The Translocation t(7;12)(q36;p13) in Childhood Acute Myeloid Leukemia

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“We are star stuff harvesting sunlight.”

Carl Sagan

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ABSTRACT

The reciprocal translocation t(7;12)(q36;p13) gives rise to acute myeloid leukemia (AML) in infants and very young children. A fusion transcript *MNX1*::*ETV6* is sometimes detected and an aberrant expression of *MNX1* is detected in 100% of patients but the mechanism of transformation has previously not been identified. In earlier studies the frequency and outcome for t(7;12) AML has varied widely and remain contested leading to most treatment protocols stratifying t(7;12) to a high-risk group, however, the NOPHO-DBH-AML-2012 protocol used in Sweden does not. The aims of this project were to determine the frequency, event-free survival and overall survival of t(7;12) in childhood AML, and identify molecular mechanisms involved in the development of AML with t(7;12). In Paper I an iPSC model of t(7;12) was developed. Using this model, a high ectopic expression of *MNX1* and the long noncoding RNAs *MNX1-AS1* and *MNX1-AS2* from the same gene locus was observed. The t(7;12) translocation gave rise to a differentiation block and the model matched the gene expression signature from t(7;12) AML patient material. In Paper II a murine model was used. High expression of MNX1 did induce leukemia, however only in transduced fetal liver cells and not bone marrow cells and primarily in immunocompromised recipient mice. In Paper III patient data and patient material was investigated. AML with t(7;12) was associated with trisomy 19 and with CNS involvement. The expression of fusion transcripts in t(7;12) AML patients was heterogenous, giving rise to several fusion transcripts involving *ETV6*. All t(7;12) AML patients had a high expression of *MNX1*, *MNX1-AS1* and *MNX1-AS2*. The frequency of t(7;12) AML was 7% in AML

patients 0-2 years old. Patients with t(7;12) AML often suffers relapse but allogeneic hematopoietic stem cell transplantation (HSCT) was an effective treatment.

The work presented in this thesis has led to the conclusions that the t(7;12) translocation drives high expression of *MNX1*, that the high expression of *MNX1* is the transforming event and that AML with t(7:12) likely has a fetal cell of origin. The frequency of t(7;12) AML was relatively low at 7% in AML patients under 2 years old in this study and patients often relapsed but allogeneic HSCT was an effective treatment.

Keywords: Acute myeloid leukemia, t(7;12)(q36;p13), MNX1, ETV6, Fusion transcripts, NOPHO.

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SAMMANFATTNING PÅ SVENSKA

Translokationen t(7;12)(q36;p13) ger upphov till akut myeloisk leukemi (AML) hos spädbarn och mycket små barn. AML med t(7;12) är associerat med en extra kromosom 19 och ett avvikande uttryck av *MNX1* detekteras hos 100 % av patienterna. I tidigare studier har frekvensen och utfallet för patienter med t(7;12) AML varierat kraftigt och de är fortfarande omtvistade. Det har lett till att de flesta behandlingsprotokoll placerar AML med t(7;12) i en högriskgrupp. Det behandlingsprotokoll som används i Sverige gör inte det. Syftet med detta projekt var att studera frekvensen och utfall för t(7;12) AML, identifiera molekylära mekanismer involverade i utvecklingen av AML med t(7;12). I artikel I utvecklades en modell av t(7;12) med hjälp av inducerade pluripotenta stamceller. Med den här modellen observerades ett högt uttryck av *MNX1* och högt uttryck av långa icke-kodande RNAn *MNX1-AS1* och *MNX1-AS2* från samma genlokus som *MNX1*. t(7;12)-translokationen gav upphov till en blockering i differentiering och modellens genuttryck matchade genuttryckssignaturen från patientmaterial. I artikel II utvecklades en musmodell för leukemin. Högt uttryck av MNX1 i celler från fetal lever gav upphov till AML. I artikel III undersöktes patientdata och patientmaterial. AML med t(7;12) var associerad med en extra kromosom 19 och med spridning till centrala nervsystemet. Flera fusionstranskript identifierades hos patienter med t(7;12) AML. Mest anmärkningsvärt fusioner med *ETV6* till *NOM1* och *LMBR1*, de två generna närmast *MNX1* på kromosom 7. Alla t(7;12) patienterna hade ett högt uttryck av *MNX1*, *MNX1-AS1* och *MNX1-AS2*. Frekvensen av t(7;12) AML var 7 % hos AML-patienter 0-2 år gamla. Patienter med t(7;12) AML drabbas ofta av återfall men benmärgstransplantation är en effektiv behandling.

Arbetet som presenteras i den här avhandlingen har lett till slutsatserna att högt uttryck av MNX1 driver AML med t(7;12) och att denna leukemi sannolikt har en cell från fosterstadiet som ursprungscell. Frekvensen av AML med t(7;12) är relativt låg med 7 % hos patienter med AML under 2 års ålder. De har en hög frekvens av återfall men benmärgstransplantation är en effektiv behandling.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. 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.

Nilsson T., Waraky A., Östlund A., Li S., Staffas A., Asp J., Fogelstrand L., Abrahamsson J. and Palmqvist L.

Int J Cancer, 2022. **151**(5): p. 770-782

II. Aberrant MNX1 expression associated with t(7;12)(q36;p13) pediatric acute myeloid leukemia induces the disease through altering histone methylation.

Waraky A., Östlund A., Nilsson T., Weichenhan D., Lutsik P., Bähr M., Hey J., Adamsson J., Morsy M.H.A., Li S., Fogelstrand L., Plass C. and Palmqvist L.

Haematologica, 2024. **109**(3): p. 725-739

III. Characterization of Pediatric Acute Myeloid Leukemia with t(7;12)(q36;p13).

Östlund A., Waraky A., Staffas A., De Moerloose B., Arad-Cohen N., Cheuk D., Fernandez Navarro J.M., Jahnukainen K., Kaspers G.J.L., Kovalova Z., Pasauliene R., Saks K., Zeller B., Norén-Nyström U., Hasle H., Fogelstrand L., Abrahamsson J. and Palmqvist L.

Manuscript

CONTENTS

ABBREVIATIONS

ADxE	Daunoxome, cytarabine and etoposide
AGM	Aorta-gonad-mesonephros
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
BM	Bone marrow
Cas9	type II CRISPR RNA-guided endonuclease Cas9
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Central nervous system
CR	Complete remission
CRISPR	Clustered regulatory interspersed short palindromic repeats
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
EFS	Event-free survival
EHT	Endothelial to hematopoietic transition
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FISH	Fluorescence in situ hybridization
FL	Fetal liver
gDNA	Genomic deoxyribonucleic acid

GFP	Green fluorescent protein
GSEA	Gene set enrichment analysis
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic Stem and Progenitor Cells
iHSPC	Induced Hematopoietic Stem and Progenitor Cells
iPSC	Induced Pluripotent Stem Cells
IT	Intrathecal
ITD	Internal tandem duplication
Lin	Lineage
MEC	Mitoxantrone, etoposide and cytarabine
Me	Methyl group
miRNA	Micro ribonucleic acid
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
MTX	Methotrexate
NGS	Next generation sequencing
NOPHO	Nordic society of paediatric haematology and oncology
OS	Overall survival
PCA	Principal component analysis
PSC	Pluripotent stem cells
RD	Resistant disease

RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
ssODN	single stranded oligodeoxynucleotide
WBC	White blood cells
WGS	Whole genome sequencing
WHO	World health organization
WTS	Whole transcriptome sequencing
YFP	Yellow fluorescent protein

GENES

ABL1	ABL proto-oncogene 1, non-receptor tyrosine kinase
ALK	ALK receptor tyrosine kinase
BCR	BCR activator of RhoGEF and GTPase
CDX2	Caudal type homeobox 2
CTLA4	Cytotoxic T-lymphocyre associated protein 4
DNMT3A	DNA methyl transferase 3a
ETV6	ETS variant transcription Factor 6
FGFR1	Fibroblast growth factor receptor 1
FLT3	Fms related receptor tyrosine kinase 3
H3K4	Histone 3 lysine 4
H3K27	Histone 3 lysine 27
JAK2	Janus kinase 2
KIT	KIT proto-oncogene receptor kinase
KLF4	KLF transcription factor 4
KMT2A	Lysine methyltransferase 2A
KRAS	KRAS, proto-oncogene, GTPase
LIN28A	Lin28 homolog A
LIN28B	Lin28 homolog B
LMBR1	Limb development membrane protein 1
MNX1	Motor neuron and pancreas homeobox 1

MNX1-AS1	Motor neuron and pancreas homeobox 1 antisense RNA 1 (head-to-head)
MNX1-AS2	Motor neuron and pancreas homeobox 1 antisense RNA 2
MYB	MYB proto-oncogene, transcription factor
MYBBP1A	MYB binding protein 1a
MYC	MYC proto-oncogene, bHLH transcription factor
MYCL	MYCL proto-oncogene, bHLH transcription factor
MYCN	MYCN proto-oncogene, bHLH transcription factor
NANOG	NANOG homeobox
NOM1	Nucleolar protein with MIF4G domain 1
NRAS	NRAS, proto-oncogene, GTPase
NPM1	Nucleophosmin 1
NUP98	Nucleoporin 98 and 96 precursor
PDCD1	Programed cell death 1
PDGFRA	Platelet derived growth factor receptor alpha
PDGFRB	Platelet derived growth factor receptor beta
POU5F1	Pou class 5 homeobox 1
RB1	RB transcriptional corepressor 1
RET	RET proto-oncogene
RUNX1	RUNX family transcription factor 1
SOX2	SRY-box transcription factor 2
SYK	Spleen associated tyrosine kinase

TP53	Tumor protein p53
WT1	WT1 transcription factor

DEFINITIONS IN SHORT

Defined genetic aberration	Is a defined genetic aberration in the 5th edition of the World Health Organization (WHO) Classification of Hematolymphoid Tumors.
Endothelial-to-hematopoietic transition	Is the process how hematopoietic cells are generated from an endothelial cell population differentiating into hematopoietic cell lineage during fetal development.
Induced Pluripotent Stem Cell	Is a somatic cell that have been reprogrammed to a pluripotent stem cell that can give rise to all three germ layers.
NOPHO-DBH-AML-2012	The current treatment protocol for pediatric AML used in the NOPHO-DB-SHIP study group i.e. patients from Sweden, Denmark, Norway, Finland, Iceland, Hong Kong, Belgium, Estonia, Israel, Latvia, Lithuania, Spain, and the Netherlands..

INTRODUCTION

STEM CELLS

Stem cells are undifferentiated cells that are defined by two characteristics that they have in common, the ability to:

1. Self-renew indefinitely
2. Differentiate into specific mature cell types

Therefore, when stem cells divide, they can give rise to two stem cells, two cells that differentiate or through asymmetric division one stem cell and one cell that differentiates [1].

CELL POTENCY AND DIFFERENTIATION

The potency of a cell is defined by its potential to differentiate into other cell types. As an egg is fertilized a zygote is produced. This one cell can give rise to all embryonic as well as extraembryonic cells such as placenta. The ability for a stem cell to differentiate into all three germ layers and extraembryonic tissues is called totipotency [2]. The zygote sits at the top of a hierarchy where the next step is a pluripotent stem cell. A pluripotent stem cell can give rise to the three germ layers of the embryo but is unable to give rise to the extraembryonic tissues. During development pluripotent stem cells continuously lose potency into multi- oligo or unipotent stem cells. The multistep process of differentiation and loss of potency is tightly regulated by cellular signaling in a timely and spacial manner [3]. As the different tissues of the embryo start to form the stem cells lose potency and become tissue specific. Small populations of tissue specific stem cells replace damaged cells as needed postnatally and all through adulthood.

EMBRYONIC STEM CELLS

Embryonic stem cells (ESC) are derived from the inner cell mass of the blastocyst. They can self-renew and stay pluripotent and therefore proliferate indefinitely and can give rise to the three germ layers endoderm, ectoderm, and mesoderm [4]. To perform studies on ESCs is possible but have caused ethical difficulties due to use of human embryos.

INDUCED PLURIPOTENT STEM CELLS

The ethical difficulties regarding ESCs have been avoided by reprogramming of somatic cells. Reprogramming of somatic cells to pluripotent stem cells was at first performed by transferring the nucleus of a somatic cell to an enucleated oocyte [5] or by fusion of a somatic cell and an ESC [6]. These experiments showed that oocytes and ESCs contained factors that maintained pluripotency and that differentiation was not irreversible. Reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) by a defined set of factors was first performed in 2006. Reprogramming to pluripotency was performed by expressing a set of four factors *Pou5f1*, *Sox2*, *Myc* and *Klf4* in mouse fibroblasts [7]. The year after somatic human cells were reprogrammed to a pluripotent state using a different set of factors consisting of *POU5F1*, *SOX2*, *NANOG* and *LIN28B* [8]. Since the introduction of iPSCs differentiation programs of iPSCs into hematopoietic cells [9], neuronal cells [10], skeletal muscle cells [11] etc have been developed.

STEM CELLS DURING FETAL DEVELOPMENT

A fertilized egg goes from one cell to a new born baby in nine months during this time cells go through a high division rate and several stages of differentiation such as epiblast stem cell and embryonic germ cell and tissue specific stem cells [12].

ADULT STEM CELLS

Throughout life, tissues go through turnover where cells are worn out or damaged. Normally these cells go through apoptosis and need to be replaced by new tissue specific cells to uphold tissue homeostasis. The task is performed by rare adult stem cells. Tissue specific adult stem cells reside in a specific niche where only a small population is maintained. Upon stimulation stem cells can divide and the progeny differentiate to replenish the tissue with mature cells as needed. Populations of stem cells that replenish blood [13], intestine [14] and skin [15] etc. have all been well studied.

HEMATOPOIESIS

The cells that constitute blood are essential to life. Individual components of blood are responsible for oxygen transportation, carbon dioxide transportation, hemostasis, immune responses etc. Blood cells are generally short lived, and hematopoiesis is the process where the cellular components of blood are formed and replenished throughout life to maintain homeostasis.

This process is maintained by differentiation of hematopoietic stem cells (HSC) into mature specialized cells. Hematopoiesis occur at different sites and gives rise to different kinds of cells during development and adulthood.

ADULT HEMATOPOIESIS

Adult hematopoiesis occurs in the bone marrow (BM) where HSCs reside [16]. HSCs can repopulate the BM and through asymmetric cell division the HSCs give rise to cells that differentiate into mature specialized cells through a multistep process. The HSC differentiates into a multipotent progenitor. This is the first branch point in the hematopoietic differentiation tree and differentiation potential is restricted. It differentiates into either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP). These are the two major branches of the hematopoietic tree. Further differentiation of CLPs give rise to B- and T-lymphocytes and NK-cells. CMPs can further differentiate into granulocyte-monocyte progenitor (GMP) and megakaryocyte-erythroid progenitors (MEP). GMPs give rise to granulocytes and monocytes. MEPs give rise to erythrocytes and megakaryocytes. Megakaryocytes produce platelets.

FETAL HEMATOPOIESIS

Throughout fetal development hematopoiesis goes through several distinct stages and takes place at several different sites. The first wave of hematopoiesis takes place in the yolk sac. The initial wave is only transient and is termed “primitive”. During primitive hematopoiesis mainly nucleated erythrocytes expressing embryonic globins are produced [17]. As the primitive hematopoiesis is transient, it is replaced by definitive hematopoiesis. The definitive hematopoiesis arises in the aorta-gonad-mesonephros (AGM). In the dorsal aorta a small endothelial cell population undergoes an endothelial-to-hematopoietic transition (EHT) and give rise to HSCs. The HSCs home to and populate the fetal liver (FL) [18]. After FL the spleen and thymus are populated with hematopoietic cells before the BM is populated. In addition, throughout embryonic development the placenta generates HSCs and is a potent HSC niche [19].

CANCER

Cancer is one of the leading causes of death with almost 10 million deaths worldwide in 2020. One in five persons will develop cancer during their lifetime and one in 10 will die from the disease [20]. In 2020 the most

diagnosed forms of cancer were breast, lung and prostate cancer and the leading causes of cancer death is lung, liver and stomach cancer [20].

Cancer is a general name for a variety of diseases in any part of the body. It is commonly characterized by a mass of cells that grow beyond their natural boundaries through uncontrolled cell growth. This leads to invasion of the neighboring tissues and at later stages spread to other remote parts of the body. Usually, the cancerous cells may over time acquire these traits through gene mutations and epigenetic alterations leading to aberrant gene expression.

PROTO-ONCOGENES AND ONCOGENES

The proto-oncogenes are a group of genes that exist in normal cells but when mutated or overexpressed are able to drive cancer formation and are then called oncogenes [21].

The idea of oncogenes initially came from viruses that were able to transform normal cells into cancerous cells [22, 23]. Transforming viral oncogenes were identified and later it was discovered that normal cells have genes that were homologous to the viral oncogenes [24]. The oncogenes drive transformation dominantly and usually are involved in cell signaling pathways that leads to typical cancer characteristics such as increased cell proliferation, a block in cell differentiation or inhibit apoptosis.

TUMOR SUPPRESSOR GENES

Early experiments where a cancer cell was fused with a normal cell gave rise to a hybrid progeny that was shown to have a high cancer potential [25]. These results were in line with that the cancer genotype was dominant and acting through oncogenes. A problem for this hypothesis arose as a study on the statistics of retinoblastoma showed that two specific mutations in the same cell, called the two-hit model, was necessary for retinoblastoma to arise [26]. Later cytogenetic and molecular studies identified this to be due to recessive loss-of-function mutations where both gene copies of *RB1* had to be inactivated [25]. This is due to that oncogenes are genes that drive oncogenesis and tumor suppressor genes instead act as brakes and both copies need to be inactivated. The tumor suppressor genes are involved in cellular processes such as inhibit cell division, inhibit cell cycle progression, DNA damage response or promote apoptosis [27].

HALLMARKS OF CANCER

Genetic aberrations that give rise to mutations in and aberrant expression of proto-oncogenes and tumor suppressor genes lead to changes in cellular characteristics that cause cancer development. In 2000 Hanahan and Weinberg published their landmark paper Hallmarks of Cancer [28]. In this paper the authors sought to describe what mutations in proto-oncogenes and tumor suppressor genes lead to and describe what characteristics all cancers have in common. They found that there were six characteristics that cells needed to acquire to become cancerous and one enabling characteristic.

The initial hallmarks of cancer were:

- Self- sufficiency of growth signals
- Insensitivity to anti-growth signals
- Tissue invasion and metastasis
- Limitless replicative potential
- Sustained angiogenesis
- Evading apoptosis

An enabling characteristic was:

- Genomic instability

Since the publication of the Hallmarks of Cancer a set of follow up papers have been published and more hallmarks and enabling characteristics of cancer have been described [29, 30].

Additional Hallmarks of Cancer:

- Reprogramming cellular metabolism
- Avoiding Immune destruction
- Unlocking phenotypic plasticity
- Senescent cells

Additional enabling characteristic:

- Tumor promoting inflammation
- Non-mutational epigenetic programming
- Polymorphic microbiomes

GENOMIC INSTABILITY

When cells divide, the DNA is copied to ensure that each daughter cell have a copy of the genome. This process is not perfect, and mutations may be introduced. Mutations may also be introduced through chemicals or irradiation. Therefore, cells have systems to monitor the genome and to repair it when DNA lesions do occur [28]. For example, mismatch repair genes that detect or repair mismatched nucleotides during DNA replication [31, 32], how double strand breaks are repaired by homologous recombination or non-homologous end joining [33] or cell cycle checkpoint genes prevent cells from initiating the next step of the cell cycle, such as mitosis, due to DNA damage [34] Together these and other systems assure that mutations are repaired or cause the cell to undergo apoptosis to maintain the integrity of the genome nucleotide sequence.

If one or more of these systems break down the cell will no longer be able to keep mutations from becoming permanent in dividing cells. This is a characteristic that not only enables cancer to arise but allows a cancer cell to undergo fast evolution that permit the cell to acquire more carcinogenic mutations at a high rate. This may lead to the cancer acquiring more properties such as resistance to chemotherapy [35].

EVADE IMMUNITY

The immune system is well known to respond to and eradicate bacteria, fungi, and viruses from the body, but it also plays a complex role in cancer. It has long been well established that tumors are infiltrated by immune cells from both the innate and adaptive branches of the immune system [36]. Immune cells that infiltrate tumors have been shown to both give rise to a tumor promoting inflammatory response but also to be part of the immune surveillance system where immune cells identify and kill cancer cells [37]. Tumors can through several mechanisms evade the immune surveillance system, such as expression of PDCD1 and CTLA4 that suppress T-cell activity. The advent of immune checkpoint inhibitors has revolutionized treatment of many forms of cancers and their development led to the noble prize in 2018 [38]. This confirms the importance of evading the immune surveillance system for cancer to arise.

PHENOTYPIC PLASTICITY AND DISRUPTED DIFFERENTIATION

In normal tissues stem cell differentiation into mature specialized cells occur under strict controlled conditions to uphold homeostasis and only a small population of non-differentiated cells reside in tissues. However, cancers are generally characterized by an accumulation of cells in a non-differentiated state. This can be achieved through different mechanisms, such as:

DEDIFFERENTIATION

Dedifferentiation takes place when differentiated cells escapes terminal differentiation and revert into a non-differentiated state.

BLOCKED DIFFERENTIATION

Unlike dedifferentiation a block in differentiation does not involve differentiated cells. Instead through regulatory changes a block in differentiation in not fully differentiated cells arise.

TRANSDIFFERENTIATION

Transdifferentiation takes place when cells that are differentiated change their phenotype into cells of a different lineage. This process is also called lineage reprogramming.

These processes are not mutually exclusive and are likely intertwined in many ways during carcinogenesis. Together or separate they can give rise to a large population of undifferentiated cells [30].

PEDIATRIC CANCER

Cancer is often seen as a disease of the elderly who due to their old age have over time accumulated enough mutations for cancer to develop. Pediatric cancer differs from cancer in adults in that it is more common to have fewer genetic aberrations in pediatric cancers. These aberrations may be recurrent and act as diagnostic markers and influence choice of therapy. This is one of the reasons why pediatric cancers have separate treatment protocols from their adult counterparts.

Even though cancer is rarer in children compared to adults it is still a major cause of death among children and each year more than 400 000 children and adolescents develop cancer [39]. The most common forms of pediatric cancer

are leukemia, brain cancer and lymphomas [40]. The type of tumor varies greatly in different age ranges where one of the more common forms of cancer in children between 1-4 years of age is neuroblastoma which is very rare in the 15-19 age range. The opposite age trend can be seen for epithelial cancers and melanomas [40].

ONCOFETAL SIGNALING PATHWAYS

During fetal development cell signaling pathways are active that are not normally active during adulthood and certain characteristics of fetal development such as cellular plasticity and high proliferative rate are characteristics shared with many cancer tissues [41]. Signaling pathways that are active during fetal development are in many cases reactivated during carcinogenesis in adults and are called oncofetal signaling pathways [42].

FETAL ORIGIN OF CANCER

Certain forms of cancer arise exclusively in infants or very young children. They often show a high expression of oncofetal pathway constituents. However, instead of a reactivation of oncofetal pathways it has been suggested that the cancer cells are enduring cells from fetal development [43]. This includes neuroblastoma, Wilms tumor, medulloblastoma and several hematological malignancies [43].

For hematological malignancies studies have been performed that identify a fetal origin. Identical twin studies have identified cases where both twins develop leukemia and have the same clonal chromosome rearrangements that give rise to either Acute Lymphocytic Leukemia (ALL) [44, 45] or Acute Myeloid Leukemia (AML) [46] and in patients where the chromosomal rearrangement have been identified at diagnosis retrospective studies on dried blood spots collected at birth have been used to identify cells harboring the rearrangement already at birth in a high frequency of patients [47-49].

LEUKEMIA

Leukemia is cancer of the hematopoietic system and differ from solid tumors in many ways. Leukemia arises as the normally tightly regulated hematopoietic processes like differentiation and proliferation become deregulated through mutations etc. This leads to an abnormal uncontrolled proliferation that in turn leads to an accumulation of blast cells in the BM. This is called malignant transformation and as leukemic blasts accumulate in

the BM and take over normal hematopoiesis can no longer transpire. Leukemia can be divided into several different subgroups depending on characteristics of the leukemia, such as chronic or acute and myeloid or lymphocytic.

ACUTE MYELOID LEUKEMIA

AML is the most common leukemia and have the shortest survival in adults [50]. AML arise in a myeloid progenitor cell with a differentiation block unable to differentiate to granulocytes, monocytes, erythrocytes, or megakaryocytes. In adults mutations may accumulate over time in a preleukemic state until leukemia develops [51]. AML is diagnosed when the BM holds ≥20% blasts or when specific defining genetic aberrations are detected. No blast number is required if one of the specific defining aberrations is detected.

ACUTE LYMPHOCYTIC LEUKEMIA

ALL is the second most common leukemia in adults. ALL arise in a lymphoid progenitor cell. ALL can be divided into 2 large categorical groups depending on if it develops from a precursor to T-cells or a precursor to B-cells [52].

PEDIATRIC LEUKEMIA

Leukemia is the most common pediatric cancer. Among pediatric leukemia ALL is more common than AML, however, AML have worse prognosis. Pediatric leukemias have few mutations and are often initiated by a small set of recurrent genetic aberrations like recurring translocations.

PEDIATRIC AML

In Sweden pediatric AML are all patients diagnosed with AML aged 0-17 years old and 10-15 children are diagnosed with pediatric AML yearly. The disease is heterogenous and there are several subtypes. Pediatric patients have few mutations but have a higher proportion of patients that have gene fusions compared to adult AML patients [53]. Mutations in *DNMT3A* and *TP53* are common in adult AML, however, mutations in *MYC*, *NRAS*, *KRAS*, *FLT3* and *WT1* are recurrent in pediatric AML [53].

INFANT OR VERY YOUNG AML

AML in infants or very young children are different from AML in older children. It is more common with higher white blood cell (WBC) counts, extramedullary disease and central nervous system (CNS) involvement [54]. The most common leukemia inducing recurrent aberration in the age group are translocations involving *KMT2A* [55] and certain recurrent aberrations are only found in very young children, such as t(7;12)(q36;p13) [56] and t(1;22)(p13;q13) [57, 58].

AML WITH T(7;12)(Q36;P13)

The reciprocal chromosomal translocation t(7;12) gives rise to AML. By the late 1990s it was identified that *ETV6* had a recurring fusion partner on chromosome 7 [59]. The t(7;12) translocation was first identified in the early 2000 [60, 61]. AML with t(7;12) is highly associated with trisomy 19 and is only found in infants or very young children [56]. An *MNX1*::*ETV6* but not *ETV6*::*MNX1* fusion transcript has been identified [62]. The fusion transcript *MNX1*::*ETV6* is only occasionally detected but an aberrant expression of *MNX1* is detected in 100% of patients [63] and MNX1 protein show a strong nuclear staining in AML with t(7;12) [64]. The mechanism of transformation in this leukemia has not been shown and is not well understood.

The translocation breakpoints are typically localized in a breakpoint cluster region distal to *MNX1* (gene on chromosome 7) [65] and within intron 1 or 2 of *ETV6* (gene on chromosome 12) leading to only relatively small parts of the chromosomes being translocated [63]. This leads to it being difficult to identify through karyotyping and may have led to the frequency of t(7;12) to be underestimated. It has been shown to be the second most frequent recurring aberration in the age group at 28% in infants [66] but another study found its frequency to be only 4% in children under 2 years old [56]. The outcome for patients with t(7;12) AML have in different studies ranged from dismal [61, 66] to good [56]. Due to that the results have varied in previous studies both the frequency and the outcome of AML with t(7;12) remains unclear.

As the outcome of AML patients with t(7;12) is not clear it has led to there being no consensus on risk group stratification in different treatment protocols. In the children oncology group treatment protocol COG AAML1831 all translocations involving 12p13 were considered an unfavorable abnormality, in the UK, Ireland and France treatment protocol MyeChild01 AML with t(7;12) was considered a poor risk abnormality and

in Japan treatment protocol JPLSG AML-20 t(7;12) was considered a high-risk abnormality [67]. However, in the NOPHO-DBH-AML-2012 treatment protocol, used in for example Sweden, AML with t(7;12) is not considered a high-risk genetic abnormality and risk stratification for AML with t(7;12) patients treated under this protocol is instead based on induction therapy response [67]. AML with t(7;12) was recently recognized as a leukemia with a defining genetic aberration in the 5th edition of the World Health Organization (WHO) Classification of Hematolymphoid Tumors. It is included as subtype AML with *MNX1*::*ETV6* fusion [68].

PEDIATRIC AML TREATMENT

The treatment of pediatric AML has made remarkable improvements over the last decades. Before the introduction of intensive chemotherapy in the late 1970s cure rates were very low [69]. Since then, the introduction of induction therapy to accomplish remission, consolidation therapy to keep the patient in remission, HSCT and intensification of therapy has led to further step wise improvements in treatment. However, there is no consensus on risk grouping or treatment and different study groups use different treatment protocols. Sweden is part of the Nordic society of paediatric haematology and oncology (NOPHO) and the current AML treatment protocol is NOPHO-DBH-AML-2012, (EudraCT 2012-002934-35).

INDUCTION THERAPY

The current treatment protocol uses two courses of induction therapy. The first course consists of Mitoxantrone, Etoposide and Cytaribine (MEC) or Daunoxome, Etoposide and Cytaribine (DxEC). Patients treated with either MEC or DxEC also receive one dose intrathecal (IT) treatment with Methotrexate (MTX). After 1 course of induction therapy BM is evaluated for minimal residual disease (MRD) as measured by flow cytometry. If MRD is high (≥ 5% leukemic cells) then course two proceeded otherwise the patient would not receive course two until regeneration of peripheral blood counts. The second course consists of Daunoxome, Cytarabine and Etoposide (ADxE) or Fludarabine, Cytarabine and Daunoxome (FLADx). Patients treated with either ADxE or FLADx also receive one dose IT treatment with MTX. After course two of induction therapy BM is again evaluated for MRD by flow cytometry [70].

COMPLETE REMISSION

Patients that have < 5% leukemic cells as determined by flow cytometry and MRD or < 5% blast cells as determined by cell morphology in BM and have

hematological regeneration after induction course two are said to have gone into complete remission (CR).

RISK STRATIFICATION

Risk stratification is performed based on genotype (Presence of *FLT3*-ITD but without *NPM1* mutation or presence of inv(16)(p13;q22).) or treatment response. Treatment response is primarily determined by flow cytometry and MRD but if there is no informative MRD then cell morphology is used. Patients that have a poor response to induction course two defined as ≥ 5% leukemic cells as determined by flow cytometry and MRD or have ≥ 5% blast cells as determined by cell morphology have resistant disease (RD). Patients that have an intermediate or good response to induction course two are grouped accordingly:

High-risk group:

- Poor response after course one (≥15% leukemic cells)
- Intermediate response after two induction courses (≥0.1%-4.9% leukemic cells)
- *FLT3*-ITD mutation in absence of *NPM1* mutation

Standard risk group:

All patients that are not allocated to the high-risk group and achieve complete remission after two induction courses are assigned to the standard-risk group.

CONSOLIDATION THERAPY

Patients who have responded to induction therapy move on to consolidation therapy. Standard risk patients receive three courses of consolidation therapy. The first course consists of Cytarabine, Mitoxantrone and MTX (HAM), the second consists of Cytarabine, Etoposide and MTX (HA_3E) and Fludarabine, Cytarabine and MTX (FLA). Standard risk patients with inv(16) only receive HA_3E and FLA. Patients who are in the high-risk group receive HAM treatment and should as soon as possible thereafter receive an allogeneic HSCT.

SALVAGE THERAPY

Treatment of patients with RD aims to achieve remission. There are several different treatment options to achieve remission and choice of treatment is

dependent on which treatment the patient has already received. If the patient responds to treatment, then HSCT should be considered.

AML WITH CNS INVOLVMENT

Leukemia with CNS involvement at diagnosis can occur in both adult and pediatric AML [71, 72]. For diagnosis of CNS involvement cytological analysis of cerebrospinal fluid (CSF) is required. It is more frequently identified in pediatric AML than adult. This may be due to that CSF in adult patients is only examined when symptoms indicate CNS involvement whereas in pediatric AML it is always investigated. CNS involvement have been linked to young age, high WBC count, extramedullary involvement other than CNS involvement, monoblastic morphology, and inv(16) in pediatric AML [73]. Currently there is no consensus on treatment of pediatric AML with CNS involvement at diagnosis but under the current NOPHO AML protocol CNS involvement at diagnosis changes the treatment from one IT treatment with MTX per course of induction or consolidation treatment to IT treatments with MTX, Cytarabine and Prednisolone twice a week until the CSF no longer contain blasts by two more injections. At least four treatments are given.

NON-RANDOM GENETIC ABERRATIONS

Non-random genetic aberrations are often involved in the development of solid tumors [74] and hematological malignancies [75]. In cancer chromosomes are often abnormal and chromosomal aberrations can be classified as numerical or structural. The numerical are chromosomal gains or losses and structural involve translocations, inversions, deletions, insertions and duplications [75]. Non-random recurrent chromosome aberrations can be detected in 75-80% pediatric AML [76].

Some non-random genetic aberrations are used as defining genetic aberrations in AML and are used for diagnosis, may predict prognosis, risk stratification and choice of therapy [67]. Defining genetic aberrations includes translocations but also molecular aberrations such as mutations of *NPM1* and *FLT3*-ITD that are commonly observed in AML [77].

CHROMOSOMAL TRANSLOCATIONS

Reciprocal chromosomal translocations are the result of an exchange of genetic material between two chromosomes. If the translocation is balanced it

does usually not result in disease as no genetic material is lost. However, in 1959 a chromosomal abnormality was identified in association with chronic myelogenous leukemia [78]. One chromosome was reported to be too small and was named the Philadelphia chromosome. The Philadelphia chromosome, though at first thought to be a deletion, was later identified to be the result of a reciprocal chromosomal translocation between chromosome 9 and 22 and was the first non-random genetic aberration to cause cancer to be identified. Molecular analysis have shown the translocation t(9;22)(q34;q11) to give rise to a chimeric gene by fusion of two genes, namely *BCR* and *ABL1* [79, 80] and the expression of the fusion gene *BCR*::*ABL1* was later shown to be able to give rise to myeloproliferative syndrome closely resembling the human illness in a murine model [81].

Since the identification of the Philadelphia chromosome balanced translocations have been identified in most types of cancers, more than 267 in AML, 155 in ALL and 75 in solid tumors have been identified [82]. As more recurrent balanced translocations were identified the same chromosomal locations were often identified. A set of genes at these locations have been described as promiscuous as they can have many different fusion partners. In pediatric AML examples of promiscuous genes are *ETV6* at chromosome 12p13 [83], *KMT2A* (Previously known as *MLL*.) at chromosome 11q23 [53] and *NUP98* [84] at chromosome 11p15.

FUSION PROTEINS

A translocation can give rise to chromosomal breakpoints within two genes and cause expression of a new chimeric in-frame mRNA. Translation of the fusion protein can be a driver of cancer through several different mechanisms.

TYROSINE KINASES

Tyrosine kinases are often activated in many different forms of cancer. They can drive tumor development and tyrosine kinase inhibitors such as imatinib have been effective in treatment of these cancers [85]. There are several different mechanisms that can lead to abnormal activation of tyrosine kinases such as point mutations but also fusion proteins as a result of translocations. An investigation of transcriptomes of 7000 solid tumors identified recurrent fusion transcripts involving tyrosine kinases in 3% of tumors [86]. Normally a tyrosine kinase receptor is activated through binding of a ligand that induces dimerization and autophosphorylation. However, in the case of fusions involving tyrosine receptor kinases the tyrosine kinase domain of a

gene often become fused to a fusion partner that have a domain that can dimerize, or it loses an inactivation domain. Both mechanisms lead to an abnormal activation that drive carcinogenesis. Examples of tyrosine kinase genes often involved in translocations are *ABL1*, *PDGFRA*, *PDGFRB*, *FGFR1*, *SYK*, *RET*, *JAK2* and *ALK* [87]. *ETV6* have been identified as a frequent fusion partner to tyrosine kinases in leukemias [87].

ALTERED TRANSCRIPTION FACTORS

Translocations involving transcription factors are common in hematological malignancies. This often involves transcription factors that are involved in regulating proliferation or differentiation during hematopoiesis and they can be inactivated, or their transcription can be inappropriately activated etc. Downstream this alters the landscape of genes being transcribed in the cell thereby driving leukemogenesis. Examples of transcription factor genes often involved in translocations are *ERG*, *MYC* and *RUNX1* [88]. *ETV6* is a common fusion partner to *RUNX1* in pediatric ALL and the fusion gives rise to repression of *RUNX1* target loci [89].

ALTERED EPIGENOME MODULATORS

Normally epigenome modulators can alter histone modifications or DNA methylation. By these mechanisms epigenetic modulators can determine which parts of the genome are accessible for transcription. *KMT2A* is an important epigenome modulator during embryogenesis and hematopoiesis and is very commonly involved in hematological translocations [90].

ENHANCER HIJACKING

When balanced translocations occur with chromosomal breakpoints in intergenic regions no DNA is lost, and no genes are disrupted. Normally, this may lead to low fertility, but no illness or syndrome arise. However, in some instances non-coding genomic elements are rearranged due to the translocation and can therefore give rise to high ectopic expression of genes normally not expressed or expressed at lower levels in that cell type. This is not restricted to only intergenic-intergenic translocations but may also occur in intergenic-gene or gene-gene reciprocal translocations.

One genetic element that may be rearranged to a new chromosomal location and drive expression of a new gene is an enhancer and this mechanism is known as enhancer hijacking or enhancer swapping. Enhancers are non-coding genetic elements that activate transcription of genes [91]. They can act independently of orientation and distance from the target gene [92].

Enhancer hijacking events have been shown to take place in hematological malignancies and solid cancers [93].

Previous studies have identified several examples of translocations involving *ETV6* in AML that do not give rise to expression of a fusion protein [94, 95]. In AML with t(12;13)(p13;q12) two fusion transcripts involving *ETV6* and *CDX2* and an ectopic expression of *CDX2* have been identified. In a murine model overexpression of CDX2 induced AML but overexpression of the fusion did not. Showing that the transforming event is not the ETV6 fusion protein but the ectopic expression of CDX2 [96].

The AML cell line GDM-1 carry the translocation t(6;7)(q23;q36). The genomic breakpoints occur within *MYB* on chromosome 6 and in the intergenic region upstream to the transcription start of *MNX1* on chromosome 7 [97]. The genes are transcribed in different directions and does not give rise to a fusion transcript, however a high ectopic expression of *MNX1* has been reported [98]. Recently It was reported that the ectopic expression of *MNX1* in GDM-1 cells was due to an enhancer hijacking event [97].

MOLECULAR PATHWAYS

MNX1

The *MNX1* gene (Previously known as HLXB9 or HB9.) is located at chromosome 7q36 and the encoded protein is a member of a family of proteins that have a homeodomain [99]. It is the only member of the MNX subgroup of homeobox domain proteins present in humans [99]. MNX1 is highly conserved. It functions as a transcription factor. It is expressed during embryogenesis and plays a vital role in the formation of the dorsal pancreatic bud and β-cell maturation, development of motor neurons and in stimulated human B-lymphocytes [100]. Germline mutations of *MNX1* have not been reported to give rise to any hematological disorders, however, several different mutations in MNX1 does give rise to sacral agenesis and anorectal malformations [101]. This is called Currarino Syndrome [102, 103].

When expressed in hematopoietic cells MNX1 have led to senescence through p53-p21 tumor suppressor network. MNX1 also caused an early block in lymphoid differentiation and in the myeloid lineage a differentiation block of erythrocytes and megakaryocytes caused accumulation of cells at the MEP stage [104].

High expression of *MNX1* have been linked to cancer in many other tissues such as glioma [105], cervical cancer [106], prostate cancer [107], breast cancer [108], hepatocellular carcinoma [109] and colorectal cancer [110].

ETV6

The *ETV6* gene (Previously known as *TEL*.) is located at chromosome 12p13 and is a member of a family of proteins that have a DNA binding ETS domain that all have the same DNA binding consensus sequence [111]. In the N-terminal ETV6 has a pointed (PNT) domain that enables self-assembling and multimerization [112]. It can bind to other ETS domain proteins such as FLI1 through the PNT domain [113] and binding of ETV6 to FLI1 inhibits transcription of FLI1 targets [114]. Multimerization of ETV6 can give rise to transcriptional repression. Etv6 is highly expressed throughout embryogenesis and Etv6 knock out mice are embryonically lethal due to faulty yolk-sac angiogenesis and intraembryonic apoptosis [115].

In hematopoiesis ETV6 plays a critical role. Normal HSCs express high levels of *ETV6* [116] and this expression is essential for normal HSC function and generation of thrombocytes by megakaryocytes [117]. However, ETV6 is not essential for other mature hematopoietic cell lineages or embryonic hematopoiesis [117]. Heterozygous germline mutations of *ETV6* have been shown to lead to bleeding disorders through thrombocytopenia and lead to an increased risk of AML, ALL and Myelodysplastic syndrome [111, 118, 119].

Single nucleotide polymorphisms (SNPs) in *ETV6* have been shown to predispose to not only hematological malignancies but also to colorectal cancer [120] and translocations involving *ETV6* have been found in several forms of cancer such as epithelioid fibrous histiocytoma [121], breast cancer [122, 123], congenital fibrosarcoma [124], thyroid cancer [125], salivary gland cancer [126] and hematological malignancy [83]. In hematological malignancies more than 40 translocations involving *ETV6* and more than 30 fusion partners have been identified [83].

MNX1-AS1 AND MNX1-AS2

Located at the same chromosome locus as *MNX1*, 7q36, are the long non-coding RNAs *MNX1-AS1* and *MNX1-AS2*. *MNX1-AS1* is located head-to-head to *MNX1* and shares a promoter region. *MNX1-AS2* is located within the *MNX1* gene, its exons are located in the introns of *MNX1* and expression of *MNX1-AS2* is regulated by another promoter region [127].

High expression of *MNX1-AS1* has been associated to cancer in many other tissues such as breast [128], colorectal cancer [129], glioblastoma [130], hepatocellular carcinoma [131], ovarian cancer [132], lung cancer [133], esophageal cancer [134]. A recent publication identified that *MNX1*, *MNX1-AS1* and *MNX1-AS2* expression can be used as candidate diagnostic and prognostic biomarkers for several types and subtypes of cancer [127].

In molecular studies *MNX1-AS1* has been shown to drive cancer progression through different mechanisms such as interacting with and inhibiting miRNAs [133] or binding to and activating YB1 [129], however, to date no studies on the function of *MNX1-AS2* have been performed.

LIN28B

The LIN28 family of proteins consists of LIN28A and LIN28B in humans. LIN28B is highly expressed during embryogenesis and plays a vital role during development. It can as one of four factors be used to reprogram somatic human cells and give rise to iPSCs [8].

LIN28 proteins have a cold shock domain and a cysteine cysteine histidine cysteine zinc knuckle domain. Both domains can bind to RNA [135]. Its most well studied mechanism of action is its inhibition of biogenesis of the Let-7 family of miRNAs. However, LIN28B transcripts have several Let-7 target sequences that enables LIN28B transcripts to be targeted for degradation by Let-7. This reciprocal inhibition of LIN28B and Let-7 enables them to work as a switch. Our knowledge in LIN28B function have been extended beyond the regulation of Let-7 biogenesis. Several studies have shown LIN28B to play a role in the regulation of specific mRNAs for example LIN28B have been shown to bind to *MYBBP1A* mRNA and cause increased translation [136]. In contrast, LIN28B have also been shown to bind *TP53* mRNA but in this case, it decreases TP53 translation [137].

LIN28B plays an important role in hematopoiesis. It is highly expressed in hematopoietic FL cells where Let-7 is expressed at low levels. In adult hematopoiesis the opposite expression pattern is seen. Overexpression of Lin28b in adult BM hematopoietic cells mediates multilineage reprogramming to a state that resembles fetal lymphopoiesis [138]. The LIN28B-Let-7 axis therefore work as a switch from fetal to adult hematopoiesis [139].

High expression of *LIN28B* have been linked to cancer in many tissues such neuroblastoma [140], leukemia [141, 142], Wilms tumor [143], breast cancer [144], colon cancer [145]. However, in an AML mouse model driven by a KMT2A fusion protein LIN28B acts as a tumor suppressor gene by repressing the oncogene MYB [136].

MYCN

MYCN encodes the protein N-MYC and is part of the MYC protein family. This family consists of NMYC, MYC (also known as c-MYC) and MYCL [146]. MYC is broadly expressed in many normal tissues and is known to be one of the most deregulated proto-oncogenes in cancer [147]. MYCN is widely expressed during embryonal development [148] and high expression of MYCN has been shown to give rise to rapid onset AML in a murine model [149].

High expression of *MYCN* have been linked to cancer in many tissues such as neuroblastoma [150], Wilms tumor [151], small cell lung cancer [152] and leukemia [153].

AIM

In this thesis the focus was to have a closer look at a gene alteration that is typically found in AML in children under the age of 24 month, namely the chromosomal translocation t(7;12). The aims of this project are to determine the true frequency, event-free survival (EFS), and overall-survival (OS) of t(7;12) in childhood AML, and identify molecular mechanisms involved in the development of AML with t(7;12).

SPECIFIC AIMS

1. Investigate the transformation capacity of the MNX1::ETV6 and ectopic expression of MNX1 in a murine transplantation model.

2. Investigate the molecular mechanisms through which the t(7;12) may drive leukemogenesis.

3. Determine the frequency, EFS and OS of the t(7;12) translocation in pediatric AML patients in the NOPHO AML-2004 and NOPHO-DBH-AML-2012 treatment protocols.

4. Investigate the presence of additional genetic and epigenetic alterations in cases with t(7;12) at diagnosis and at with next generation sequencing (NGS).

PATIENTS AND METHODS

This section is an overview of patient samples and methods used. Detailed descriptions of methods can be found in the respective paper.

PATIENTS

PATIENT DATA

The patient data included data from children aged 0-2 years old from the NOPHO-DB-SHIP study group from Sweden, Denmark, Norway, Finland, Iceland, Hong Kong, Belgium, Estonia, Israel, Latvia, Lithuania, Spain, and the Netherlands. Patients were diagnosed between January 2004 and November 2020 and patient follow-up times were updated in December 2023. Patients were treated according to the NOPHO-AML-2004 or NOPHO-DBH-AML-2012 protocols. The clinical data was retrieved through the NOPHO AML registry.

PATIENT MATERIAL

Patient material was retrieved from the NOPHO biobank consisting of mRNA and genomic DNA (gDNA). Normal cord blood came from discharged units collected for the Swedish National Cord Blood Bank (Sahlgrenska University Hospital, Gothenburg, Sweden). CD34 positive cells from cord blood was isolated and mRNA was extracted and used as controls for WTS analysis.

ETHICS CONCERING HUMAN STUDIES

The research has been conducted in accordance with the Declaration of Helsinki of the World Medical Association. The studies involving human data and material was approved by the Regional Ethics Committee of Gothenburg (DNR 313-18). Precautions have been taken to ensure that the patient data cannot be traced back to specific individuals.

MURINE MODEL

GENE INTRODUCTION SYSTEM

Molecular cloning was used to introduce human *MNX1*, *ETV6* or *MNX1*::*ETV6* into the MSCV viral vector. The viral vectors express yellow fluorescent protein (YFP) or green fluorescent protein (GFP) as a marker for transduced cells. An HA-tag (36bp) was introduced at the 5' end of each gene and via a linker sequence of 24 bp attached to the separate gene sequences where the first ATG of the gene is removed. Phoenix cells were used as primary virus producers and E86 cells were used as secondary virus producers. 5-FU treated BM cells or FL cells, isolated on day E14.5, from B57BL/6J mice were transduced to express high levels of the introduced gene for further experiments.

TRANSPLANTATION

Transduced BM or FL cells were sorted using fluorescence-activated cell sorter (FACS). Cells were sorted for GFP and/or YFP expression to achieve a poly-clonal cell population. The cells were introduced into the bloodstream of the recipient mice using tail vein injections. From the bloodstream the introduced cells homed to the BM and populated the hematopoietic system. If mice were irradiated prior to tail vein injection, then non-transfected rescue BM was also introduced. Either B57BL/6J or NOD.Cg-Kit^{W-41J} Tyr^{+} Prkdc^{scid} Il2rg^{tm1Wjl}/ThomJ (NBSGW) mice were used as recipient mice. Mice were monitored by drawing blood every other week. The blood was analyzed with Sysmex (WBC, hemoglobin etc.) and flow cytometry (GFP/YFP expressing cells in peripheral blood.). Blood was monitored every continued until leukemia developed, or the experiment ended. Signs of leukemia included pallor, inactivity, weight loss, hunched posture, high proportion GFP/YFP expressing cells in peripheral blood, leukocytosis and anemia. The mice were sacrificed due to leukemia or when the experiment ended and organs as well as cells were collected.

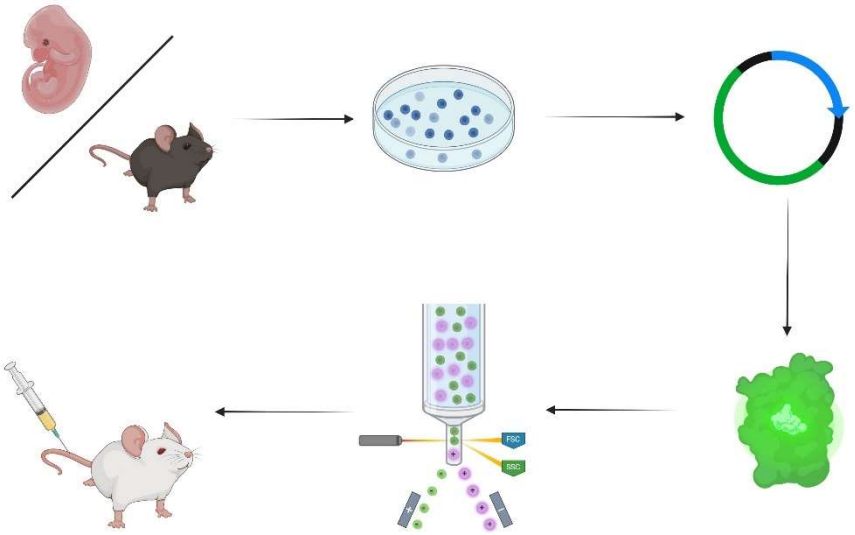

Figure 1. *Schematic representation of the murine model. BM or FL was isolated and transduced with an MSCV viral vector expressing a gene of interest and GFP/YFP. Cells were sorted using FACS and polyclonal transduced cells were introduced into recipient mice using tail vein injections. Image created with BioRender.com.*

ADVANTAGES AND DISADVANTAGES CONCERNING MURINE MODELS

The most important advantage for the murine model is that it is an *in vivo* leukemia model. Studies performed *in vitro* can be performed to show that the cells have leukemogenic properties but will not be able to fully reconstitute all aspects of leukemogenesis. This is because a leukemia is not independent of the body it arises in that is a complex system of tissues and cells that interact with each other. Therefore, a mammalian *in vivo* model system is much closer to an AML patient than *in vitro* experiments. However, it is a mouse model and mice are not humans, even though related. A disadvantage of this model is that it relies on forcibly overexpressed genes that pushes the system. The model system studies AML induced by overexpression *MNX1*, this is only one effect of the translocation and only the effect of MNX1 and what happens downstream of MNX1 can be studied using this model. Other direct effects of t(7;12) translocation cannot be

studied using the current murine model. Patients with t(7;12) most often have trisomy 19, this aspect of the disease cannot be studies using this model.

ETHICS CONCERING ANIMAL STUDIES

The animal studies were approved by the Regional Animal Ethics Committee of Gothenburg (IDNR 4009). When conducting research with animal models it is of utmost importance that the research questions are valid and that the results of the experiments lead to increased knowledge to the scientific community. This is due to that the impact of the research must outweigh the potential suffering of the mice. To minimize the number of mice needed for each part of this project as much material as possible from each mouse was obtained and thereby maximized the results each mouse could generate.

IPSC MODEL

In paper I, a human iPS cell line ChiPSC22 (RRID:CVCL_RN02) was used. The ChiPSC22 cell line was produced by reprogramming dermal fibroblasts from a 32-year-old donor using a defective polycistronic retrovirus technology to deliver *POU5F1*, *SOX2*, *KLF4* and *MYC*. The CRISPR/Cas9 technology was used to introduce the reciprocal translocation t(7;12) in the iPSCs. The cells with or without t(7;12) were then differentiated into hematopoietic cells using STEMdiff Hematopoietic kit (Stemcell Technologies Inc.) according to the manufacturers protocol.

INTRODUCTION OF T(7;12) USING CRISPR/CAS9

Two crRNAs were designed to target and give rise to breakpoints in chromosome 7 at NC_000007.14:g.156984781 and in chromosome 12 at NC_000012.12:g.11797368. The crRNAs were annealed to tracrRNA and introduced into cells together with Cas9 protein. 150 nucleotides long single stranded oligodeoxynucleotides (ssODNs) were also introduced and were designed to cover 75 nucleotides of each chromosome at the fusion site. The ssODNs worked as a template for chromosomal repair and allowed the cleaved chromosomes to be repaired as a reciprocal translocation.

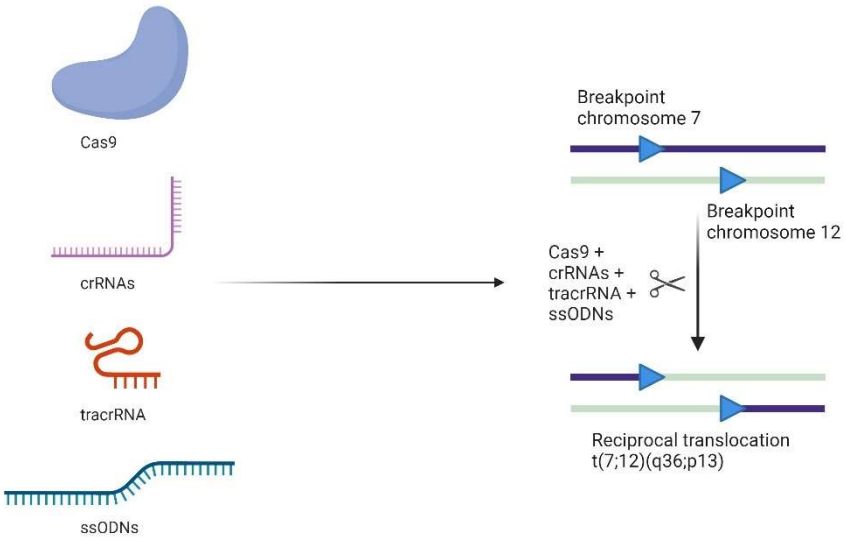

Figure 2. *Schematic representation of introduction of Cas9, crRNAs, tracrRNA and ssODNs in iPS cells recreating the t(7;12) translocation. Image created with BioRender.com.*

ADVANTAGES AND DISADVANTAGES CONCERNING IPSC MODELS

An important advantage for the iPSC model is that it is a model of human cells. Afterall the AML intended to study is human and therefore having a model system based on human cells is important. Another important advantage of the iPS cells is that it is a model that does not rely on overexpression of specific genes. It is a model enabling us to study the effects of the translocation. This can be seen as an advantage as well as a disadvantage because using a model where the translocation is studied in isolation and no other genetic alterations is not what is usually seen in patients. Patients with t(7;12) most often also have trisomy 19, this aspect of the disease cannot be studies using this model. However, as the translocation most likely is the leukemia initiating event studying this in isolation is very important.

ETHICS CONCERING IPSC MODEL

The research has been conducted in accordance with the Declaration of Helsinki of the World Medical Association. The study was approved by the research ethics committee at University of Gothenburg (DNR 313/18).

METHODS

NEXT GENERATION SEQUENCING

NGS was used to sequence genomes and transcriptomes. To sequence RNA it was first reverse transcribed into DNA. The next step is library preparation where the DNA is fragmented. To sequence the DNA fragments adapters are added to the ends of the fragments. The adapters have complementary sequences to oligonucleotide sequences bound to a flow cell and single stranded DNA binds to oligonucleotides. Clonal amplification by bridge amplification forms clusters of DNA strands. Sequencing primers that bind to adapter sequences are added. Nucleotides with conjugated fluorescent molecules are used with different fluorophores for different nucleotides. The sequencing reaction is performed by sequencing one nucleotide at a time. When a specific nucleotide is incorporated its fluorescent signal is recorded by a camera. This process is repeated cyclically with all the different nucleotides. If paired end sequencing is performed, then the reverse strand is sequenced after the forward strand is finished.

WHOLE GENOME SEQUENCING

Whole genome sequencing (WGS) was performed on gDNA isolated from AML patients. Sequencing was performed at the Center for Medical Genomics, Department of Clinical Genetic and Genomics, Sahlgrenska University Hospital, Sweden. Paired-end sequencing with 150 bp read length was performed using NovaSeq 6000 system. Read data was aligned to human reference genome GRCH38 (hg38) using bwa mem (v.0.7.17-r1188). Genomic breakpoints were identified using MANTA (v.1.6.0). Breakpoints were manually inspected using IGV (v.2.16.2)

WHOLE TRANSCRIPTOME SEQUENCING

Whole transcriptome sequencing (WTS) was performed on mRNA isolated from AML patients. Sequencing was performed at the SNP&SEQ Technology Platform in Uppsala, Uppsala University, Dept. of Medical

Sciences, Uppsala Biomedical Centre (BMC), D11:2, Box 1432, 751 44 Uppsala, Sweden. Paired-end sequencing with 150 bp read length was performed using NovaSeq 6000 system. Read data was aligned to human reference genome GRCH38 (hg38) using STAR (version 2.7.7a) Differential gene expression analysis was performed in R using the package "DeSeq2" [154]. Genes with log2. (foldchange) 1 and false recovery rate (FDR) adjusted p-value < 0.05 were considered differentially expressed genes. Fusion transcript analysis was performed using Arriba (v2.4.0).

WTS ANALYSIS OF DATA FROM THE TARGET DATA BASE

Read files from 12 t(7;12) patients and 62 normal human BM were obtained from the children's oncology group (COG)–National Cancer Institute (NCI) TARGET AML initiative data set. Differential gene expression was performed using the R package "DeSeq2" [154]. Genes with log2 (foldchange) 1 and false recovery rate (FDR) adjusted p-value < 0.05 were considered differentially expressed genes.

GENE SET ENRICHMENT ANALYSIS

Gene set enrichment analysis (GSEA) [155, 156] was used as a tool to analyze gene expression data. Instead of analyzing individual genes GSEA uses gene sets that describes a common biological function, set of upregulated or downregulated genes due to activity of a transcription factor etc that were significantly differently expressed between groups.

For GSEA gene expression values were obtained from the normalized counts of "DeSeq2" [154]. GSEA was used with the Human Molecular Signature Data Base (MSigDB) Hallmarks gene set for pathway enrichment analysis. A pathway in GSEA analysis was regarded significant at nominal p-value < 0.05.

FLOW CYTOMETRY AND FLUORESCENCE ACTIVATED CELL SORTING

Flow cytometry and FACS was used to identify cells that had been transduced using GFP/YFP as markers as well as expression of specific cell surface markers to analyze differentiation into different populations. GFP and YFP are fluorescent proteins, and the cell surface markers were identified using fluorescently labeled antibodies that bind specifically to that cell

surface marker. Presence of the florescent marker was then determined using a flow cytometer.

Cells were sorted for GFP/YFP before introduction into recipient mice cells to ensure transplantation of transduced cells. Peripheral blood from mice was monitored for presence of GFP/YFP by flow cytometry every other week. After mice were sacrificed, spleen and peripheral blood cells were analyzed using flow cytometry.

Detecting GFP and/or YFP expressing cells (The murine model.) as well as panels of antibodies was used to determine cell surface marker expression (The murine and iPSC model.). The cell surface marker expression was determined by incubating fluorescently labeled antibodies with cells in suspension. The fluorescent markers were then detected by flow cytometry.

FLUORESCENCE IN SITU HYBRIDIZATION

FISH can be used to determine the presence and genomic location of specific DNA sequences in cells. DNA probes that is complementary to the sequence of interest is fluorescently labeled and hybridized to the genomic DNA. Fluorescence-microscopy is then used for detection. This method lends itself well to detection of specific translocations by using DNA probes close to the breakpoints that “break apart” if the translocation is present.

In paper I and paper III FISH was used to identify the t(7;12) translocation. It was performed using a double fusion break apart probe specific for t(7;12) (q36;p13) (MetaSystems Probes). FISH was performed with standard procedures at the Cytogenetic laboratory, Department of Clinical Genetics and Genomics, Sahlgrenska University Hospital.

MOLECULAR GENETIC ANALYSIS IN PAPER I

In paper I a dataset from a previous study was downloaded and used to compare the gene expression signature used to identify patient t(7;12) AML to gene expression in t(7;12) iPSCs. In the paper Evaluation of Gene Expression Signatures Predictive of Cytogenetic and Molecular Subtypes of Pediatric Acute Myeloid Leukemia by Balgobind et. al. 237 paediatric AML samples were collected at diagnosis. They were hybridzed to Affymetrix U133 Plus 2.0 arrays. This dataset (GSE17855) was then uploaded to GEO database [157]. The microarray dataset GSE17855 was downloaded from the GEO database. AML gene expression profile analysis of this data set was

performed using R packages “Affy” and “Limma”. The gene expression signatures of the different pediatric AMLs were then compared to the RNAseq results from the CRISPR/Cas9 t(7;12) model to ensure that the iPSC model mimicked the gene expression signature from patient leukemias.

STATISTICS

The following statistical analyses were used in the publications that underline this thesis.

STUDENT’S T-TEST

Student’s t-test was used in paper I and paper II to compare the means between two groups and determine if the difference is significant. This test can be used if the distributions of the groups are normal, also called gaussian, and to have equal variance in both groups. Student’s t-test can be paired or unpaired, where samples in the groups are matched if using a paired test and if using an unpaired test, they are not.

MANN-WHITNEY U-TEST

Mann-Whitney u-test was used in paper III to compare the medians between two groups and determine if the difference was significant. Mann Whitney u-test can be used even if the distribution of the groups does not follow a normal distribution or is unknown.

LOG-RANK TEST

The Log-rank test was used in paper II and paper III to compare OS and EFS between groups generated from Kaplan-Meier estimates.

CHI-SQUARE TEST

Chi-square test was used in paper III to compare the frequency between groups in a contingency table and determine if any difference was significant. The chi-square test was used when the values of all cells of the contingency table was at least 5. If this requirement was not met, then Fishers’ exact test was used instead.

FISHERS’ EXACT TEST

Fishers exact test was used in paper III to compare the frequency between groups in a contingency table and to determine if any difference was

significant. The Fishers' exact test was used when at least one cell in the contingency table was less than 5.

PRINCIPAL COMPONENT ANALYSIS

Principal component analysis (PCA) was used in paper I, paper II and paper III. It is used to reduce the dimensionality and simplify large data experiments, such as WTS, while still retaining as much information as possible. The principal components are new variables constructed from the input data where the first principal component explains as much variance from the original data set as possible, the second principal component explains as much variance as possible from the remaining information in the original data etc. PCA of WTS data was performed using the R package "DeSeq2".

RESULTS

AN INDUCED PLURIPOTENT STEM CELL T(7;12) ACUTE MYELOID LEUKEMIA MODEL SHOWS HIGH EXPRESSION OF MNX1 AND A BLOCK IN DIFFERENTIATION OF THE ERYTHROID AND MEGAKARYOCYTIC LINEAGES (PAPER I)

The mechanism that gives rise to leukemia induced by t(7;12) is not well understood. This is in part because of that there are no good quality models to study the leukemogenic properties of t(7;12). To this end induced pluripotent stem cell (iPSC) lines that carry the translocation by use of CRISPR/Cas9 technology were generated.

The t(7;12) iPSCs were able to differentiate into all three germ layers, thereby confirming that iPSCs with t(7;12) still had pluripotent stem cell properties. That iPSCs with t(7;12) could differentiate into hematopoietic stem and progenitor cells (iHSPC) could be shown by expression of the hematopoietic markers CD34, CD43 and CD45. Using a larger panel to identify subpopulations identified a differentiation block in iHSPCs with t(7;12) as there was a significant decrease in cells expressing CD235a and CD41a. In colony formation assays the block in differentiation manifested as an accumulation of cells at erythroid as well as myeloid progenitor stages. Pathway analysis of WTS uncovered significant downregulation of genes involved in megakaryocyte differentiation and significant upregulation of genes involved in myeloid pathways in iPSCs with t(7;12).

Gene expression signatures derived from patient data to discriminate between pediatric AML caused by different translocations were used to compare to the gene expression of this cell model. This model matched the gene expression signature from t(7;12) patient material well and most importantly a robust high expression of *MNX1*, a hallmark of AML with t(7;12) could be replicated in the cell model. Interestingly an ectopic expression of the long noncoding RNAs *MNX1-AS1* and *MNX1-AS2* from the same gene locus as *MNX1* was also detected.

In conclusion the iPSC model of the t(7;12) AML subtype provides an important tool for further studies of the mechanism of t(7;12) leukemogenesis and to develop new treatment.

ABERRANT MNX1 EXPRESSION ASSOCIATED WITH T(7;12) PEDIATRIC ACUTE MYELOID LEUKEMIA INDUCES THE DISEASE THROUGH ALTERING HISTONE METHYLATION (PAPER II)

In this study the transforming event in AML with t(7;12) and potential ways to inhibit its effects were investigated. The translocation can lead to a fusion transcript of *MNX1*::*ETV6* but also give rise to high ectopic expression of *MNX1*. The transformation capacity of MNX1, ETV6, MNX1::ETV6 and ETV6::MNX1 was investigated. ETV6, MNX1::ETV6 and ETV6::MNX1 did not induce leukemia while a high expression of MNX1 did give rise to AML in a murine model, however, this could only be observed using FL cells and not cells from BM. Transplantation of BM from mice with primary leukemia gave rise to a more rapid onset secondary leukemias. This AML was highly penetrant in immunocompromised mice but only partly penetrant in immunocompetent mice. That the cells that can give rise to leukemia is restricted to cells from FL in the murine model may explain why AML with t(7;12) only occurs in infants or very young patients.

The ectopic expression of MNX1 led to changes in histone methylation and gave rise to an increase in H3K4mono, di- and trimethylation and a reduction in H3K27me3. The changes in histone methylation most likely acted through MNX1 interaction with the methionine cycle and through methyltransferases. ATAC-sequencing illustrated that the changes in histone methylation gave rise to changes in genome wide chromatin accessibility and WTS confirmed changed gene expression.

The high MNX1 expression gave rise to an enrichment of DNA damage response genes. High expression of MNX1 also induced a high number of γH2AX foci, thereby confirming an increase in DNA damage. High expression of MNX1 also led to an exhaustion of the Lin-/Sca1+/c-Kit+ immature cell population and shifted differentiation to the myeloid lineage. The effects of MNX1 could be inhibited by treatment with the pan-methyltransferase inhibitor Sinefungin and FL cells that were pretreated with Sinefungin could not give rise to leukemia in mice.

Comparison of WTS results between the murine leukemia model and t(7;12) AML patient material from the TARGET database showed almost 50% overlap of differentially expressed genes and comparison of GSEA results from the murine leukemia model and the human iPSC with t(7;12) model system differentiated into HSPCs showed an enrichment in similar pathways.

In conclusion, the ability to overexpress MNX1 drives leukemia development in hematopoietic cells isolated from FL and therefore illustrates the importance of overexpression of MNX1 for leukemia development in the t(7;12) AML.

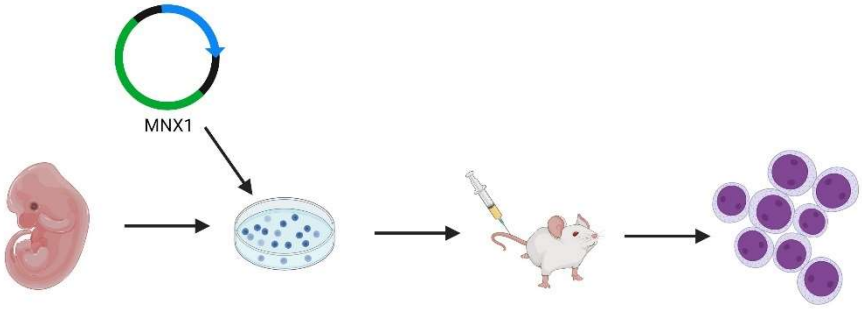

Figure 3. *Schematic representation of how FL cells transduced with MNX1 introduced into immunocompromised mice gives rise to AML. Image created with BioRender.com.*

CHARACERIZATION OF PEDIATRIC ACUTE MYELOID LEUKEMIA WITH T(7;12) (PAPER III)

In this study children with t(7;12) AML treated according to NOPHO-AML-2004 or NOPHO-DBH-AML-2012 treatment protocols were investigated. In Sweden, Denmark and Iceland the frequency of t(7;12) in AML under 2 years old was 7% and under 1 year old it was 11%. AML with t(7;12) was associated with trisomy 19 as previously described and was also associated with CNS involvement.

In the full clinical cohort 12 patients with t(7;12) AML were reported in between January 2004 and November 2020. One patient died prior to treatment. Ten out of the remaining 11 patients responded well to induction treatment. The patient who did not respond well was assigned to high-risk therapy including allogeneic HSCT. This patient was still in remission at the end of the study. The 10 remaining patients received standard therapy, however, six relapsed and underwent allogeneic HSCT. Of the patients that relapsed one died due to a second relapse, none of the remaining five patients have suffered a second relapse. Comparing OS and EFS between t(7;12) AML patient with other AML in same age group could not identify any significant differences.

Molecular analysis was performed with WTS on six patients with t(7;12) AML. The *MNX1*::*ETV6* fusion transcript was not identified in any of the investigated patients, however, other novel fusion transcripts involving *ETV6* were identified. Interestingly, fusion transcripts with *NOM1* and *LMBR1*, the two genes closest downstream from *MNX1* on chromosome 7 were identified. The genomic breakpoint was consistently found to be in intron 1 in *ETV6* on chromosome 12 and on chromosome 7 it was most often found in *NOM1*. One patient had part of *ETV6* inserted upstream of *MNX1* thus making expression of *MNX1*::*ETV6* impossible. PCA performed on WTS data showed that patients with t(7;12) grouped together and heterogenous expression of fusion transcripts had no effect on global gene expression.

In accordance with previous reports, there was a high ectopic expression of *MNX1* in all AML patients with t(7;12). Together with *MNX1* a high expression of two long non-coding RNAs from the same gene locus as *MNX1*, namely *MNX1-AS1* and *MNX1-AS2* was observed. GSEA of WTS

data identified a high enrichment of MYC target genes in AML with t(7;12) and WTS data also identified a high expression of *MYCN*.

Analysis of WGS data from six AML patients with t(7;12) identified that two patients were heterozygous for an SNP in the 5' region of *MNX1* and that three patients were heterozygous for SNPs in exon 2 of *MNX1-AS1*. However, analysis of the WTS data from the same patients only identified mRNA expression of one of the two possible transcripts.

In conclusion, the frequency of t(7;12) AML was 7% in patients 0-2 years old treated according to NOPHO-AML-2004 or NOPHO-DBH-AML-2012 treatment protocols. AML with t(7;12) is associated with CNS involvment. Patients with t(7;12) AML suffers relapse at a high rate but allogeneic HSCT is an effective treatment. OS was not significantly different compared to other AML patients from the same age group. Patients expressed heterogenous fusion transcripts involving *ETV6*, but WGS analysis identified a large part of *ETV6* in close proximity to *MNX1*. All t(7;12) AML patients had high expression of *MNX1* that was accompanied by a high expression of *MNX1-AS1* and *MNX1-AS2*, suggesting an enhancer hijacking event.

DISCUSSION

This thesis aimed to gain a greater understanding of AML with t(7;12) by developing and studying models of the disease, such as an iPSC model and a murine model, as well as patient data, patient outcome and patient material. In an iPSC model t(7;12) gave rise to a high expression of MNX1, a block in differentiation and the model could replicate a gene expression signature similar to that of AML with t(7;12) (Paper I). The transformation capacity of MNX1 and MNX1::ETV6 was investigated in a murine model. MNX1::ETV6 was found to be unable to induce leukemogenesis while MNX1 was able to give rise to leukemia, however only when FL cells were transduced and not BM and primarily in immunocompromised recipient mice (Paper II). The patient characteristics, EFS and OS of patients with t(7;12) was investigated and molecular analysis by WTS and WGS on patient material from patients with t(7;12) was performed (Paper III).

TRANSFORMATION CAPACITY

Early studies on patient material from AML with t(7;12) identified expression of fusion transcript *MNX1*::*ETV6* and *MNX1*, however, the fusion transcript in patient material could only be detected occasionally whereas a high ectopic expression of *MNX1* was detected in 100% of patients. However, a thorough investigation of the transforming capacity of the genes involved had not been performed prior to these studies.

The transformation capacity of MNX1, ETV6, MNX1::ETV6 and the reciprocal fusion ETV6::MNX1 was investigated using a murine model. A high expression of ETV6, MNX1::ETV6 or ETV6::MNX1 was not able to induce leukemia but transducing HSPCs from FL with MNX1 was able to induce leukemia with low penetrance in immunocompetent recipient mice. However, high expression of MNX1 in FL cells introduced in immunocompromised recipient mice induced leukemia at high penetrance. The induced leukemia matched criteria for AML and was more rapidly fatal in secondary recipients compared to primary transplanted mice. This established that a high expression of MNX1 but not the fusion MNX1::ETV6 can transform hematopoietic cells and give rise to AML.

Expression of *MNX1* and *MNX1*::*ETV6* in patient material was also investigated using WTS. Surprisingly the fusion transcript *MNX1*::*ETV6*

could not be identified in any patient. However, a low expression of other fusion transcripts involving *ETV6* most notably with *NOM1* and *LMBR1* as fusion partners could be identified. *NOM1* and *LMBR1* are the two genes closest distal of *MNX1*. Despite this heterogeneous expression of fusion transcripts PCA of the WTS data from the patients with AML clearly grouped patients with t(7;12) in a group separate from the other patients with AML from the same age group. This established that in spite of the lack of *MNX1*::*ETV6* expression and heterogenous expression of other fusion transcripts, patients with t(7;12) are similar to each other and dissimilar to other forms of AML in the same age group. Using a previously established expression signature to identify AML with t(7;12) it could be ascertained that the patients with AML with t(7;12) from this study were phenotypically similar to patients with t(7;12) from earlier studies. In stark contrast to the heterogenous expression of fusion transcripts all patients diagnosed with t(7;12) AML had a high ectopic expression of *MNX1*.

Normally in AML with t(7;12) the translocation places a large part of *ETV6* distal to *MNX1* and thereby making expression of the *MNX1*::*ETV6* fusion transcript possible. WGS could identify that one patient diagnosed with t(7;12) did not carry the regular translocation but instead had a large part of chromosome 12 inserted into chromosome 7. What set this patient apart from other patients with t(7;12) is that it placed the large part of *ETV6* proximal to the non-disrupted *MNX1* and thereby making expression of the fusion transcript *MNX1*::*ETV6* an impossibility. Despite this the patient displayed several characteristics of t(7;12) such as being diagnosed with AML before 2 years of age and the leukemic cells also had trisomy 19 and the same gene expression profile as the other t(7;12) AML patients.

In recent years a high expression of *MNX1* has been identified in many forms of malignancies, such as such as glioma [105], cervical cancer [106], prostate cancer [107], breast cancer [108], hepatocellular carcinoma [109] and colorectal cancer [110], and has emerged as a driver of carcinogenesis by increasing proliferation [110] and giving rise to an aggressive phenotype [106]. Here it is established that high expression of MNX1 drives leukemogenesis in t(7;12) AML.

ENHANCER HIJACKING

Before these studies it was well established that AML with t(7;12) patients have a high ectopic expression *MNX1*. This was confirmed as all AML with

t(7;12) patients from NOPHO biobank and the TARGET database had a high expression of *MNX1*. It is however not well understood what drives the expression of *MNX1*. In an early paper on t(7;12) AML the *MNX1*::*ETV6* fusion transcript had been identified in a small number of patients and assumed to be the leukemogenic driver. They believed the chromosomal breakpoint on chromosome 7 to be situated within the *MNX1* gene and therefore speculated that the expression of *MNX1* came from the non-translocated gene copy of *MNX1* [66]. Since then, it has been identified that the breakpoint in chromosome 7 is not found within the *MNX1* gene but is found within a breakpoint region distal to *MNX1*, leaving the *MNX1* gene on the translocated chromosome still intact and thereby possibly expressed [63]. In paper III WGS performed on patient material were able to confirm that the *MNX1* gene was not disrupted by t(7;12).

Recently a study was published on the cell line GDM-1 that carries the reciprocal translocation t(6;7)(q23;q36) [97]. This translocation does not disrupt the *MNX1* gene locus either and the cell line have a high expression of *MNX1*. The translocation places the intact *MNX1* gene locus in close proximity to the *MYB/AHI1* gene locus. MYB is known to play an essential role in hematopoiesis [158]. Using the GDM-1 cell line they were able to show that the high expression of *MNX1* was driven by hijacking an enhancer normally used to drive *MYB* expression to now drive expression of *MNX1* in hematopoietic cells. Thereby enhancer hijacking events as a mechanism to drive high expression of *MNX1* was established. This led us to hypothesize that this also takes place in AML with t(7;12).

By generating an iPSC model of t(7;12) the effect of the translocation itself could be studied separating its effect from the effects of other genetic aberrations present such as trisomy 19 as well as potentially other aberrations. Interestingly in the iPSCs with t(7;12) no ectopic expression of *MNX1* was seen, however, upon differentiation into iHSPCs a robust high expression of *MNX1* arose. Using this model, a high expression of two long non-coding RNAs *MNX1-AS1* and *MNX1-AS2* from the same gene locus was identified. This shows that it is the t(7;12) translocation that causes high expression of *MNX1* as well as *MNX1-AS1* and *MNX1-AS2* from the same gene locus.

In paper III gDNA and mRNA from patient material to study the effects of t(7;12) AML was used. As stated earlier all AML patients studied from NOPHO and the TARGET database had a high ectopic expression of *MNX1*

and all patients also had a high expression of *MNX1-AS1* and *MNX1-AS2*. Further analysis of the WGS data from patients identified that two patients were heterozygous for an SNP in the 5' UTR of *MNX1* and three patients were heterozygous for SNPs in the second exon of *MNX1-AS1*. To determine if the high expression of *MNX1* and *MNX1-AS1* was transcribed from one or two alleles the SNPs were used to compare the WTS data to the identified SNPs. In the heterozygous patients only expression of one of the two copies of *MNX1* and *MNX1-AS1* could be identified, presumably from the translocated chromosome.

In a collaborative follow-up study on iPSCs with t(7;12) it has been identified that the mechanism leading to the high *MNX1* expression is an enhancer hijacking event. This study is in the process of being published.

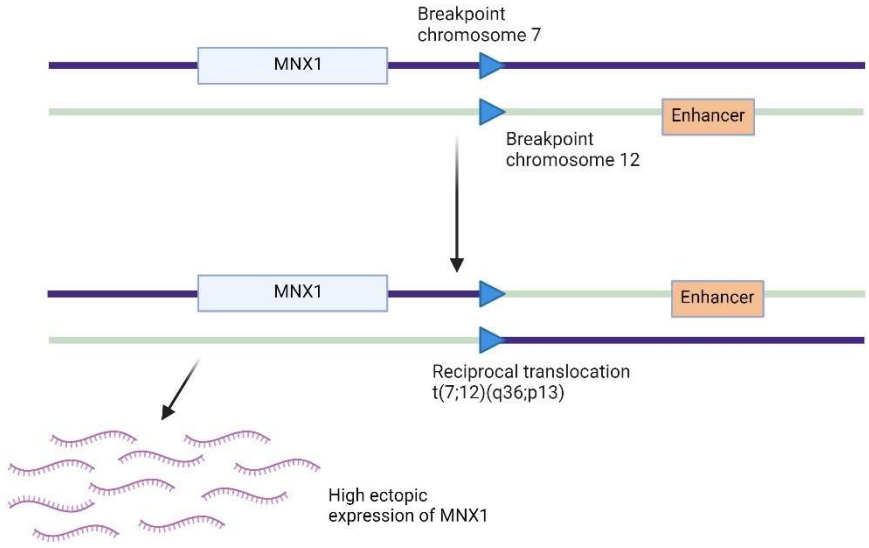

Figure 4. *Schematic representation of how the t(7;12) translocation leads to an enhancer hijacking event that drives the high ectopic expression of MNX1. Image created with BioRender.com.*

CELL OF ORIGIN FOR AML WITH T(7;12)

In Paper III all AML patients with t(7;12) all patients presented with AML before 18 months of age and the majority of patients presented before one year of age. This is in accordance with previous reports on AML with t(7;12) and that AML with t(7;12) only arise in very young children and has a higher frequency the younger the patients are raises the question of if there is a prenatal origin of AML with t(7;12).

That the murine model of AML with high expression of *MNX1* developed cannot transform adult HSCs shows that MNX1 is unable to transform adult hematopoietic cells. Instead, BM cells transduced with *MNX1* gave rise to DNA damage and a dramatic apoptotic induction. Transduction of *MNX1* in FL did also give rise to DNA damage but in contrast did not give rise to apoptosis. Most importantly transduction of *MNX1* in FL was able to transform hematopoietic cells in the murine model providing evidence to that AML with t(7;12) arise during fetal hematopoiesis.

Fetal hematopoiesis and adult hematopoiesis differ in many ways such as proliferation rate and capacity to differentiate. One of the best studied molecular differences between fetal and adult hematopoiesis is Lin28b expression [139]. It has previously been shown that Lin28b govern the switch from fetal to adult hematopoiesis and its high expression in FL inhibits the switch to adult hematopoiesis. High expression of *LIN28B* have been linked to other cancers that arise in very young children such as neuroblastoma [140] and Wilms tumor [143]. A high expression of *MYCN* and a significant enrichment of *MYC* target genes was identified in t(7;12) AML. High expression of *MYCN* have like *LIN28B* been linked to other cancers that arise in very young children such as neuroblastoma [150], and Wilms tumor [151]. MYCN is also like LIN28B expressed throughout embryonic development. Interestingly LIN28B has been shown to enhance MYCN protein levels by suppression of let-7 [140] and MYCN has also been shown to regulate the expression of *LIN28B* [159], thus possibly forming a positive feedback loop. In adult cancer oncofetal reprogramming leads to reactivation of fetal signaling pathways and expression of genes not normally expressed in adult tissues [42], in AML with t(7;12) it is more likely that the leukemia originated in a fetal cell and retained these signaling pathways.

Taken together the high expression of *LIN28B* and *MYCN* in t(7;12) AML, the age of patients at leukemia presentation and that only transduced FL and

not BM cells transduced with *MNX1* induces leukemia indicates that the cell of origin is of fetal origin.

EVADING IMMUNE RESPONSE

Of interest is also that in the murine model, it was only possible to induce leukemia at high penetrance in recipient mice that were immunocompromised. This may be due to that it resembles the fetal/neonatal state as hematopoietic system during this developmental period does not have the same developed immune system as the adult hematopoietic system. This indicates that a fully developed immune system could be able to eradicate AML with t(7;12) and may be a reason why AML with t(7;12) is not seen in older children or adults. Because of this, AML with t(7;12) would not need a way to evade adult immunity it develops during a period when it does not encounter it.

Further evidence to this is that graft versus leukemia effect of haploidentical HSCT after relapse has previously been documented. In that report they were able to detect the fusion transcript 25 days post-transplant, however on day 53 and 92 post-transplant they could no longer detect leukemic cells [160]. In addition to this strong graft versus leukemia effect is the high success rate seen after stem cell transplant in the NOPHO treatment protocols. However, it is important to note that even though a graft versus leukemia effect is seen, this effect may be due to that the leukemic cells are foreign to the transplanted cells and not only due to that the transplanted cells provides a fully developed immune response. Therefore, to gain a better understanding for the mechanism through which AML with t(7;12) interact with the immune system further studies are needed.

CHARACTERISTICS OF AML WITH T(7;12)

In previous studies the frequency of AML with t(7;12) has varied greatly from 4% in children younger than 2 years old in Espersen et. al. to 28% in children younger than 1 year old in von Bergh et. al. [56, 66]. It has been suggested that this difference is due to that only small parts of the chromosomes are translocated in AML with t(7;12) and may therefore have been missed and the frequency of AML with t(7;12) underestimated. Therefore, WTS was used to screen AML patients with no AML defining genetic abnormality under 2 years from NOPHO-AML-2004 and NOPHO-

DBH-AML-2012 protocols for high expression of *MNX1*. In this study the frequency of AML with t(7;12) was 7% in AML patients under 2 years old and 11% in AML patients under 1 year old. This is a study on a small population and the frequency may therefore differ from other populations. However, the study by Espersen et. al. studied patients diagnosed from 1993 to 2014 and the first studies recognizing t(7;12) as a recurrent translocation in AML were published in the early 2000s. Expectedly the Espersen study identified no patient with t(7;12) AML between 1993 and 2004. This explains the very low frequency reported as there likely were patients with t(7;12) AML between 1993 and 2004 that were missed. On the other hand the high frequency reported in von Bergh et. al. may in part be explained by that they reported the frequency among infants as AML with t(7;12) is more common among very young patients. However, this study was not population based and is likely an overestimation due to the very small number of patients studied (5 out of 18 patients.). For these reasons we expect the true frequency of AML with t(7;12) to be close to the 7% reported here. In accordance with previous results in Paper III 11 out of 12 patients with t(7;12) are reported to have trisomy 19. AML with t(7;12) was also associated with CNS involvement which has an impact on the IT therapy the patients receive.

AML with t(7;12) was recently recognized as a leukemia with a defining genetic aberration in the 5th edition of the WHO Classification of Hematolymphoid Tumors. It is classified as subtype AML with *MNX1*::*ETV6* fusion [68]. This is in line with how other subtypes are classified, however, in previous studies the *MNX1*::*ETV6* fusion transcript has only been detected occasionally and in Paper III *MNX1*::*ETV6* was not detected in any patient. Instead, t(7;12) AML patients expressed heterogeneous fusion transcripts involving *ETV6*. In stark contrast to the low and heterogeneous expression of fusion transcripts all t(7;12) AML patients had a high ectopic expression of *MNX1* and in Paper II MNX1 and not MNX1::ETV6 is shown to induce leukemia. Perhaps ectopic expression of *MNX1* should be the classifying criteria of this AML.

OUTCOME AND RISK-GROUP

Several studies report that AML patients with t(7;12) have a low EFS [56, 66]. However, OS has differed in previous studies. The outcome for AML patients with t(7;12) have been reported to be very poor [66], however, the study performed by Espersen et. al. reported a good OS for t(7;12) AML patients treated under NOPHO protocols [56].

In most current pediatric AML treatment protocols AML with t(7;12) is considered a high-risk group and is often stratified to consolidation with HSCT in primary therapy. NOPHO-DBH-AML-2012 is an exception. It is important to avoid HSCT if possible due to the long-term risks. The results presented here on more patients with longer follow up are in line with the results previously presented by Espersen et. al. [56]. AML patients with t(7;12) have a high relapse rate but allogeneic HSCT is a very effective treatment. The OS of 10 out of 11 patients is excellent and shows that there is no need to place all t(7;12) AML patients in a high-risk group. Therefore, the NOPHO-DB-SHIP study group will continue to risk stratify AML t(7;12) according to induction therapy response and not genotype.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis evidence has been provided that has led to the following conclusions:

- High expression of *MNX1* is driven by the t(7;12) translocation.
- High ectopic expression of *MNX1* drives transformation in AML with t(7;12).
- AML with t(7;12) likely has a fetal cell of origin.
- The frequency of AML with t(7;12) was established to be relatively low at 7% in AML patients under 2 years old.
- Patients with t(7;12) AML often relapsed but allogeneic HSCT is an effective treatment.

The studies performed in the production of this thesis have provided answers to the aims set out to answer, however, they have also provided several questions for future studies.

CAN AGE AT LEUKEMIA PRESENTATION PREDICT OUTCOME

The patients with t(7;12) are very young. In Espersen et. al., where patients treated under NOPHO protocols as well as all published data on t(7;12) patients were analyzed, no patient older than 24 months was identified [56]. In conjunction it was found in the murine model that adult BM transduced with *MNX1* is unable to give rise to leukemia, however FL cells transduced with *MNX1* can give rise to leukemia. As discussed earlier, together these results indicate that leukemic cells with t(7;12) can only arise in cells of fetal origin. This would mean that the patients with t(7;12) AML have leukemic cells present already at birth. This would give the leukemic cells time to acquire more genomic aberrations as well as time to spread from birth until clinical presentation of the leukemia. This could mean that patients with t(7;12) whose AML present later in life have a worse prognosis than patients that present early in life.

To be able to perform a study where the outcome of patients diagnosed in different age ranges is compared to each other would need a higher number of total t(7;12) AML patients than one pediatric AML study program, such as NOPHO-DB-SHIP study group, can provide. Therefore, a collaborative effort with other AML study groups to collect patient data for a larger study on AML with t(7;12) have been initiated.

DOES TRISOMY 19 EFFECT OUTCOME FOR PATIENTS WITH T(7;12)?

In the studies presented here there was a focus on the t(7;12) translocation and the effects it gives rise to. However, most patients with t(7;12) AML also have trisomy 19. Eleven out of 12 patients with t(7;12) AML in the NOPHO registry had trisomy 19. That trisomy 19 is this prevalent makes comparisons between t(7;12) AML patients with or without trisomy 19 very difficult and to date it is not known what drives this nor is it known if the presence or absence of trisomy 19 has an effect on prognosis for the patient. By performing the large collaborative study on AML with t(7;12) previously described there will be enough patients to enable a comparison of t(7;12) AML with or without trisomy 19 and study the effects on AML characteristics and outcome.

WHAT IS THE CONTRIBUTION OF LIN28B?

The studies presented here show that t(7;12) likely have a fetal cell of origin. In the murine model it was shown that FL overexpressing *MNX1* gives rise to leukemia and previously it has been shown that FL hematopoiesis is driven by expression of Lin28b [139]. The expression of Lin28b goes down after birth and is only expressed at low levels in BM derived hematopoietic cells. In AML patients with t(7;12), however, expression of *LIN28B* is high. Can *MNX1* overexpressed together with overexpression of *Lin28b* induce leukemia transformation in adult BM? Experiments to develop murine models to perform these experiments are ongoing.

CAN MNX1 OVEREXPRESSION INDUCE LEUKEMIA TRANSFORMATION IN BONE MARROW CELLS WITH TP53 KNOCKED-OUT?

In the mouse model overexpressing MNX1 in normal FL cells give rise to leukemia. Previous results also show that the resulting leukemia with high MNX1 expression gave rise to increased DNA damage. Another study published on MNX1 they showed that MNX1 overexpression led to senescence in human HT1080 and murine NIH3T3 cells and that this onset of senescence was dependent on tp53 [104]. Therefore, we hypothesized that BM with impaired tp53 signaling could give rise to MNX1 driven leukemia. To investigate this, a mouse model of *tp53* knockout have been acquired and *tp53*-/- BM cells have been isolated to transduce to overexpress MNX1 to use in a murine model. These experiments are ongoing.

WHAT IS THE CONTRIBUTION OF MNX1-AS1 AND MNX1-AS2 TO T(7;12) AML?

In the studies performed high expression levels of *MNX1*, *MNX1-AS1* and *MNX1-AS2* were identified in t(7;12) AML. The high expression of these genes is due to the translocation through enhancer hijacking. In the murine model it was shown that FL cells transduced with *MNX1* gives rise to AML in immunocompromised mice. However, the effect of high *MNX1-AS1* and *MNX1-AS2* expression on the development of leukemia is not investigated. The contribution of *MNX1-AS1* and/or *MNX1-AS2* to t(7;12) AML may in future studies be investigated using the murine model systems and future experiments could include:

- Does overexpression of *MNX1-AS1* and/or *MNX1-AS2* give rise to leukemia on their own in adult BM or FL in the murine model?
- Does overexpression of *MNX1-AS1* and/or *MNX1-AS2* together with MNX1 overexpressed give rise to a more aggressive leukemia using the murine model system?
- Does overexpression of *MNX1-AS1* and/or *MNX1-AS2* together with MNX1 enable leukemia development in transduced adult BM in the murine model system?

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