An induced pluripotent stem cell t(7;12)(q36;p13) acute myeloid leukemia model shows high expression of MNX1 and a block in differentiation of the erythroid and megakaryocytic lineages

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Funding information
Barnancerfonden, Grant/Award Numbers: PR2014-0125, PR2019-0013, TJ2019-0053; Cancerfonden, Grant/Award Number: CAN2017/461; Västra Götalandsregionen, Grant/Award Number: ALFGBG-431881; Swedish Research Council, Grant/Award Number: 2018-05973; Swedish National Infrastructure for Computing (SNIC) at UPPMAX, Grant/Award Number: SNIC 2021/22-754

Abstract
Acute myeloid leukemia (AML) results from aberrant hematopoietic processes and these changes are frequently initiated by chromosomal translocations. One particular subtype, AML with translocation t(7;12)(q36;p13), is found in children diagnosed before 2 years of age. The mechanisms for leukemogenesis induced by t(7;12) is not understood, in part because of the lack of efficient methods to reconstruct the leukemia-associated genetic aberration with correct genomic architecture and regulatory elements. We therefore created induced pluripotent stem cell (iPSC) lines that carry the translocation t(7;12) using CRISPR/Cas9. These t(7;12) iPSC showed propensity to differentiate into all three germ layers, confirming retained stem cell properties. The potential for differentiation into hematopoietic stem and progenitor cells (HSPC) was shown by expression of CD34, CD43 and CD45. Compared with the parental iPSC line, a significant decrease in cells expressing CD235a and CD41a was seen in the t(7;12) iPSC-derived HSPC (iHSPC), suggesting a block in differentiation. Moreover, colony formation assay showed an accumulation of cells at the erythroid and myeloid progenitor stages. Gene expression analysis revealed significant down-regulation of genes associated with megakaryocyte differentiation and up-regulation of genes associated with myeloid pathways but also genes typically seen in AML cases with t(7;12). Thus, this iPSC t(7;12) leukemia model of the t(7;12) AML subtype constitutes a valuable tool for further studies of the mechanisms for leukemia development and to find new treatment options.

KEYWORDS
AML, chromosome translocation, leukemia, pluripotency, stem cells

Abbreviations: AML, acute myeloid leukemia; CFU, colony forming unit; FISH, fluorescence in situ hybridization; HSPC, hematopoietic stem and progenitor cells; iHSPC, induced hematopoietic stem and progenitor cell; iPSC, induced pluripotent stem cell; PCA, principal component analysis; SNP, single nucleotide polymorphism.

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Acute myeloid leukemia (AML) is the result of aberrant hematopoietic processes, such as enhanced proliferation, blocked differentiation and dysregulated apoptosis of hematopoietic stem and progenitor cells (HSPC), frequently initiated by chromosomal translocations. In children, AML is associated with higher rates of relapse and death as compared with acute lymphoblastic leukemia. In the current study we have focused on a chromosomal alteration that is found in AML only in children diagnosed before 2 years of age, namely the chromosomal translocation t(7;12)(q36;p13). This AML subtype has been associated with poor prognosis and the reasons for this and potential targeted treatment strategies remain to be shown. The position of the translocation breakpoints is typically localized in a breakpoint cluster region distal to MNX1 gene on chromosome 7 and within intron 1 or 2 of the ETV6 gene on chromosome 12. No difference in outcome has been seen between these two variants. The ETV6 gene encodes a transcriptional repressor that plays a critical role in hematopoiesis and in embryonic development. The MNX1 gene encodes a homeodomain protein that is highly conserved and functions as a transcription factor. It is expressed during embryogenesis and is essential for the formation of the dorsal pancreatic bud and B-cell maturation. The translocation t(7;12) suggests that a fusion gene between MNX1 and ETV6 can be generated and MNX1-ETV6 gene transcripts can also be detected in leukemia cells from approximately half of AML patients with t(7;12). Since the complete MNX1 coding region is typically translocated, a reciprocal fusion transcript is not detected. All t(7;12) AML patients typically display an increased expression of the wild type MNX1 gene, which has been associated with a block in differentiation of HSPC, and therefore suggested to play a major role in leukemia development. The mechanism behind this ectopic expression of MNX1 has not been established but enhancer high-jacking and alternative splicing might explain this phenomenon. High expression of MNX1 has also been reported in other malignancies, such as prostate cancer, hepatocellular carcinoma and colon carcinoma.

The MNX1-ETV6 fusion and/or the aberrant MNX1 expression and their respective role in leukemia transformation is presently not fully understood at the molecular level, mainly due to scarcity of available patient leukemia material and lack of suitable experimental models. Efficient methods for modeling leukemia and to recreate leukemia-associated genetic aberrations, such as chromosome translocations, are crucial for investigating how leukemia is initiated. Today, most such models are murine and based on introduction of a gene or a gene fusion of interest under the control of a constitutive active or inducible promoter using lentiviral or retroviral transduction rather than the chromosomal translocation itself. This might introduce unwanted off-target effect by the viral integration into the genome. Also, the gene fusion may lack properties carried by the translocation itself, such as the genomic architecture with correct regulatory elements. The technique to generate induced human pluripotent stem cells (iPSC) from somatic cells of human origin has provided new tools for disease modeling. The iPSCs are relatively easy to manipulate genetically utilizing the CRISPR/Cas9 technology and can be differentiated into the relevant cell type of interest, in the case of leukemia HSPC. We have here modified a protocol for translocation formation using CRISPR/Cas9 to efficiently create iPSC with t(7;12). Characterization of these iPSC with t(7;12) showed cell differentiation and growth properties as well as a gene expression signature similar to leukemic cells from patients with t(7;12) AML. This model will therefore be a valuable tool for further studies of the mechanisms behind the leukemogenic effects of this translocation.
from the bone marrow utilizing the CD34 MicroBead Kit UltraPure (Milteny Biotech) 48 h after thaw as above. The CD34+ cells were kept in StemPro medium for 24 h before reprogramming with the CytoTune-iPS 2.0 Sendai Reprogramming kit, following the CD34+/CD38- isolation. From day 3, the same feeder-free procedure as above was pursued. The resulting iPSC clones were tested for pluripotency with the BD Stemflow Human and Mouse Pluripotent Stem Cell Analysis Kit (BD Biosciences).

The human iPSC line ChiPSC22 (RRID:CVCL_RN02) (Cellartis/Takara Bio Europe) was maintained in the feeder-free DEF-CS culture system under standard conditions. The cells were transfected to CornMatrigel (BD Biosciences) and mTeSR1 medium (StemCell Technologies Inc.) for 2 to 3 passages before differentiation to hematopoietic cells utilizing STEMdiff Hematopoietic kit (Stemcell Technologies Inc.) according to protocol. At day 12 of differentiation, the hematopoietic cells were harvested and analyzed by immunophenotyping, RT-qPCR and CFU assays. For flow cytometry, cells were recovered overnight in StemSpan SFEM II + CC100 (Stemcell Technologies Inc.) and the CD34+CD43+CD45+ cell pool was sorted on a FACS Aria IIu (BD Biosciences) before RNA preparations for RNAseq and RT-qPCR. For continuous culture, unsorted cells were kept in StemSpan SFEM II + CC100 for up to 14 days. Cells were counted and diluted to 0.8 × 10^5/ml every 48 h by centrifuging cells, saving 25% of the old medium and adding 75% fresh medium. Cell divisions were calculated from the formula
\[ n = \frac{s^p}{2^p} \]
where \( s \) = number of cells at start and \( p \) = number of cells at end (48 h later). All cell culture work was conducted under strictly controlled mycoplasma-free conditions. The ChiPSC22 cell line has recently been authenticated using short tandem repeat profiling (August 2021).

2.2 | CRISPR/Cas9 based translocation procedure

CRISPR RNAs were designed for chromosome 7 and 12, respectively, by “Custom Alt-R CRISPR-Cas9 guide RNA design tool” (Integrated DNA Technology, IDT) and chosen based on the best “on-target potential” and “off target risk” combination from a list of 100 for every sequence tested. This resulted in the following breakpoints on chromosome 7: NC_000007.14:g.156984781 and chromosome 12: NC_000012.12:g.11797368, respectively. The crRNAs were synthesized at IDT and annealed to an ATTO550 tagged tracrRNA (IDT) to yield guideRNAs. Cas9 was introduced as a protein to make a Cas9: gRNA ribonucleoprotein (RNP) complex before delivery into the cells. Sequences for the crRNAs are listed in Table S1. The 150 nt ssODNs (single stranded oligodeoxynucleotides) were designed to cover the fusion sites by joining 75 nt each from the two chromosome partners to generate der (7) and der (12). The ssODNs were synthesized at IDT as UltramerOligonucleotides (Table S1). The 4D-Nucleofector (Lonza) was used with 16 well nucleocuvettes and program CA-137. For each well a reaction was set up with 350 000 cells in 15 μl nucleofector solution P3 and a premix of RNP complexes and ssODNs in 6 μl. Directly after the pulses 75 μl medium was added and the cells transferred to a previously Coat-1 coated well in a 24-well plate containing 1 ml DEF-CS medium. Cells from two transfections were pooled in one well. Twenty-four hours posttransfection the ATTO-550+ cells were sorted using blue laser with a 585/42 nm filter on a FACS Aria IIu (BD Biosciences). The cells were suspended in DEF-CS medium, left to recover for 24 to 48 h as a bulk before seeding in Coat-1 coated 96-well plates at a dilution corresponding to 0.5 cell/well. Surviving cells were expanded and approximately 2 to 3 weeks after single cell seeding cells were taken for analysis of successful translocation. The lysis procedure from the Guide-it mutation detection kit (Takara Bio Europe) was used for colony screen with direct PCR. Primer sequences are listed in Table S1. gBlocks (IDT) were synthesized as positive controls for the correct translocation by PCR (Table S1). The iPSC lines positive for a correct translocation by PCR and FISH were tested for differentiation potential to all three germ layers with STEMdiff Trilineage Differentiation kit (Stemcell Technologies Inc.) and analyzed by immunocytochemistry. (Antibodies are listed in Table S2). Pluripotency was also tested via “Confirmation of pluripotency by spin embryoid body formation” (Takara Bio). Eighteen days after the start of spin EB formation, cells were taken for analysis of germ-layer specific gene expression by RT-qPCR (Table S3).

2.3 | Cyto genetic characterization

Fluorescence in situ hybridization (FISH) analysis, karyotyping-banding and single nucleotide polymorphism (SNP)-array were performed with standard procedures at the Cyto genetic laboratory, Department of Clinical Genetics and Genomics, Sahlgrenska University Hospital. FISH was done using a Double Fusion Break Apart probe specific for t(7;12) (q36:p13) (MetaSystems Probes). SNP-array was performed using the CytoScan HD Array (ThermoFischer Scientific). The software Nexus Copy Number Discovery (BioDiscovery) was used for data analysis.

2.4 | Immunophenotyping

For flow cytometry analysis, the cells were incubated with antibodies for 20 min at room temperature in PBS/1% BSA/2 mM EDTA, washed and resuspended in the same solution with the addition of 7-AAD (BD Via-Probe, BD Biosciences) just before analysis on a BD FACScan (BD Biosciences). The BD FACSDiva software was utilized for recording and analyzing data. Doublets and dead cells were gated out before final analysis and isotype antibodies were used as negative controls (Figure S1 and Table S2). For Figure 3A, the free software Flowing was used.

Cytospins were made from 50 000 to 100 000 cells suspended in PBS, spun onto glass slides for 5 min at 400 rpm in a Shandon Cytospin 2 centrifuge, air dried and stained in 4% formaldehyde solution. Cells on the coverslips were permeabilized with 0.1% Triton X-100, washed and blocked with 5% fetal bovine serum +0.01% Tween20 in PBS. Antibodies (Table S2) were diluted, and staining performed according to manufacturer’s recommendations. The slides were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen/ThermoFisher Scientific).
FIGURE 1 Legend on next page.
2.5 | CFU assay

Methocult H4435 Enriched (Stemcell Technologies) was used for the colony forming unit (CFU) assays where 5000 or 20 000 cells were plated/petri dish and incubated for 14 days according to the manufacturer’s protocol.

2.6 | Gene expression analysis

Total RNA was isolated using the miRNAesy Plus micro kit (Qiagen) and cDNA synthesized with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR or SuperScript VILO cDNA Synthesis Kit (Invitrogen/ThermoFisher Scientific). TaqMan Gene Expression Assays (Table S3) and TaqMan Universal MasterMix II (Applied Biosystems/ThermoFisher Scientific) were used for probe-based qPCR assays. A SYBR Green assay was used for the MNX1(ex1)-ETV6(ex3) fusion detection (forward primer: CCCGACTTCAACTGCTTGCA, covering the splice site, and reverse primer: TGCTGTCATTGGGCTTAAAAG), with primers synthesized at IDT and Power SYBR Green PCR Master Mix (Applied Biosystems/ThermoFisher Scientific). The reactions were performed on the ABI 7900HT Sequence Detection System (Applied Biosystems/ThermoFisher Scientific) with GUSB as the reference gene. Gene expression was calculated using the deltaCt method and is presented as $2^{-\Delta\Delta C_t}$.

RNA sequencing was performed at the Center for Medical Genomics, Department of Clinical Genetic and Genomics, Sahlgrenska University Hospital, Sweden, on a NextSeq 500 instrument (Illumina). The library preparation was done with TruSeq Stranded Total RNA Sample Preparation kit with Ribo-Zero Gold (Illumina). For RNA-seq analysis, alignment was carried out to reference human genome GRCh38 (hg38) using STAR (version 2.9.0a) with the following parameters: outSAMtype BAM SortedByCoordinate. Quantification was carried out using HTSeq-count (Python package HTSeq, python v 3.8.1) software using the following parameters -f bam -r pos -s no -m union with the reference annotation file. The human reference genome and annotation files were obtained from the Ensembl database. The sequencing coverage and quality statistics of the RNA sequencing data is listed in Table S4. Differential gene expression was performed using the R package “Deseq2”.

Genes with log2 (foldchange) 1 and FDR <.05 were considered differentially expressed genes (Table S5A).

Principal component (PCA) analysis and Pearson correlation heatmap analysis were performed in order to examine the level of differences in the gene expression profiles between control and t(7;12) samples. For pathway analysis two pathway databases were used; the Reactome pathway database and the Gene Ontology GO-biological process database. A pathway was regarded significantly enriched when FDR <0.05, P value <.05 and >10 genes of the respective pathway were present. Gene Set Enrichment Analysis (GSEA) was used with the Gene Ontology GO-biological process gene set. 

A pathway in GSEA analysis was regarded significant at nominal $P$ value <.05 and FDR <0.05 (Table S5B).

2.7 | Gene expression comparison

For gene expression comparison analysis the significant differentially expressed genes from the RNA sequencing analysis of the t(7;12) induced HSPC was used (FDR <0.05, $P$ value <.05, gene list in Table S5C). Microarray gene expression data from pediatric AML cases was retrieved from the GEO database (GSE17855) and microarray and RNA Sequencing data was retrieved from the NCI TARGET database (https://target-data.nci.nih.gov/Public/AML/). AML gene expression profile analysis of this data set was performed using R packages “Affy” and “Limma.” The t(7;12) gene signature set was further validated using RNA sequencing data retrieved from the TARGET data set FHCRC-LowDepth.

3 | RESULTS

3.1 | Leukemic cells from an AML patient with t(7;12)(q36;p13) are blocked in their potential to achieve pluripotency

Our first attempt to make a human disease model to study the mechanisms behind the t(7;12) AML development was to create an iPSC line directly from a patient sample with this translocation. The bone marrow sample from this patient contained 90% leukemic blasts positive for the t(7;12), but also had additional chromosomal aberrations typically found in this type of AML, in this case trisomy 8 and trisomy 19. Viably frozen bone marrow cells were subjected to reprogramming in two independent experiments, where the second was performed on CD34+ enriched bone marrow cells. Forty-three iPSC clones were established, and the clones were defined as iPSC based on their expression of pluripotency markers, OCT3/4 and SSEA-4, both positive on >99% of the cells. Interestingly, both FISH and G-banding showed that all clones had a normal karyotype and lacked the t(7;12) translocation. Thus, leukemic cells from this AML patient with t(7;12) might have lost their potential to achieve pluripotency and the derived iPSC must therefore have originated from the very few normal nonleukemic cells in the bone marrow sample.

**FIGURE 1** Human iPSC cells with translocation t(7;12). (A) Human iPSC with t(7;12)(q36;p13) was created and the translocation was confirmed in all five clones with a FISH probe (Metasystems, Dual Fusion Break Apart probe) specific for t(7;12)(q36;p13) and indicated by arrows. Representative picture from clone A. (B) The presence of a functional fusion was confirmed by RT-qPCR for the MNX1-ETV6 transcript. Results are presented as $2^{-\Delta C_t}$ vs endogenous reference gene GUSB (n = 5, all t(7;12) clones). (C) Conventional karyotype was performed on a subset of the clones as a complement to the FISH analysis. A representative metaphase from clone B is shown, with the translocation t(7;12) indicated (arrows). (D) SNP-array was performed on all t(7;12) clones. No additional gains or losses of genetic material were detected. Representative SNP array from clone B is shown.
Efficient recreation of the t(7;12)(q36;p13) in human iPSC using CRISPR/Cas9

We consequently determined to use another approach, described by Torres-Ruiz et al.\textsuperscript{13} where they successfully generate several iPSC lines with a translocation t(11:22) with the CRISPR/Cas9 technology. Their protocol was slightly improved by including a fluorescent tag on the crisprRNA enabling FACS sorting of the successfully transfected cells increasing the yield of correct single cell clones. We used the human iPSC line ChiPSC22 (hereafter denoted parental iPSC) for
Introduction of the translocation. To mimic our patient’s translocation, we chose to introduce the breakpoint in intron 2 of the ETV6 gene on chromosome 12. The breakpoint on chromosome 7 was placed in a region denoted CCB, Candidate Common Breakpoint, 13 to 26 kb downstream of MNX1. The presence of the translocation was confirmed with PCR over the fusion site. Several different single-cell derived iPSC clones were chosen for further analysis and were regarded as biological replicates. In total five different iPSC lines with the correct translocation were established, denoted t(7;12) iPSC (individual cell lines t(7;12)-A-E, respectively). The presence of the translocation was confirmed with PCR over the fusion site. Several different single-cell derived iPSC clones were chosen for further analysis and were regarded as biological replicates. In total five different iPSC lines with the correct translocation were established, denoted t(7;12) iPSC (individual cell lines t(7;12)-A-E, respectively).

**FIGURE 3** Legend on next page.
translocation was further verified by FISH, and mRNA expression of the MNX1-ETV6 fusion transcript could be detected, although at a very low level (Figure 1A,B). Thus, a functional chromosomal rearrangement was verified. No other chromosomal alterations were seen when the t(7;12) iPSC lines were analyzed with conventional karyotyping and SNP array (Figure 1C,D).

3.3 | Hematopoietic stem and progenitor cells differentiated from t(7;12) iPSCs show increased proliferation and are blocked at the megakaryocytic and erythroid progenitor stage

To confirm retained stem cell properties after introduction of the translocation, directed and spontaneous differentiation via embryoid bodies were investigated. The t(7;12) iPSCs showed similar propensity as the parental iPSC to differentiate into all three germ layers, as shown by expression of markers specific for endo-, ecto- and mesoderm (Figure 2A,B). To be able to study the function and effects of the translocation in the appropriate cellular context, the iPSC lines were differentiated into HSPC. The resulting induced HSPC (iHSPC) displayed the expected combined expression of CD34, CD43 and CD45 (Figure 3A). When analyzed with a broader panel of markers for subpopulation of hematopoietic cells, the t(7;12) iHSPC showed a significant decrease in cells expressing CD235a and CD41a, and an increased expression of CD45RA compared with the parental iHSPC (Figures 3B and S1). This finding suggests a block in differentiation into the erythroid and megakaryocytic lineages and a shift towards the myeloid lineage. CFU analysis showed increased colony formation, in particular for BFU-E and GM colonies, but also total cell numbers in the t(7;12) iHSPC compared with the parental iHSPC, indicating that cells were detained at the progenitor stage and with an increased proliferative capacity (Figure 3C). However, CFU secondary replating did not give rise to any colonies but continuous culturing of the iHSPC, we could see an increased proliferative capacity of the t(7;12) compared with the parental iHSPC (Figure 3D and Table S6).

3.4 | High expression of the MNX1 gene is induced in t(7;12) iHSPC

The translocation t(7;12) indicates that a fusion gene between MNX1 and ETV6 can be generated and the MNX1-ETV6 gene fusion transcript was also detected in our iHSPC harboring the t(7;12) (Figure 4A). To investigate whether the translocation had any influence on the expression of full-length MNX1, expression was analyzed with RT-qPCR and immunocytochemistry in the parental and t(7;12) iHSPC (Figure 3B,C). The MNX1 gene was barely expressed or expressed at very low levels in the undifferentiated iPSCs, both in the

| Figure 3 | Analysis of iHSPC. (A) Flow cytometry showed efficient differentiation to hematopoietic cells by high CD34, CD43 and CD45 expression. Analysis was done on viable single cells and gating was set on isotype control plots. The gated areas were used for sorting of triple positive population. Numbers shown are percentage of viable single cells and in the second graph the triple positive proportion (example from clone E). (B) Immunophenotyping by flow cytometry revealed that t(7;12) iHSPC lines have a significant decrease in number of CD235a+ and CD41a+ cells as well as an increase in CD45RA+ compared with the parental iHSPC. All cell lines were analyzed after repeated differentiation rounds, one to five times per line, CD235a; n = 19 ± 7, CD41a; n = 10 ± 6, CD45RA; n = 7 ± 3, where n denotes total number of analyses for t(7;12) and the parental lines, respectively. **P < .0001; see also Figure S1. (C) CFU analysis showed significantly more GM and E colonies as well as an increased total number of colonies in the t(7;12) iHSPC lines compared with the parental iHSPC. All five clones, A-E, were analyzed once each and the parental line three times (E: erythroid, M: macrophage, G: granulocyte, GM: granulocyte, macrophage, GEMM: granulocyte, erythrocyte, macrophage, megakaryocyte). **P < .01. (D) An increased proliferative capacity for t(7;12) iHSPC lines compared with the parental iHSPC was seen by continuous culture after the 12 day differentiation protocol was over. All five clones, A-E, were analyzed once each and the parental line twice. ****P < .0001, ***P < .001

| Figure 4 | MNX1-ETV6 and MNX1 expression in iHSPC. (A) The MNX1-ETV6 fusion transcript was expressed only in the t(7;12) iHSPC analyzed by RT-qPCR. Four clones analyzed, total n = 7 (A; n = 2, C; n = 2, D; n = 2, E; n = 1). Results are presented as 2 ΔCt vs endogenous reference gene GUSB. (B) A high expression of MNX1 was detected in t(7;12) iHSPC but was not detectable in the parental iHSPC with RT-qPCR. Same clones analyzed as in 4A. (C) Immunocytochemistry on cytosplins showed protein expression of MNX1 in the t(7;12) iHSPC but not in the parental iHSPC (scale bar = 100 μm). All clones analyzed, results shown for t(7;12) line B and parental line.
parental and the t(7;12) cell lines, and could not be detected at the protein level. Upon differentiation into HSPC, the t(7;12) iHSPC showed a high mRNA expression of both the MNX1-ETV6 fusion as well as MNX1 compared with the parental iHSPC (Figure 4A,B), and MNX1 could also be detected at the protein level (Figure 4C).

3.5 Gene expression analysis of t(7;12) iHSPC revealed enrichment in pathways commonly affected in AML

The next step to investigate our t(7;12) iHSPC clones was to perform global transcriptome analysis. RNA sequencing was performed on iPSC differentiated into iHSPC, and differentially expressed genes and affected signalling pathways were identified by comparing the t(7;12) iHSPC to iHSPC from the parental iPSC. The read quality of all samples was deemed good, with the number of QC-passed reads per sample ranging from 31.6 to 45.0 million and the fraction of uniquely mapped reads ranging from 81% to 89%. Pearson correlation heatmap and PCA showed a clear separation of the four sequenced t(7;12) iPSC clones (A-D) from the parental iPSC on the first principal component and strong clustering of t(7;12) and parental iPSC samples, respectively (Figure S2A,B). Analysis of the most significant differentially expressed genes showed an abundance of genes that were downregulated in the t(7;12) iHSPC compared with the parental iHSPC (Figure 5A). Of the differentially expressed upregulated genes, MNX1 was the most significant. Elevated expression of the long non-coding RNA, MNX1-AS1 and MNX1-AS2, from the same gene locus as MNX1 was also observed.

Pathway analysis based on the Reactome pathway database and the Gene Ontology GO-biological process database, revealed a significant enrichment in immune, cell cycle and DNA repair pathways. Among the significant regulated immune pathways were myeloid differentiation and megakaryocyte differentiation pathways, in addition to leukocyte migration, cytokine production and T-cell differentiation pathways (Figure S2C,D). Gene Set Enrichment Analysis (GSEA) was
FIGURE 6  Comparison of gene expression signatures. (A) Pie chart illustrating the overlap of differentially expressed genes from RNA sequencing of t(7;12) iHSPC compared with pediatric leukemia gene expression signatures from patients with different AML subtypes. (B) RT-qPCR of representative genes to validate the RNA sequencing results. RNA prepared from FACS sorted triple positive populations of cells, three different t(7;12) lines; A,C,D and the parental line differentiated at three different occasions. Results are presented as $2^{-\Delta\Delta CT}$ vs endogenous reference gene GUSB. ****P < .0001, ***P < .001, **P < .01, *P < .05
performed using the Gene Ontology GO-biological process gene set and the all genes with normalized counts from the parental iHSPC and the t(7;12) iHSPC (Table S5B) to determine whether we could see any statistically significant differences between them. This analysis showed a significant enrichment of several immune pathways including monocytic and megakaryocytic differentiation in line with the Reactome pathway analysis (Figure S5B,C).

### 3.6 Comparison with gene expression signatures in pediatric acute myeloid leukemia

In order to assess the similarity between our iPSC model and AML with t(7;12), we compared the differentially expressed genes found in t(7;12) iHSPC with differentially expressed genes found in different pediatric AML using data retrieved from the TARGET database. This global comparison revealed an overlap where approximately 30% of the differentially expressed genes were in common with AML with t(7;12) but this degree of overlap was also seen with other subtypes of pediatric AML with recurrent chromosomal aberrations. We could not identify a specific pathways, using either Reactome or GO-analysis, that was significant and overlapping between (7;12) iHSPC and t(7;12) AML samples from patients. Since AML subtypes with recurrent chromosomal aberrations typically have characteristic gene expression signatures based on a limited set of genes that are enough for their identification, we compared such signatures obtained from 237 pediatric AML cases with our differentially expressed genes from t(7;12) iHSCP (Table S5C). This comparison revealed an overlap of 84% with t(7;12) AML and this degree of overlap could not be seen when compared with other pediatric AML subtypes (Figure 6A and Table S7). Thus, among the genes that were most discriminating and differentially expressed in t(7;12) AML compared with other pediatric AML subtype almost all were also differentially expressed in our (7;12) iHSPC, and could also be verified by RT-qPCR (Figure 6B and Table S7). Furthermore, using an independent RNAseq data set from the TARGET database we could verify that the same genes were differentially expressed in t(7;12) AML cases and that these genes also differ in their expression from most other types of pediatric AML (Figure S3).

### 4 DISCUSSION

In this study we have created a human iPSC model of pediatric AML harboring the t(7;12) (q36;p13) using CRISPR/Cas9 technology. The iHSPC that were derived from these iPSC showed features typically seen in this type of leukemia, such as an induced block in differentiation and increased proliferative capacity. We could also verify that the translocation lead to ectopic and high expression of the homeodomain protein MNX1 in t(7;12) iHSPC, which is also a hallmark typically found in AML samples from patients.

Using iPSC derived from leukemia samples from patients has been suggested as a source to make suitable disease models, and some successful examples have also been reported. Our finding that leukemic cells from AML patients with t(7;12)(q36;p13) seem to be blocked in their potential to achieve pluripotency is interesting. This implies that these leukemic cells also have a block in dedifferentiation capacity, and that this could be attributed to the epigenetic and genetic aberrations inherent to this type of AML. This is also in line with findings in other studies, where it has been shown that the ability to make iPSC from acute leukemia samples from patients is far from always possible. To overcome this we instead used a modified CRISPR/Cas9 protocol to create iPSC clones with the (7;12)(q36;p13). This comes with the advantage that we now have several iPSC clones that only harbor the translocation and no other genetic alterations that are typically seen in this AML, for example, trisomy 8 and trisomy 19. This makes it possible to further dissect the importance of individual genetic alterations where gene-editing can be done step-wise, introducing one modification after another, studying the effect of each modification alone and in combination.

When we differentiated the t(7;12) iPSC to iHSPC we saw a relative block in their ability to differentiate into the megakaryocytic and erythroid lineage and an accumulation at the progenitor stage. This findings corroborate well with what has been reported in another experimental model, where MNX1 transduced primary murine HSPC underwent profound differentiation arrest and accumulated at the megakaryocyte and erythrocyte progenitor stage. Gene expression analysis of t(7;12) iHSPC also revealed pathway enrichment of genes associated with myeloid differentiation and megakaryocyte differentiation as well as leukocyte migration and cytokine production. In the majority of these the effects were negative, thus reflecting a down-regulation, or lack of activation, of genes and a subsequent blockade of the pathways regulating cell differentiation. This is a hallmark of AML in general, and to find drugs that can overcome this block and induce differentiation has been one major focus to treat AML. We also observed an increased proliferative and an increased colony formation capacity of t(7;12) iHSPC compared with parental iHSCP have. However, these cells did not give rise to new colonies in secondary replating, suggesting they do not have enhance self-renewal capacity in vitro.

Our comparison of differentially expressed genes in t(7;12) iHSPC and human AML revealed a clear enrichment of genes that are both typically expressed in t(7;12) AML compared with other forms of AML and that were also differentially expressed in the t(7;12) iHSPC. For this comparison, we used expression signatures introduced by Balgobind et al, showing the potential to classify pediatric acute myeloid leukemia. Using gene expression microarray data from pediatric AML cases, Balgobind et al could correctly predict the most prevalent cytogenetic subtypes of pediatric acute myeloid leukemia with high accuracy. When comparing differentially expressed genes from the t(7;12) iHSPC to this more restricted set of differentially expressed genes from patient material we could see a large overlap between patients with t(7;12) and our generated t(7;12) iHSPC. This gene set was also verified when we analyzed a data set from TARGET representing a larger cohort of pediatric AML cases.

This shows that t(7;12) iHSPC reconstitutes a core gene expression signature similar to that of t(7;12) AML. Several of these identified core genes can also give important leads to the mechanism
behind this type of leukemia. On a global level, there was however 
no unique RNA expression pattern or specific pathway overlapping 
significantly between t(7;12) IHSCP and t(7;12) AML patients. It has 
been shown that pediatric AML with different recurrent chromosomal 
aberrations share the majority of differentially expressed genes when 
compared with normal bone marrow.20 This together with the fact 
that we were comparing results from cells cultured in vitro perhaps 
make such an effort futile.

The dramatic increase in MNX1 expression in the t(7;12) IHSCP is 
linked to the effects seen on differentiation and proliferation, 
although the mechanism behind the high expression of the 
MNX1 gene in t(7;12) AML is not known. Our findings however indicate 
that this is most likely directly linked to the chromosomal translo-
cation itself and is presumably an enhancer hijacking event of a 
hitherto unidentified enhancer. The activity of this enhancer is most 
likely restricted to hematopoietic cells since we only saw high MNX1 
expression in the t(7;12) iHSCP and not t(7;12) iPSC. We also saw that 
Lin28B is expressed in t(7;12) AML and in our t(7;12) iHSCP and this 
upregulation is potentially an intriguing finding. Lin28B has previously 
been shown to govern fetal hematopoiesis and its expression is 
severely reduced postpartum in normal hematopoietic cells.21 Since 
the majority of patients harboring t(7;12) AML are diagnosed before 
6 months of age and is typically not found in AML patients older than 
2 years of age, development of t(7;12) AML may be restricted to fetal 
hematopoiesis.2 Another interesting gene with high expression in t(7, 12) AML and our t(7;12) iHSCP is AGR2. AGR2 is a protein that 
plays a role in cell migration, cellular transformation, is a known p53 
inhibitor and has been connected to drug resistance.2223 It has been 
shown that MNX1 induces senescence in human HT1080 and murine 
NIH3T3 cells through the p53 pathway8 why inhibition of p53 might 
be a prerequisite for malignant transformation by MNX1.

In conclusion, our human t(7;12) iPSC model will be a valuable 
tool to continue to investigate the transforming effects by MNX1 in 
hematological malignancies. An interesting observation is that the 
t(7;12) AML is basically only found in infants or very young children. 
This suggests that MNX1 might be such a strong inducer or driver of 
leukemia transformation that it does not need any additional genetic 
lesions and therefore presents itself early. However, the exclusiveness 
of this AML to infants also suggests that the HSPC that can be trans-
formed are only present during fetal development or early infancy and 
that leukemia initiation therefore can only occur in a window of early 
hematopoiesis, making our iPSC model extra suitable to address these 
questions. Furthermore, this model will be ideal to reveal the mecha-
nism behind the activation of the MNX1 gene, to target its down-
stream effectors, and to investigate possible treatment strategies by 
drug screening.

AUTHOR CONTRIBUTIONS

Lars Palmqvist, Tina Nilsson and Ahmed Waraky designed the 
research study. Tina Nilsson, Ahmed Waraky, Anders Östlund, Susann 
Li, Anna Staffas performed laboratory work and data analysis. Jonas 
Abrahamsson and Linda Fogelstrand provided patient samples. Tina 
Nilsson, Ahmed Waraky, Anders Östlund, Anna Staffas, Julia Asp, 
Linda Fogelstrand and Lars Palmqvist analyzed the combined data and 
wrote the paper. All authors approved of the final version. The work 
reported in the paper has been performed by the authors unless 
clearly specified in the text.

ACKNOWLEDGEMENTS

This study was supported by grants from the Cancerfonden 
(CAN2017/461), the Barncancerfonden (PR2014-0125 and 
PR2019-0013, T2019-0053) and Västra Götalandsregionen (ALFGBG-
431881). The computations were enabled by resources in project 
(SNIC 2021/22-754) provided by the Swedish National Infrastructure 
for Computing (SNIC) at UPMMAX, partially funded by the Swedish 
Research Council (2018-05973). The authors would like to acknowled-
ge Clinical Genomics Gothenburg, Science for Life Laboratory, 
Sahlgrenska Akademin, Göteborgs Universitet and Center for Medical 
Genomics, Department of Clinical Genetic and Genomics, Sahlgrenska 
University Hospital, Sweden, for providing sequencing assistance and 
bioinformatic services.

CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the 
supplementary material of this article, from Gene Expression Omnibus 
(GEO) database, accession number GSE182168. Further details and 
other data that support the findings of this study are available from 
the corresponding author upon request.

ETHICS STATEMENT

The study was performed in accordance with the Declaration of 
Helsinki, and parents of the patient have given written informed con-
sent. The research ethics committee at University of Gothenburg 
approved the study, Dnr 746/03 and 238/07.

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SUPPORTING INFORMATION
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How to cite this article: Nilsson T, Waraky A, Östlund A, et al. An induced pluripotent stem cell t(7;12)(q36;p13) acute myeloid leukemia model shows high expression of MNX1 and a block in differentiation of the erythroid and megakaryocytic lineages. Int J Cancer. 2022;151(5):770-782. doi:10.1002/ijc.34122