pre-B-ALL	Hyperdiploid	18	NO	
29	Pro-B-ALL	t(4;11) MLL-AF4	6	YES (4%)	4/4 (100%)
30	Pro-B-ALL	t(4;11) MLL-AF4	11	YES (7%)	
31	Pro-B-ALL	t(4;11) MLL-AF4	6	YES (7%)	
32	Pro-B-ALL	t(4;11) MLL-AF4	4	YES (8%)	
33	M5-AML	t(9;11) MLL-AF9	180	NO	0/6 (0%)
34	M5-AML	t(9;11) MLL-AF9	12	NO	
35	M2-AML	t(9;11) MLL-AF9	7	NO	
36	M5-AML	t(10;11) MLL-AF10	15	NO	
37	Pre-B-ALL	t(11;19) MLL-ENL	7	NO	
38	AML	t(11;19) MLL-ENL	9	NO	

^aNumber in parentheses represents percentage of MSCs.

the presence of monoclonal rearrangements of the heavy chain of Ig genes in genomic DNA from MLL-AF4⁺ leukemic blast cells from the available cases. As expected, they displayed at least one monoclonal V(D)JH rearrangement identifiable by PCR and gene scanning. Patient A had one incomplete monoclonal DJH (DH2*2-JH5*02; Fig. 3 A). Patient B had two identifiable monoclonal rearrangements, one complete VDjh (VH6-1*01/IGHD2-2*01/IGHJ4*03; not depicted) and one incomplete DJH (DH1-26-JH1; Fig. 3 B). Patient C had one incomplete monoclonal DJH rearrangement (DH3-9/JH4.2;

Fig. 3 C). [Fig. S3](#) displays the electropherograms confirming products sequences.

Once such rearrangements were characterized in the MLL-AF4⁺ leukemic blasts, we searched for their presence in the MLL-AF4⁺ MSCs from BM from the same infants with MLL-AF4⁺ B-ALL (Fig. 3, A–C, 1). For this purpose, we tested dilutions of the MLL-AF4⁺ leukemic blast cells in normal MSCs by using conventional PCR amplifications with consensus primers of VH, DH, and JH segments (van Dongen et al., 2003). In both cases, the most sensitive PCR

approach was provided by DJH rearrangements. The monoclonal patterns of both monoclonal amplifications were always identifiable in MLL-AF4⁺ leukemic blast cell dilutions with normal MSCs with a sensitivity of 10^{-2} – 10^{-3} (Fig. 3), sensitivity which is considered the usual standard for IgH rearrangements (van Dongen et al., 2003), especially when the nonleukemic background is composed by cells with Ig

genes in germline configuration such as normal BM-MSCs (López-Pérez et al., 2000, 2001). However, when this sensitive methodology was used in DNA from MLL-AF4⁺ BM-MSCs, the amplification of the monoclonal PCR product was always negative. Collectively, whereas monoclonal Ig gene rearrangements were consistently detected in MLL-AF4⁺ leukemic blasts, no monoclonal rearrangements

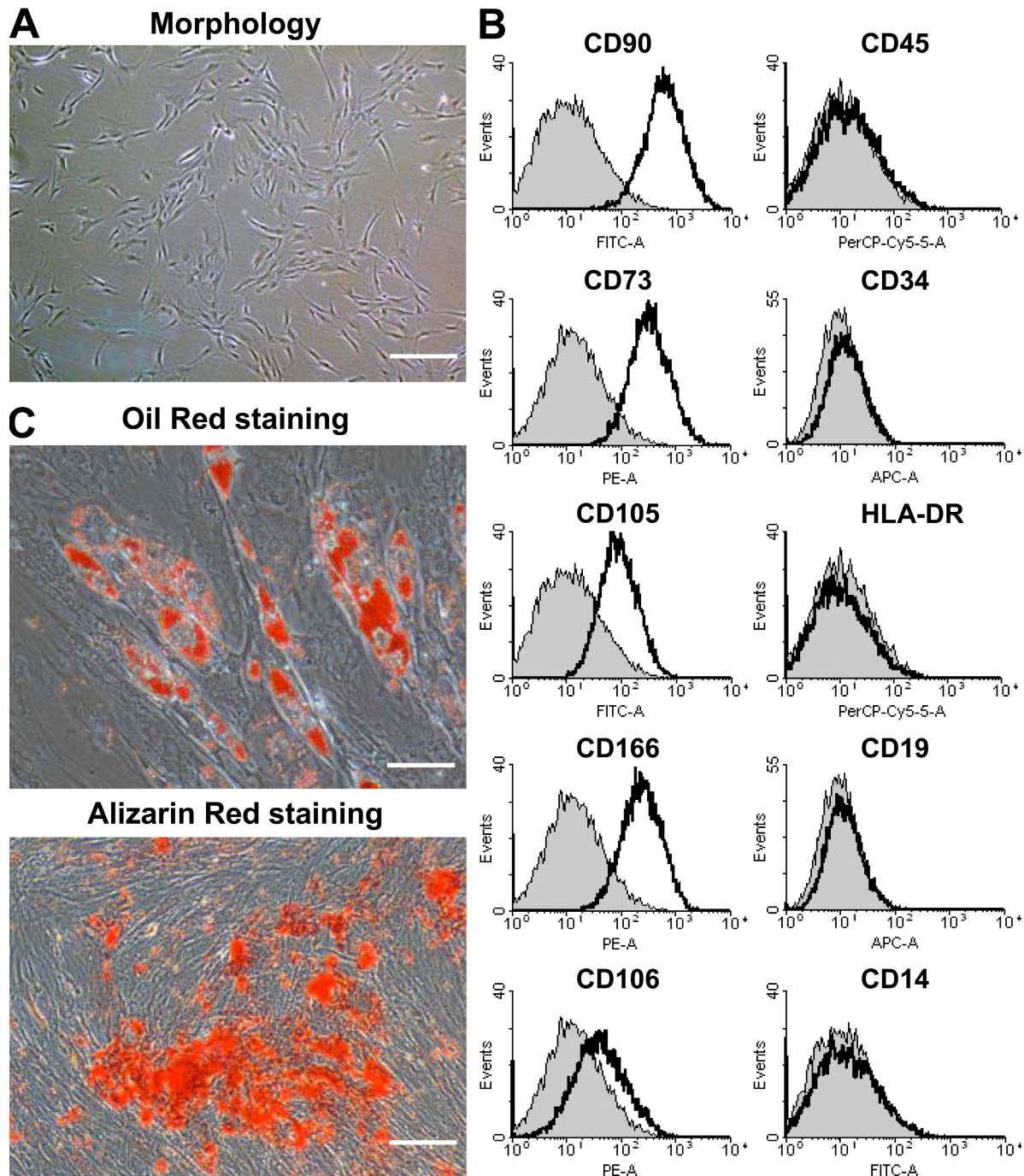


Figure 1. Characterization of MSCs derived from childhood leukemia patients. (A) Morphology. (B) FACS analysis of MSC surface markers. Gray area indicates the isotype control and the empty area shows specific antibody staining. (C) Adipogenic (oil red staining) and osteogenic (alizarin red staining) differentiation potential of MSCs. Data from $n = 38$ children suffering from acute leukemia. Bars, 100 μ m.

could be detected in BM-MSCs from any MLL-AF4⁺ B-ALL patient, ruling out potential contamination of the MSC cultures by leukemic cells and suggesting a close early developmental relationship between MSCs and the leukemic blasts rather than plasticity or de-differentiation of B-ALL blasts.

Ectopic expression of MLL-AF4, MLL-AF9, and TEL-AML1 in BM-MSCs exerts no effect on MSC culture homeostasis

To further explore whether distinct leukemia-associated fusion oncogenes exert differential effects in MSC culture homeostasis, the lentiviral ectopic expression of MLL-AF4, MLL-AF9, and TEL-AML1 fusions (Fig. 4 A) in normal BM-MSC was

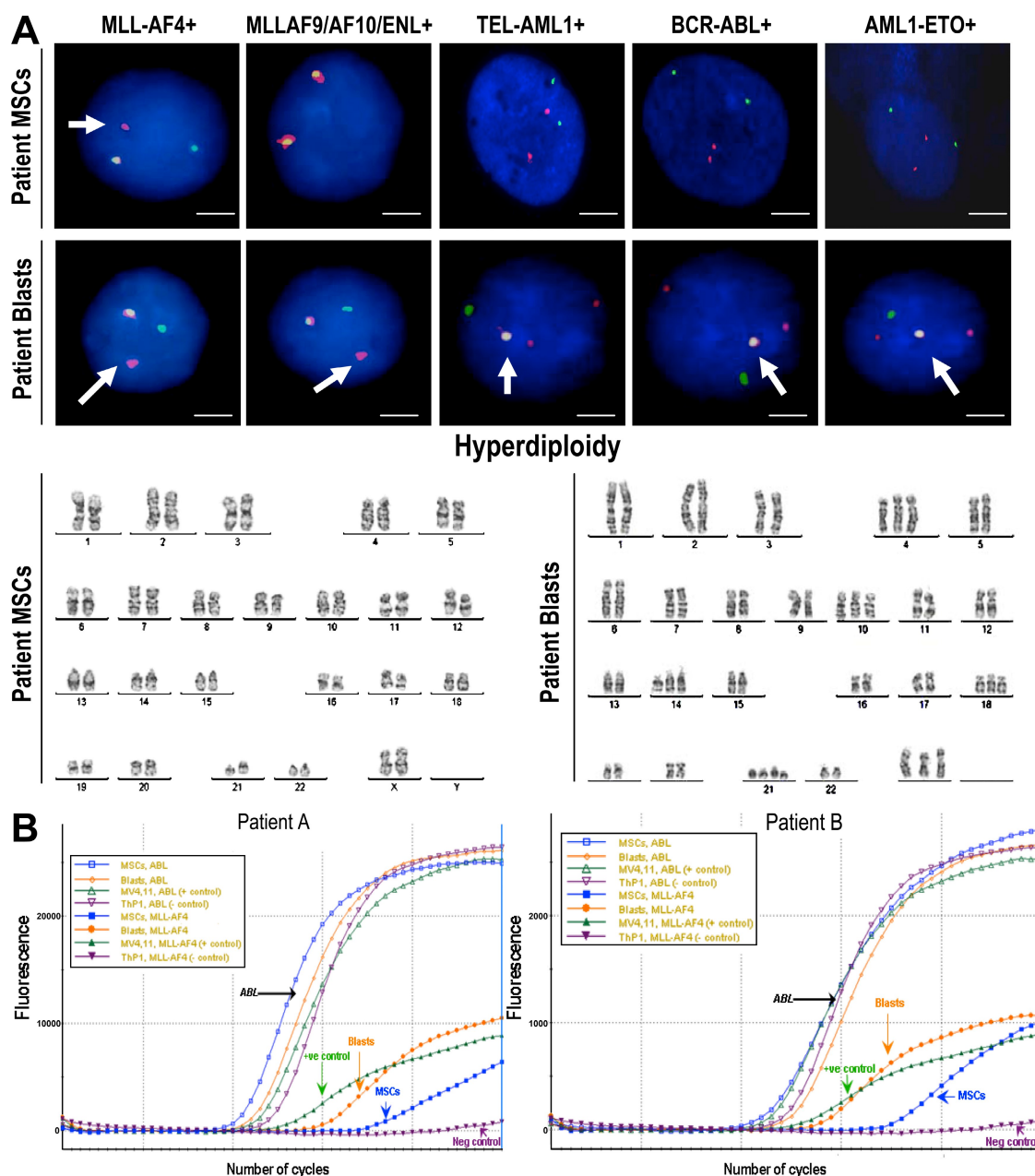


Figure 2. MLL-AF4 fusion gene is present and expressed in BM-MSCs from infants with MLL-AF4⁺ pro-B-ALL. (A) FISH performed in patient-derived MSCs (top row) and leukemic blasts (bottom row; $n = 38$). Leukemia-specific fusion genes were always observed in the leukemic population. Using a split apart probe, MLL rearrangements are identified by the presence of one red signal, one green signal, and one yellow signal (germline). Using locus-specific probes, the fusions TEL-AML1, AML1-ETO, and BCR-ABL are determined by the presence of yellow fusion signals (and the derivative chromosome), whereas cells without the translocation have two green (either BCR, TEL, or ETO) and two red signals (either ABL or AML1). The white arrows depict the rearranged allele. G-banding karyotyping was performed in the BM-MSCs from children with hyperdiploid (>51 chromosomes) B-ALL ($n = 10$). Bars, 100 μm . **(B)** Representative RT-Q-PCR experiments performed in duplicate from two patients showing MLL-AF4 transcript expression in MSCs from infants with B-ALL MLL-AF4⁺.

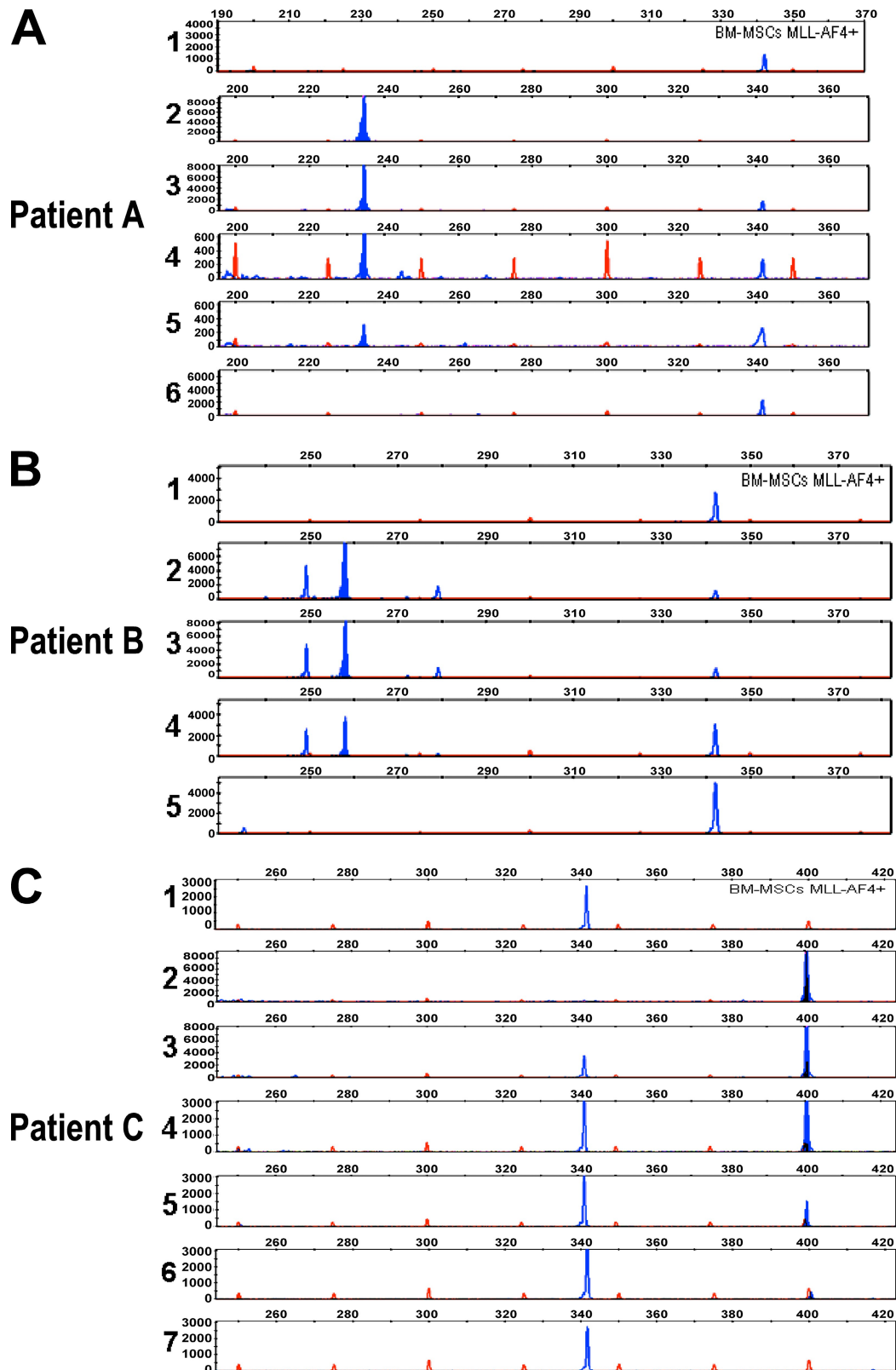


Figure 3. V(D)JH monoclonal rearrangements were present in MLL-AF4⁺ leukemic blast cells but absent in BM-MSCs from infants with MLL-AF4⁺ B-ALL. (A) VDJH monoclonal rearrangements of patient A. (A, 1) DNA from MSCs from the patient. (A, 2) undiluted diagnostic leukemic blast sample. (A, 3–6) 10, 6, 1, and 0.1% dilution, respectively, of DNA from diagnostic leukemic blasts into DNA from normal MSCs. The monoclonal rearrangement (234 bp; filled blue peak) is detectable in MLL-AF4⁺ leukemic blast cells, whereas it is absent in MLL-AF4⁺ MSCs. As it can be seen in A (6), the detection

compared. Lentiviral-mediated transduction efficiency of normal BM-MSCs was between 19 and 69% for the different fusion genes and was 100% for the GFP control (Fig. 4 B). Transgene expression was demonstrated by GFP expression (Fig. 4 B), RT-PCR, and Western blot (Fig. 4 C). As expected, in contrast to HSCs, germline MLL is not expressed in MSCs (unpublished data). Transduced MSC cultures were followed up for 62 d, and culture homeostasis was analyzed for the different fusion genes. Ectopic expression of the indicated leukemic fusion oncogene did not alter the proliferation (Fig. 4 D) and cell cycle distribution (Fig. 4 E) of the BM-MSC cultures. Similarly, ectopic expression of the fusion oncogenes had no effect on cell death or apoptosis: >90% of the transduced MSCs were alive and healthy 9 wk after transduction (Fig. 4 F). Similar to normal MSCs, those transduced with fusion oncogenes underwent senescence by passage 9 (62 d) as assayed by β -galactosidase assays (Fig. 4 G). Additionally, MLL-AF4⁺ BM-MSCs lacked expression of CD133, a stem cell marker previously proposed to be induced by MLL-AF4 in leukemic blasts (Thomas et al., 2005; Fig. S4). These data indicate that MLL-AF4, MLL-AF9, and TEL-AML1 expression is compatible with a mesenchymal phenotype and does not alter the culture homeostasis.

DISCUSSION

Previous studies have found that the BCR-ABL fusion gene and lymphoma-specific genetic aberrations are present in a proportion of endothelial cells derived from chronic myelogenous lymphoma and lymphoma patients, respectively (Gunsilius et al., 2000; Streubel et al., 2004), claiming the existence of a bipotent hemangioblastic precursor capable of producing endothelial and blood cells. The presence of chromosomal abnormalities on MSCs from hematological malignancies is controversial. Recent studies have shown that MSCs from multiple myeloma and myelodysplastic syndrome are abnormal and display genomic aberrations identified by gene expression or CGH-based arrays (Corre et al., 2007; Lopez-Villar et al., 2009), indicating that the stroma may play a role in tumor pathogenesis.

Leukemic fusion genes may arise, during in utero development, in a population of mesodermal prehematopoietic precursors that would give rise to HSCs and MSCs. BM-MSCs from leukemic children are candidate cell targets for fusion gene screening because they are ontogenically close to in utero development. However, the question of whether BM-MSCs from childhood leukemia harbor and express leukemia-specific fusion genes has not been addressed.

Here, we searched for leukemia-specific fusion genes in BM-MSCs from children with cytogenetically different acute leukemias. Leukemic fusion genes were not detected in BM-MSCs from any subtype of childhood leukemia, but in infant MLL-AF4⁺ B-ALL, where MLL-AF4 was detected and expressed in BM-MSCs from all patients studied. Our findings suggest that MSCs in infant MLL-AF4⁺ B-ALL are tumor related. MLL-AF4 is known to arise in utero during human development, and this infant B-ALL is characterized by its dismal prognosis and very short latency; thus a close developmental relationship between the MSCs and the leukemic blasts in this subtype of infant B-ALL diagnosed shortly after birth is plausible. Whether MLL-AF4 arises in a common precursor capable of diverging into blood and mesenchyme or it occurs as an independent event in leukemic blasts and in the stroma still needs to be elucidated. Of note, it may be plausible that the alternative leukemic fusion genes may be found in MSCs not only in this subtype of dismal infant MLL-AF4⁺ ALL but also in other children who develop mesenchymal cancer (leukemias and sarcomas) in utero.

In line with the plastic behavior of cells during embryonic development, MSCs have been reported to have a promiscuous gene expression pattern, being in a standby state in which many gene families are expressed at a low level, thereby making the cell readily capable of shifting fates (Tremain et al., 2001). In all samples analyzed, monoclonal Ig gene rearrangements were consistently detected in MLL-AF4⁺ leukemic blasts but could never be detected in MLL-AF4⁺ BM-MSCs. The absence of monoclonal rearrangements in MLL-AF4⁺ BM-MSCs rules out the possibility of cellular plasticity or de-differentiation of B-ALL blasts and suggests that MLL-AF4 might arise in a potential mesodermal common precursor. Additionally, the fact that all MLL-AF4⁺ MSCs were euploid precludes the possibility of cell fusion.

Importantly, MLL-AF4⁺ MSCs did not seem to have proliferative advantage, and ectopic expression of MLL-AF4 in normal BM-MSCs exerted no effect on MSC culture homeostasis. This indicates that MLL-AF4 expression is compatible with a mesenchymal phenotype and that MLL-AF4 itself is not sufficient for MSC transformation, suggesting the potential need for secondary cooperating oncogenic hits and a differential impact of MLL-AF4 in the hematopoietic system and mesenchyme. The mechanisms by which the BM-MSCs in infant B-ALL acquire this leukemia-specific genetic aberration remain to be elucidated. Whether the MLL-AF4 fusion is similar between BM-MSCs and leukemic blasts suggestive

sensitivity is between 0.1 and 1%. (B) DJH monoclonal rearrangements of patient B. (B, 1) DNA from MSCs from the patient. (B, 2–5) 10, 6, 1, and 0.1% dilution, respectively, of DNA from diagnostic leukemic blasts into DNA from normal MSCs. The DJH monoclonal rearrangement (258 bp; filled blue peak) is detectable in MLL-AF4⁺ leukemic blast cells whereas it is absent in MLL-AF4⁺ MSCs. (C) DJH monoclonal rearrangements of patient C. (C, 1) DNA from MSCs from the patient. (C, 2) undiluted diagnostic leukemic blast sample. (C, 3–7) 10, 6, 1, 0.1, and 0.01% dilution, respectively, of DNA from diagnostic leukemic blasts into DNA from normal MSCs. The DJH monoclonal rearrangement (400 bp; filled blue peak) is detectable in MLL-AF4⁺ leukemic blast cells whereas it is absent in MLL-AF4⁺ MSCs. As can be seen in C (5 and 6), the detection sensitivity is between 0.1 and 1% for patients A and B and between 0.01 and 0.1% for patient C. It should be noted that in all patients there is an unspecific amplification peak (empty blue peak) of 342 bp that is used as indicator (positive control) of DNA amplification (IgH germline alleles).

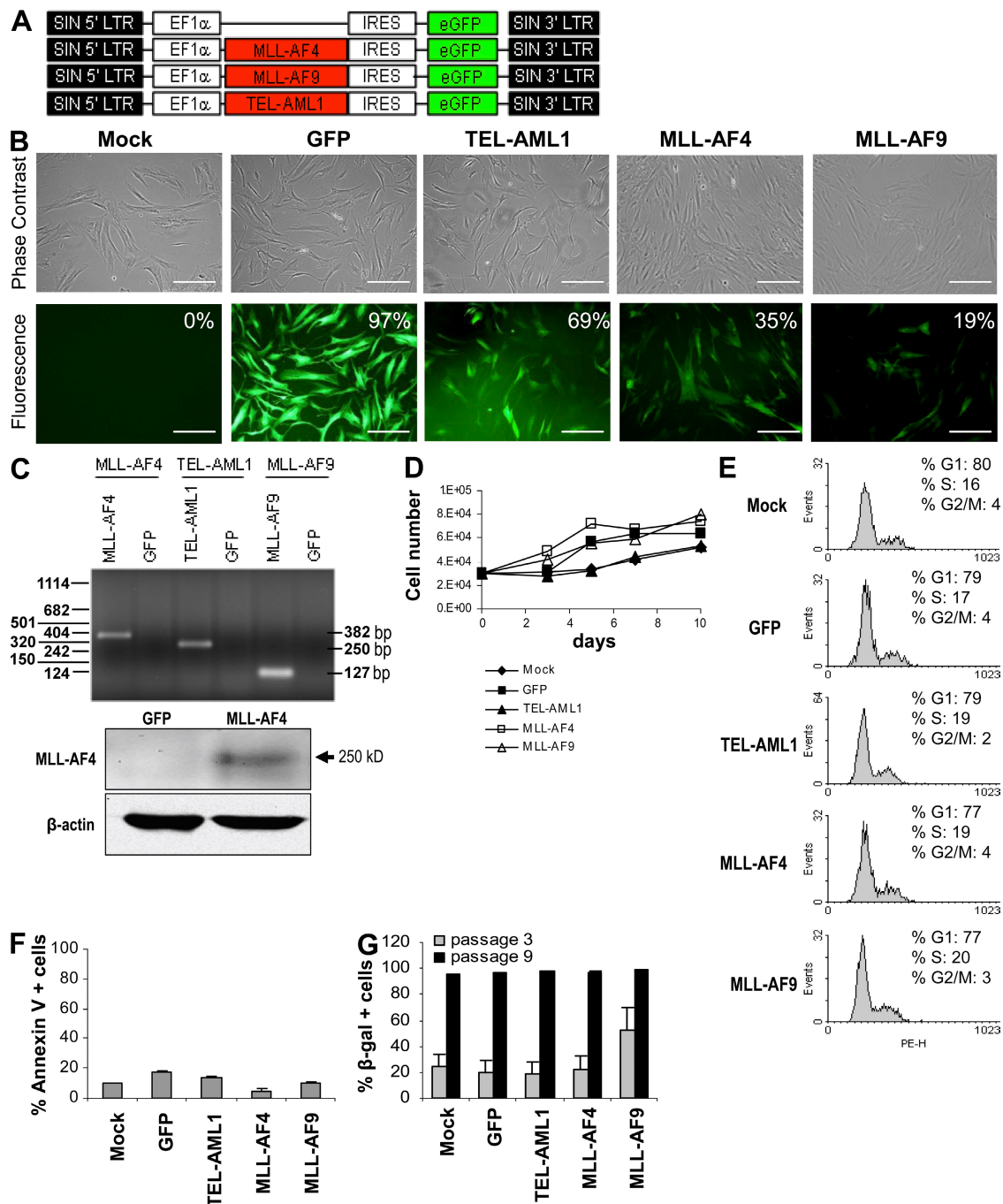


Figure 4. In vitro effects of ectopic expression of TEL-AML1, MLL-AF4, and MLL-AF9 in the homeostasis of normal BM-MSC cultures.

(A) Schematic representation of the bicistronic lentivectors used. (B) Phase contrast morphology and GFP expression in lentiviral-transduced normal BM-MSCs. Bars, 20 μ m. Mock indicates no infection; GFP indicates transduction with an empty vector. Transduction efficiency was measured by flow cytometry as percentage of GFP⁺ MSCs 3 d after transduction. (C) RT-PCR (top) and Western blot (bottom) showing the expression of the indicated fusion genes in transduced MSCs. (D) Growth curves showing similar growth properties among the different MSC cultures ($n = 3$). (E) Cell cycle distribution of mock and fusion gene-expressing MSCs. (F) Annexin V binding assays showing low levels of apoptosis of MSCs regardless of the leukemic fusion gene overexpressed. (G) Senescence-associated β -galactosidase assays of the indicated MSCs at passage 3 and 9. Experiments were performed twice with identical outcome.

of an unequivocal common precursor also remains to be elucidated. It is worth mentioning that even identical fusions might give rise to distinct transcripts through alternative splicing, whereas identical transcripts might also display dif-

ferent phenotype because the transcript/protein may exert differential effects in a cell-dependent manner, perhaps partially explaining the lack of transformation by MLL-AF4 itself in BM-MSCs.

MATERIALS AND METHODS

Patients and samples. 38 children diagnosed with cytogenetically different ALL or AML were enrolled in this study. Acute leukemias were grouped as follows: TEL-AML1⁺ B-ALL ($n = 12$), BCR-ABL⁺ B-ALL ($n = 5$), AML1-ETO⁺ M2-AML ($n = 1$), hyperdiploid B/T-ALL ($n = 10$), MLL-AF4⁺ pro-B-ALL ($n = 4$), MLL-AF9⁺ AML ($n = 3$), MLL-AF10⁺ AML ($n = 1$), and MLL-ENL⁺ B-ALL/AML ($n = 2$). Table I summarizes the diagnosis, cytogenetics, and age for each patient. Median age for each group was as follows: 51 ± 16 mo for TEL-AML1⁺, 65 ± 10 mo for BCR-ABL⁺, 47 ± 35 mo for hyperdiploid, 38 ± 61 mo for MLL-rearrangements with a partner different than AF4, and 6 ± 3 mo for MLL-AF4⁺. BM samples were harvested at diagnosis. Leukemic blasts were used for routine cytogenetic and molecular diagnostic screening, and MSCs were isolated, characterized, and expanded for cytogenetic, molecular, and functional studies. This study was approved by the Institutional Review Board of Hospital Niño Jesús, and samples were obtained upon informed consent from the parents.

Isolation and expansion of BM-MSCs. Mononuclear cells from BM were isolated by centrifugation (400 g, 25 min) using Ficoll-Paque Plus (GE Healthcare) density gradient. Mononuclear cells were seeded at a density of 3×10^4 cells per cm² in MesenCult medium and MSC-supplements (STEMCELL Technologies) and incubated at 37°C in a 5% humidified CO₂ atmosphere. After 24 h, nonadherent cells were discarded and fresh medium was added. When cell culture achieved >85% of density, adherent cells were trypsinized, washed, and replated at a concentration of 5×10^3 cells per cm².

Characterization of MSC cultures. The immunophenotype of cultured BM-MSCs was analyzed by flow cytometry as previously described (García-Castro et al., 2008). In brief, 2×10^5 cells were incubated for 30 min with the fluorochrome-conjugated monoclonal antibodies CD90, CD73, CD105, CD166, CD106, CD45, CD34, HLA-DR, CD19, and CD14 (BD) or their respective isotype controls. Next, stained cells were washed in PBS and analyzed in a FACSCanto II cytometer (BD). CD133 (clone 293C3; Miltenyi Biotec) expression was also analyzed in MSCs overexpressing ectopic MLL-AF4.

MSC differentiation studies were performed by plating the MSCs in specific differentiation inductive media for 2 wk, as previously described (Rodríguez et al., 2009). For adipogenic differentiation, cells were cultured in Adipogenic MSCs Differentiation BulletKit (Lonza) and differentiated cultures were stained with oil red O (Sigma-Aldrich). For osteogenic differentiation, cells were cultured in Osteogenic MSCs Differentiation BulletKit (Lonza) and differentiated cultures were stained with alizarin red S (Sigma-Aldrich).

FISH and karyotyping. FISH was performed on leukemic blasts and patient-matching MSCs as described (Bueno et al., 2009; Catalina et al., 2008), using commercially available probes (Vysis Inc.). BCR-ABL, TEL-AML1, and AML1-ETO fusions were detected using locus-specific LSI Dual Color Translocation probes. MLL rearrangements were analyzed using the LSI MLL Dual Color Break Apart Rearrangement Probe. At least 500 nuclei were analyzed. The slides were analyzed in a fluorescence microscope equipped with appropriate filters using the ISIS-software (Metasystems).

Aneuploidy was assessed by conventional G-banding as previously described (Catalina et al., 2009). Chromosomes were visualized using a modified Wright's staining. At least 20 metaphases were analyzed using a conventional microscope and the IKAROS-software (Metasystems). The MV4;11 (MLL-AF4⁺), THP-1 (MLL-AF9⁺), and REH (TEL-AML1⁺) cell lines were used as positive controls for FISH studies.

Quantitative RT-PCR detection of MLL-AF4 transcript in patient-derived MSCs. Real-time quantitative RT-PCR was done as previously described (Gabert et al., 2003). Primers and Taqman probes for detecting specific MLL-AF4 fusion transcripts were synthesized according to Jansen et al. (Jansen et al., 2005). In the PCR reaction, two different MLL-anchoring forward oligonucleotides (MLL-F1 and MLL-F2), two different AF4-anchoring reverse oligonucleotides (AF4-R1 and AF4-R2), and two different

probes (MLL-T1 and MLL-T2) were used simultaneously. ABL transcript was used for normalization (Beillard et al., 2003). Primers and probes sequences are described elsewhere (Beillard et al., 2003; Gabert et al., 2003; Jansen et al., 2005). The MV4;11 (MLL-AF4⁺) and THP1 (MLL-AF9⁺) cell lines were used as positive and negative controls, respectively.

Vectors, lentiviral production, and transduction. The following dual-promoter fusion gene-expressing lentiviruses were used in this study: pRRL-GFP, pRRL-MLLAF4-GFP (Fig. S2), pWPI-MLLAF9-GFP, and HR-SINCSGW-TELAML1 (provided by T. Enver, Oxford University, Oxford, England, UK; Fig. 4 A). Viral particles pseudotyped with the VSV-G protein were generated on 293T cells using a standard calcium-phosphate transfection protocol and were concentrated by ultracentrifugation. Normal MSCs from healthy donors were infected overnight with concentrated viruses. The following day, the viral supernatant was removed and transduced MSCs were washed with MSC media and allowed to expand for up to 10 wk.

MSC culture homeostasis analysis. MSC cultures were assessed daily for changes in growth rates and morphology. Growth curves were performed by assessing the cell number in triplicate MSC cultures. Cell cycle analysis was performed by flow cytometry after propidium iodide staining of 70% ethanol-fixed cells. Apoptotic cells were analyzed by flow cytometry using PE-Annexin V according to the manufacturer's instructions (BD). To determine senescence-associated β -galactosidase activity, MSCs were fixed and incubated overnight with X-gal solution (pH 6.0) as previously described (Wei and Sedivy., 1999).

RT-PCR. Total RNA extraction and RT-PCR reactions were done as previously described (Montes et al., 2009). RT-PCR conditions were as follows: cDNA synthesis at 37°C for 2 h, prePCR denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, for 35 cycles. Primer sequences were as follows: TEL-AML1, 5'-ATCATG-CACCTCTGATCCT-3' and 5'-ACGCTCGCTCATCTTGCTT-3'; MLL-AF4, 5'-CAGAGCAAACAGAAAAAGTG-3' and 5'-GTTCTG-GAAGGGACTGTGGA-3'; and MLL-AF9, 5'-GCACTCTCTCCAAT-GGCAATA-3' and 5'-GCCTTGTCACATTCACCATTC-3'.

V(D)JH Ig gene monoclonal rearrangements. Genomic DNA from leukemic blasts from infants diagnosed with MLL-AF4⁺ pro-B ALL was isolated from BM samples at diagnosis using standard methods. Complete VDJH and incomplete DJH rearrangements were amplified and identified using the BIOMED-2 strategy (van Dongen et al., 2003). For amplification of complete VDJH rearrangements, a set of family-specific primers of the FR1 and FR2 regions and one JH consensus primer were used in two multiplexed PCR reactions. Amplification of incomplete DJH rearrangements was performed in two different reactions using family-specific primers for DH1 to DH6 and DH7 families, respectively, together with the consensus JH primer. All reactions were performed in 50 μ L mixture containing 50–100 ng of DNA and 10 pmol of each primer. All these amplifications were performed using genomic DNA. The monoclonal nature of the rearrangements was confirmed by the identification of single amplification peaks by genescanning analysis following described criteria. (López-Pérez et al., 2001; van Dongen et al., 2003). All products were sequenced as previously described (Gonzalez et al., 2005). PCR products were separated by PAGE and visualized with ethidium bromide. Monoclonal PCR products were purified with ExoSap (USB Corp.) and directly sequenced in an ABI 3130 DNA sequence analyzer using BigDye Terminators with the v1.1 Cycle Sequencing kit (Applied Biosystems; Gonzalez et al., 2005; Fig. S3).

Western blotting. Whole-cell extracts from GFP-MSCs and MLL-AF4-MSCs were resolved on 6% SDS-PAGE gels and blotted onto nitrocellulose membranes (Bio-Rad Laboratories). MLL-AF4 fusion protein (~250 kD) was detected with the enhanced chemiluminescence detection system (GE Healthcare) using an anti-MLL antibody (1:750 dilution; clone N4.4; Millipore).

Online supplemental material. Fig. S1 shows representative pictures displaying adipogenic (oil red) and osteogenic (alizarin red) differentiation from normal BM-derived MSCs. Fig. S2 shows that the human MLL-AF4 cDNA has been sequenced. Fig. S3 shows electropherograms confirming the V(D)JH product sequences. Fig. S4 shows the lack of CD133 (Prominin-1) expression in primary MLL-AF4-expressing BM-MSCs. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091050/DC1>.

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