Characterization of a novel *MEF2D-BCL9* fusion-positive acute lymphoblastic leukemia cell line

B-cell acute lymphoblastic leukemia (B-ALL) is often characterized by gene fusions of transcription factors such as *ETV6-RUNX1* or tyrosine kinases such as *BCR-ABL1*. Novel gene fusions harboring the transcription factor *MEF2D* with various partner genes have been identified in 2016.[1] The estimated frequency of *MEF2D* fusions in children and adolescents with newly diagnosed ALL is 2-6%, of which the *MEF2D-BCL9* fusion is most commonly found.[1–4] Patients harboring a *MEF2D* fusion have a poor prognosis (5-year event-free survival of ~55%). They present with unfavorable clinical characteristics including an older age of onset (median ranging between 9 and 14 years) and a high white blood cell count (median 27.3x10^6 cells/mL).[1,3,4] Molecular characteristics are differential CD5 and CD10 expression compared to other B-ALL subtypes, along with increased *HDAC9* expression and a higher frequency of deletions and mutations in *CDKN2A/B* and *PHF6*, respectively.[1,3,4] Primary leukemic cells of B-ALL patients generally do not replicate *ex vivo*, which makes functional studies difficult. However, occasionally prolonged culturing of leukemic cells is successful; well-known examples are Reh (*ETV6-RUNX1*), SupB15 (*BCR-ABL1*), and RCH-ACV (*TCF3-PBX1*)[5–7] cell lines. A retrospective study recently identified *MEF2D* fusions in 19 non-publicly available B-ALL cell lines with limited molecular and functional characterization.[2] In this study, we characterize an in-house made patient-derived *MEF2D* fusion positive B-ALL cell line, named M4A1-M2B9, and compare the characteristics to its corresponding primary material. The patient-derived cell line is comparable to the primary material when examining mutations in the exome, gene expression, the immunophenotype, and sensitivity to drugs that do not affect division and/or cell cycle. These results imply that M4A1-M2B9 is a valuable resource to examine novel therapeutics targeting MEF2D fusion proteins or downstream targets activated by MEF2D, as well as to study immunotherapy options for B-ALL.

In accordance with the Declaration of Helsinki, written informed consent to use excess diagnostic material for research purposes was obtained from parents or guardians, as approved by the medical Ethics Committee of the Erasmus Medical Center. All used reagents and primers are described in the *Online Supplementary Table S1*. We used paired-end stranded total RNA illumina sequencing (RNAseq) after riboRNA depletion with fragments of 150 bp to detect gene expression differences. Paired-end whole exome sequencing (WES) was used to detect variants and copy number alterations in the coding regions. Sequencing data are deposited at the European Genome-Phenome Archive (EGA) (RNA sequencing: EGAD00001009759, WES: EGAD00001009758). Primary cells were expanded after intrafemoral injection in NOD scid γ mice, leukemic burden was tested every 2-4 weeks in peripheral blood and the cells were harvested from the spleen upon overt leukemia. Cells were seeded in a 96-wells plate and medium was replaced every 3-4 days until visible cell growth. Afterwards, cells were routinely cultured. Cell survival of primary human mesenchymal stromal cells (MSC derived from a B-ALL patient at day 79) was measured after 4 days of co-culturing by harvesting and staining the cells followed by flow cytometry (Beckman Coulter). The viability after 4 days of drug or ligand exposure was measured using MTT. In all experiments, the blast percentage of the primary samples was ≥90% at the start of the experiment. M4A1-M2B9 has been deposited at DSMZ for future distribution.

A 15-year-old male patient was diagnosed with B-ALL harboring a *MEF2D-BCL9* fusion, a biallelic *CDKN2A/B* deletion, and presenting a high white blood cell count of ~150x10^6 cells/mL *(Online Supplementary Figure S1A-C)*. The leukemic cells had a pretreatment karyotype of 46~48,XY,del(1)(q21),-9,del(9)(p1?2),+1~3mar,inc[6]/46,XY[15] and a cytoplasmic μ chain-positive pre-B immunophenotype ($CD19^+/CD20^-/CD10^+/CD45^{dim}/CD34^-/CD38^+$). The patient was treated according to ALL10 high-risk protocol. The patient initially responded poorly to therapy having a high minimal residual disease (MRD) level (>1%) at day 33, however, at day 79 the MRD level was positive but not quantifiable. The patient received stem cell transplantation and has been in continuous complete remission for >6 years till last follow-up.

We established a patient-derived cell line in which we studied the dependence on external factors, specifically serum dependence, stromal support, and addition of cytokines. M4A1-M2B9 was derived from bone marrow leukemic cells which were used for intrafemoral injection, the mouse was sacrificed after 53 days and 86% of the harvested spleen-derived cells were blasts. Within 1 month of culturing in primary medium, the spleen-derived cells were dividing and were split every 3-4 days. In 2.5 months, the cells grew from 0.4 million to 80 million (Figure 1A). The presence of the *MEF2D* fusion was confirmed by reverse transcriptase polymerase chain reaction (PCR). We compared the growth speed of M4A1-M2B9 in our primary medium, containing 20% fetal calf serum

(FCS) and supplemented with insulin, transferrin, and selenium (ITS), with our standard cell line medium containing either 10% or 20% FCS lacking ITS. M4A1-M2B9 is serum-dependent, the cells died in the culture with 10%

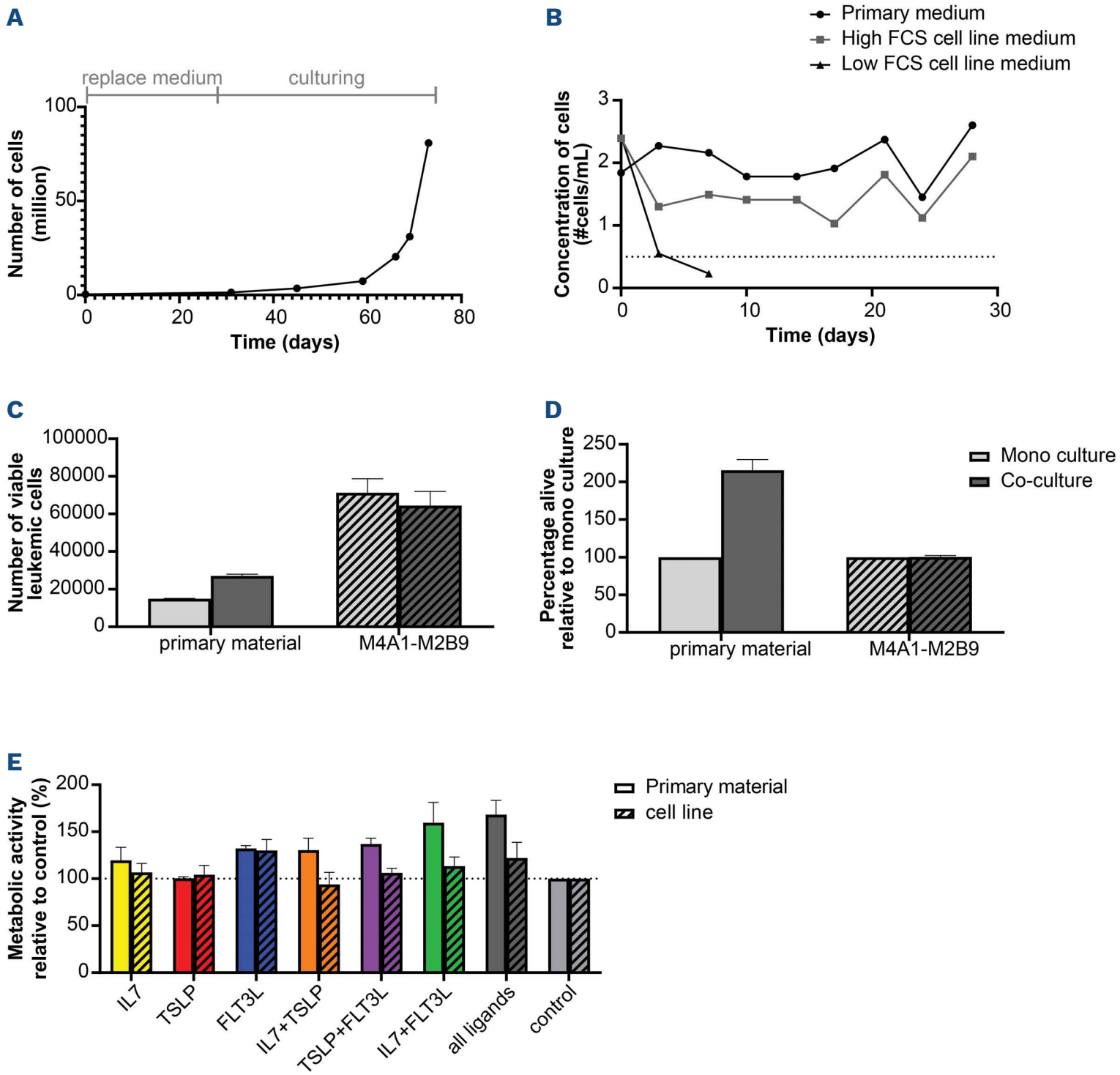
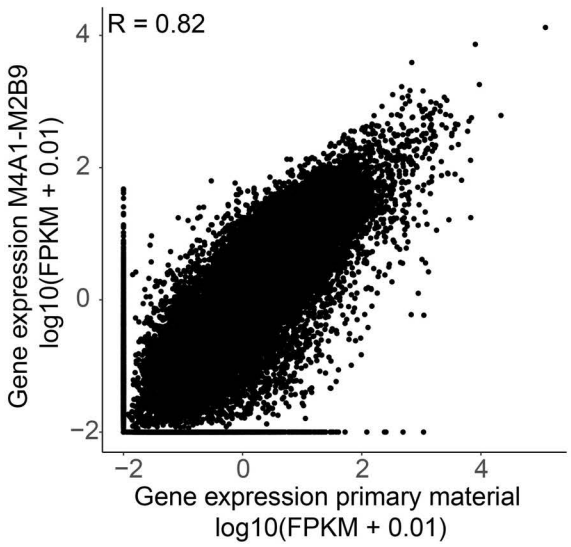


Figure 1. Growth and survival of patient-derived xenograft *MEF2D-BCL9* cell line. (A) The growth curve of cells freshly obtained from a patient-derived xenograft (PDX) when cultured on primary medium (RPMI 1640 medium supplemented with 20% fetal calf serum [FCS], insulin, transferrin and sodium selenite [ITS], glutamine and gentamycin) in the first 2.5 months. For the first month half of the medium was replaced every 3-4 days, afterwards the cell concentration was determined every 3-4 days by manual cell count using trypan blue staining and the cells were split accordingly. (B) Examining the dependence of the cells on factors present in primary medium by comparing growth in primary and cell line medium (RPMI 1640 medium supplemented with 20% or 10% FCS, penicillin-streptomycin and Amphotericin). Cells cultured on primary medium were spun down and placed on cell line medium containing either 20% or 10% FCS. Cells were counted manually every 3-4 days and cultured at a density of $0.5x10^6$ cells/mL. The average doubling time of the cells cultured in primary medium is 42.2 hours, the average doubling time of cells cultured in the cell line medium with high FCS is 56.8 hours. (C, D) Examination of the dependence of mesenchymal stromal cells (MSC) support using primary material and the cell line. Primary material and the cell line were cultured in a 48-wells plate for 4 days at 37°C and 5% CO_2 either in monoculture or on a MSC layer (16.500 cells/well seeded at day -1) by replacing MSC medium with 410 µL cell suspension at a concentration of $1x10^6$ cells/mL or $0.5x10^6$ cells/mL, respectively. The number of viable leukemic cells after 4 days was measured using flow cytometry (C) and the percentage survival relative to the monoculture (D) are plotted. Bars show mean ± standard error of the mean (SEM) of 2 independent experiments. (E) Examination of the benefit of cytokines using primary material and M4A1-M2B9. Primary material and the cell line were cultured for 4 days at 37°C and 5% CO_2 in the presence or absence of cytokines (IL7 at 8 ng/mL, TSLP at 20 ng/mL or FLT3L at 32 ng/mL or a combination thereof) at a concentration of $1.6x10^6$ cells/mL and $0.5x10^6$ cells/mL, respectively. The metabolic activity relative to unstimulated cells was measured after addition of MTT, incubation for 2-6 hours, dissolving the crystals using acidified isopropanol and subsequently the absorbance at 562 nm and 720 nm was obtained using the VersaMax plate reader. Bars show mean ± SEM of 2 independent experiments.

A



C

Chr.	Base pair start site	Gene	VAF primary	VAF PDX	VAF cell line	HGVS.c	HGVS.p
chr2	182834937	*FRZB*	60.0	54.8	33.8	c.890G>C	p.Gly297Ala
chr7	93252700	*VPS50*	53.8	45.6	41.2	c.150A>T	p.Glu50Asp
chr19	1047370	*ABCA7*	50.5	52.4	57.9	c.2059G>A	p.Val687Met
chr18	21847038	*MIB1*	48.0	*IGV (36)*	55.4	c.2306G>T	p.Arg769Leu
chr7	143394816	*EPHA1*	47.3	48.5	48.8	c.2344G>A	p.Glu782Lys
chr15	87929204	*NTRK3*	45.7	45.6	50.5	c.2120C>T	p.Thr707Met
chr1	37945808	*INPP5B*	43.0	48.9	47.6	c.100C>T	p.Arg34Cys
chr3	35739473	*ARPP21*	40.9	28.6	43.5	c.1744C>A	p.Leu582Ile
chr3	29587148	*RP11-9J18*	37.6	32.3	40.4	c.*849C>T	
chr17	10498729	*MYH1*	37.5	*IGV (26)*	54.8	c.4078G>A	p.Glu1360Lys
chr10	93329716	*MYOF*	35.3	40.8	45.6	c.4930C>T	p.Arg1644*
chr5	72194740	*MAP1B*	34.4	43.9	48.6	c.1436C>T	p.Pro479Leu
chr17	69043592	*ABCA9*	33.8	58.0	47.3	c.697T>A	p.Ser233Thr
chr5	148136985	*SPINK5*	33.3	71.9	51.2	c.3279C>T	p.Asp1093Asp
chr4	166792180	*SPOCK3*	30.6	57.1	50.8	c.*344T>C	
chr3	165067369	*SI*	25.0	49.4	51.2	c.606A>T	p.Gln202His
chr16	28342755	*NPIPB6*	10.3	10.8	16.7	c.1130G>A	p.Arg377Gln
chr5	95284235	*MCTP1*	0	*IGV (0)*	18.3	c.341G>A	p.Arg114Lys
chr2	164722089	*COBLL1*	0	9.77	30.4	c.982G>A	p.Asp328Asn

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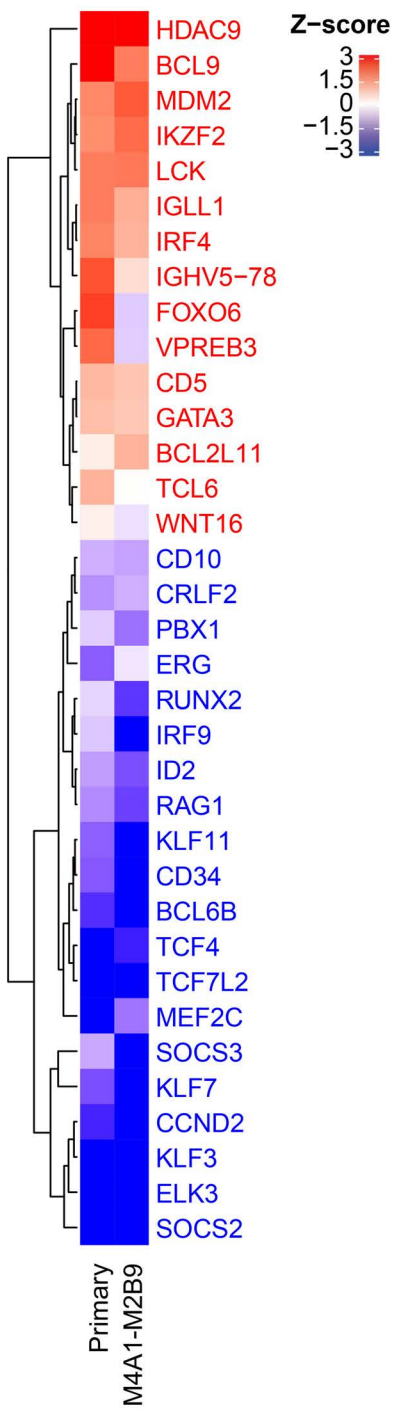

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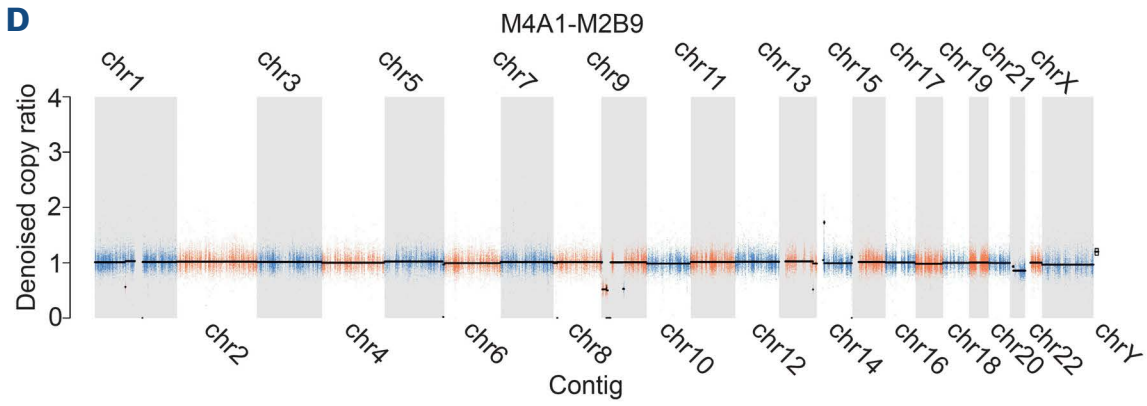

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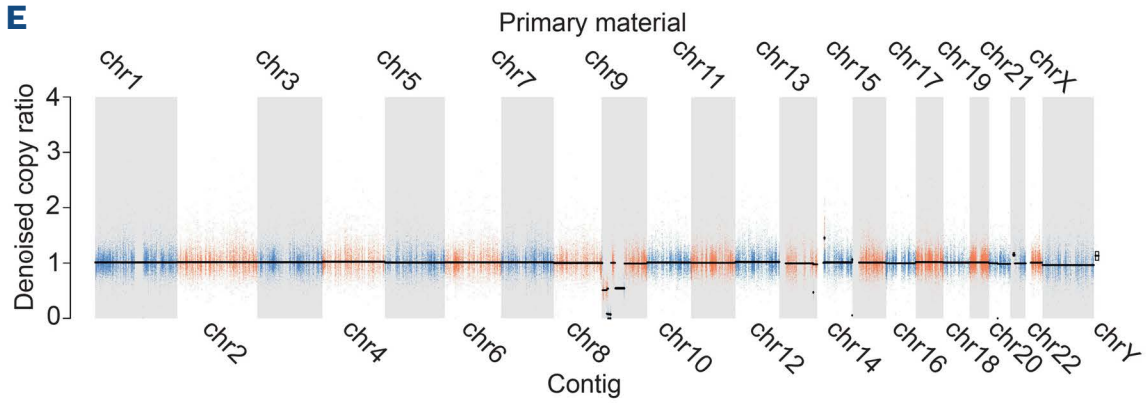

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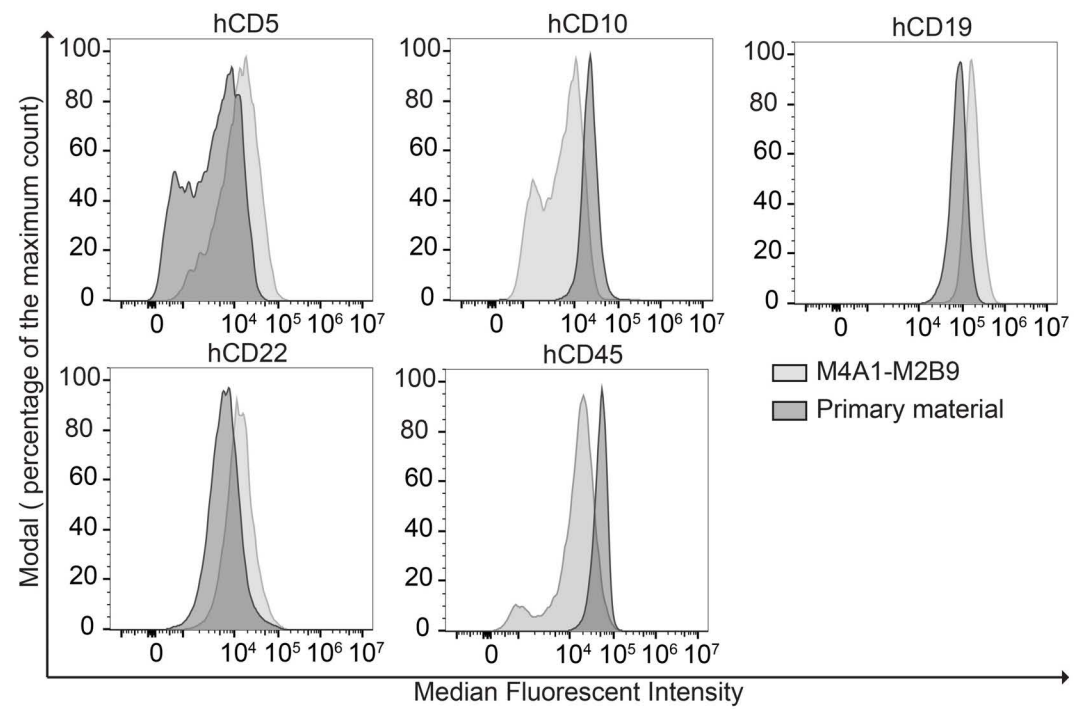

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Figure 2. Comparison of gene expression, genomic variants and cell surface protein between primary material and cell line. (A) Global gene expression comparison between primary material and cell line by plotting the log10 FPKM (fragments per kilobase million) + 0.01 per gene. (B) Heatmap of Z-scores of up- and down-regulated genes compared to the control B-cell acute lymphoblastic leukemia (B-ALL) cohort (N=424, derived from various treatment protocols). The up- (red) or down- (blue) regulated genes are selected based on the gene expression signature of *MEF2D* fused patients described by Ohki *et al.*[4], gene names are colored accordingly. *IGHG1* and *IGHV5-51* were not plotted due to the lack of expression in our cohort. Heatmap was made using the ComplexHeatmap package in R. Z-score was calculated, Z-scores >3 are set to 3, Z-score <-3 are set to -3. (C) Variant analysis of the cell line was compared with the germline and the primary tumor. First, the reads were filtered using Mutect2. Subsequently, the variants were filtered with a cut-off of at least 10 variant reads and the variant should be present in a transcript with one of the following biotypes: protein coding region, long non-coding RNA, nonsense-mediated decay, or intron retention. Next, all remaining variants were filtered based on their variant allele fraction (VAF) ≥10. After that, the remaining variants were checked manually in Integrative Genomics Viewer and if absent in the germline and present in tumor and/or cell line the variants and estimated VAF were noted. In the primary sample, there are 12 variants with a VAF <25% and 15 variants with a VAF >25%. All variants with a VAF >25% were also found in the cell line with a VAF of 34-58% (see Table, blue). In addition, 1 variant which was present in the primary sample with a VAF <25% was also found in the cell line with a VAF >10% (see Table, orange). Moreover, 2 variants were found in the cell line, which were not present or below the detection level in the primary material (see Table, red). Of these variants one originates from the patient-derived xenograft (PDX), while the other is only in present in the cell line. (D-E) Copy number alteration analysis for the primary material and the cell line, respectively. The upper figure shows the copy number of the primary material per chromosome and the lower figure the copy number of the cell line. The alterations, such as a deletion on chromosome 9 and 14, were unchanged. (F) Histograms of primary material (blast percentage of 93%) and cell line material showing the cell surface markers as determined by flow cytometry, primary cells in dark gray, cell line in light gray. Debris was discarded and alive cells were selected. X-axis, fluorescence intensity; Y-axis, modal.

FCS but grew in primary medium and cell line medium containing 20% FCS (Figure 1B). In addition, we studied the survival and cell proliferation of the primary material and M4A1-M2B9 in an *ex vivo* leukemic niche using a co-culture with MSC.[8] As expected, the primary material showed improved survival after co-culture on MSC, whereas M4A1-M2B9 showed equal survival with or without MSC support (Figure 1C, D). Moreover, we evaluated the effect of adding cytokines to M4A1-M2B9 and the primary material. IL7, TSLP and FLT3L were added as single supplement or in combination, as these cytokines stimulate common B-ALL activated pathways.[9] Both M4A1-M2B9 and primary material benefited up to 30% from the addition of FLT3L, however, the primary material had a benefit of 70% from the cytokine cocktail and benefited from multiple cytokine combinations (Figure 1E).

In addition to the dependence on external factors, we studied genetic and immunophenotypic differences between the primary material and M4A1-M2B9. Global gene expression was similar for primary material and the cell line (Figure 2A) and resembled the *MEF2D*-specific gene expression signature identified by Ohki *et al.*[4] (Figure 2B). Moreover, high expression of *HDAC9* (Z-score=3.96/4.27), *BCL9* (Z-score=3.01/1.98), and *CD5* (Z-score=1.09/0.91), and low expression of *CD10* (Z-score=-1.04/-1.20) was observed in the primary material and M4A1-M2B9 (Figure 2B; *Online Supplementary Figure S1D*). WES was used to detect leukemia-specific sequence variants and copy number alterations. Based on the variants and their allele frequencies, we conclude that the major clone of the initial material expanded in the patient-derived xenograft (PDX) (Figure 2C). The copy number alterations of the primary material and M4A1-M2B9 were comparable, and both contained a bi-allelic *CDKN2A/B* deletion (Figure 2D, E). In order to determine whether the cell surface marker expression remained stable upon cell line establishment, we performed immunophenotyping on viable cells using the cell surface markers CD5, CD10, CD19, CD22 and CD45. M4A1-M2B9 was 100% CD5-positive, while the primary material harbored 75% CD5-positive and 25% CD5-negative cells (Figure 2F). CD5 is often highly expressed on the cell surface of *MEF2D* fused B-ALL cells.[4] In addition, CD10 and CD45 were expressed in the primary material, but were weaker and partially lost in the cell line. Expression of CD19 and CD22 varied slightly between the primary material and M4A1-M2B9 but both were stably expressed (Figure 2F). Together these results suggest that our *MEFD2-BCL9*-positive cell line represents the major clone of the primary material. In order to gain a better understanding of the state of cells in which the establishment of clonal selection occurred, the experiments should have been performed with the cells directly harvested from the PDX as well. Unfortunately, the number of blasts yielded from the spleen was insufficient to perform these experiments.

Finally, we determined whether the drug sensitivity of M4A1-M2B9 resembles the drug sensitivity of the primary leukemic cells. Sensitivity to drugs that do not directly affect the cell cycle nor DNA or RNA synthesis, such as prednisolone, dexamethasone, asparaginase and different pan-HDAC inhibitors, differed minimally between the primary material and M4A1-M2B9 (Figure 3A-D; *Online Supplementary Figure S2*). As expected, drugs which influence cell cycle or DNA/RNA synthesis such as vincristine, cytarabine, and daunorubicin affected the proliferating M4A1-M2B9 more than the primary material (Figure 3E-G).

In conclusion, the patient-derived *MEF2D-BCL9*-positive cell line is highly representative for the major clone of the primary material, showing similar gene expression, mu-

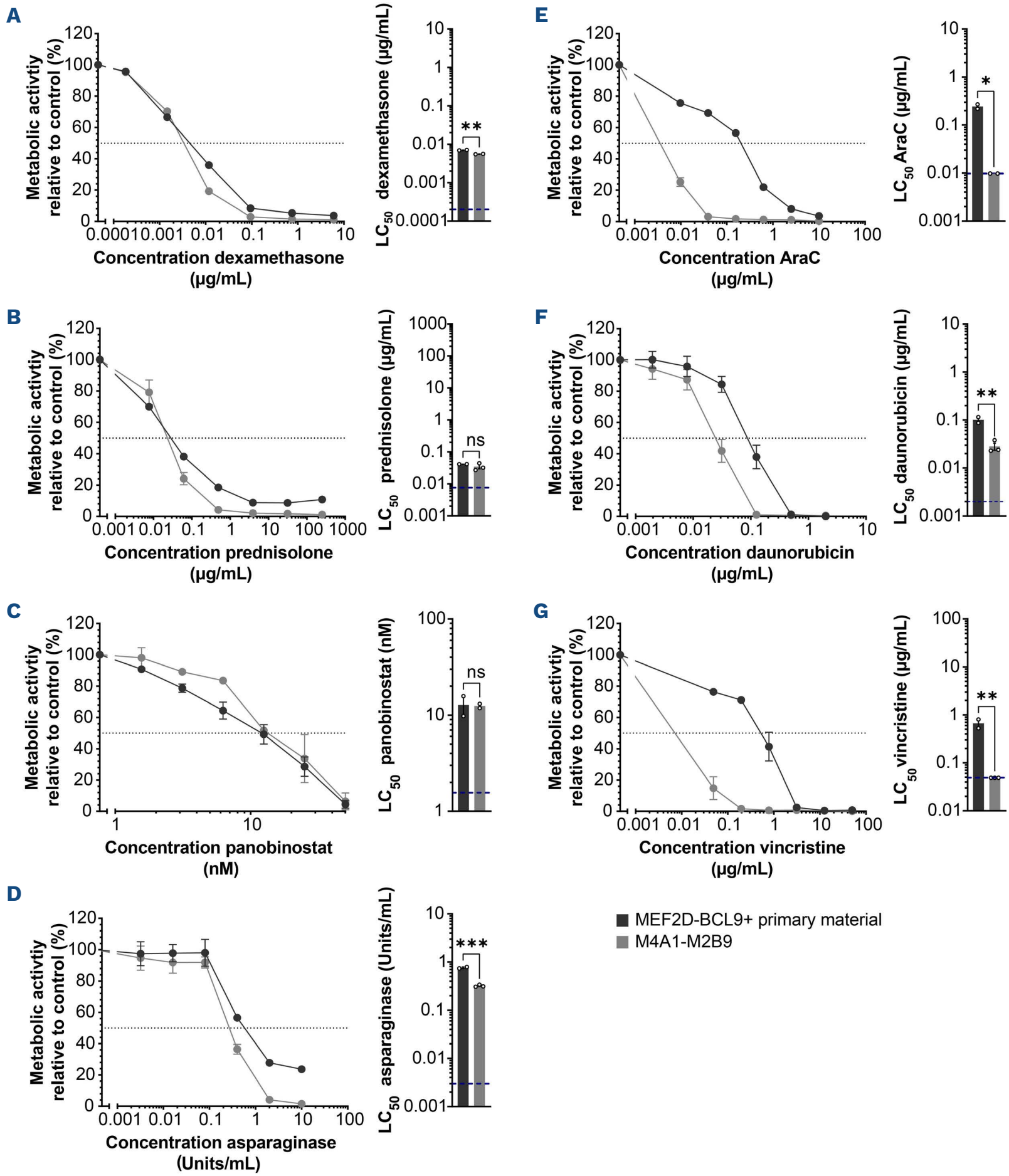

Figure 3. Drug sensitivity comparison between primary material and the cell line. Primary material and cell line were cultured for 4 days at 37°C and 5% CO_2 either in the presence or absence of the drug starting at a concentration of $1.6x10^6$ cells/mL and $0.5x10^6$ cells/mL, respectively. Survival was measured using MTT and the metabolic activity was compared to untreated cells. Primary material is shown in dark gray and the cell line in light gray. (A-D) Comparison of drug sensitivity using compounds that do not affect the cell cycle or DNA/RNA synthesis between the primary material and cell line. These drugs include 2 glucocorticosteroids, dexamethasone (1:8 dilution) and prednisolone (1:8 dilution), a metabolic drug, asparaginase (1:5 dilution), and a pan-HDAC inhibitor panobinostat (1:2 dilution). (E-G) Comparison of drug sensitivity using compounds that affect the cell cycle or DNA/RNA synthesis between the primary material and the cell line. These drugs include AraC (1:4 dilution), daunorubicin (1:4 dilution), an anthracycline, and vincristine (1:4 dilution), which inhibits mitotic spindle formation. (A, B) X-axis, concentration of the drug, Y-axis, metabolic activity relative to untreated cells. For patient material and cell line the graphs show mean ± standard error of the mean (SEM) of 2 or 2-3 independent experiments, respectively. The bar plots show the mean lethal concentration 50 (LC_{50}) ± SEM of 2 or 2-3 independent experiments, respectively, the blue line in the half maximal inhibitory concentration (IC_{50}) plots indicate the lowest measured concentration. An unpaired *t*-test was used to compare the LC_{50} values between the primary material and the cell line (ns: not significant $P > 0.05$; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

tations, copy number alterations, and immunophenotype. Because the cell line proliferates, M4A1-M2B9 may better represent the drug sensitivity of leukemic cells in the patient, especially of drugs that affect cell cycle or DNA/RNA synthesis. M4A1-M2B9 facilitates functional studies, for example the development of targeted therapies directed against the MEF2D fusion protein or downstream targets, or CAR T products directed against CD5, which are currently being studied for patients with T-cell ALL.[10] As MEF2D fusion protein-positive pediatric B-ALL is associated with high-risk features, such as high age, high white blood cell count, high MRD, and poor outcome,[1,3] targeted therapy might improve the treatment of patients with a *MEF2D* fusion.

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https://doi.org/10.3324/haematol.2022.281712

Received: November 18, 2022.
Accepted: March 31, 2023.
Early view: April 13, 2023.

Disclosures

No conflicts of interest to disclose.

Contributions

This project was conceived by JMB and MLdB and conceptualized together with IvO and FMH. Experimental and computational analyses were performed by IvO, FMH, AQH, AB and SvdB. Data interpretation was performed by IvO, FMH, MLdB and JMB. The manuscript was drafted by IvO, FMH, MLdB and JMB. All authors approved the final version of the manuscript.

Funding

The authors would like to thank Foundation Pediatric Oncology Center Rotterdam (SKOCR), the Dutch Cancer Society grant KWF-10482, and the KiKa Foundation Kika-264 grant for their support.

Data-sharing statement

The data that support the findings of this study are available on request from the Data Access Committee Princess Maxima Center (e.g. https://ega-archive.org/datasets/EGAD00001009759 [RNAseq], https://ega-archive.org/datasets/EGAD00001009758 [WXS]). The data are not publicly available due to them containing information under controlled access.

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