CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS

AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE BIOMEDICINA MOLECULAR

“Papel de la cortactina en la transmigración de precursores leucémicos en leucemia linfoblástica aguda de linaje de B”

TESIS

Que presenta

M. en C. MARTHA VELÁZQUEZ AVILA

Para obtener el grado de

DOCTORA EN CIENCIAS

EN LA ESPECIALIDAD DE

BIOMEDICINA MOLECULAR

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Ciudad de México **Enero 2018**

RESEARCH CENTER AND ADVANCED STUDIES OF THE

NATIONAL POLYTECHNIC INSTITUTE

ZACATENCO UNIT

DEPARTMENT OF MOLECULAR BIOMEDICINE

“The role of cortactin in the transmigration of leukemic precursors in B-lineage acute lymphoblastic leukemia”

THESIS

Presented by

MSc. MARTHA VELÁZQUEZ AVILA

To obtained the degree of

PHILOSOPHY DOCTOR

IN THE SPECIALITY OF

MOLECULAR BIOMEDICINE

Thesis director:

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Mexico City **January 2018**

AGRADECIMIENTOS

Al Consejo Nacional de Ciencia y Tecnología (CONACYT), por la beca otorgada durante el periodo 2013-2017 para la realización de éste posgrado.

Al Instituto Mexicano del Seguro Social (IMSS), por la beca otorgada durante el periodo 2014-2018.

A la Dra. Rosana Pelayo por haberme abierto las puertas de su laboratorio y por todo su apoyo, por su calidez humana y asesoría a lo largo de éste proyecto.

Al Dr. Michael Schnoor por permitirme formar parte de su equipo y por su apoyo en el desarrollo de éste proyecto.

A la Dra. María Antonieta Chávez, Dr. Vianney Ortíz, Dr. Leopoldo Santos y Dr. Nicolás Villegas por formar parte de mi comité y por todas sus observaciones.

A mis compañeros de laboratorio de Linfopoyesis en la UIMEO del Hospital de Oncología, CMN SXXI, IMSS: M. en C. Dalia Ramírez, M. en C. Jussara Ríos, M. en C. Jennifer Enciso, M. en C. Sandra Padilla, M. en C. Juan Carlos Balandrán y M. en C. Armando Vilchis por su apoyo y por hacer del laboratorio un lugar placentero y de intercambio de conocimiento constante.

A mis compañeros de laboratorio del departamento de Biomedicina molecular en CINVESTAV: M. en C. Sandra Chánez, M. en C. Karla Castro, Dra. Hilda Robles, M. en C. Eliana Saldívar, M. en C. Selene Zúñiga, Biol. Idaira Guerrero, QFB. Ramón Castellanos, Dr. Alexander Ponce, y Dr. Alí Citalán por todo su apoyo para la elaboración de éste proyecto y por una amena convivencia dentro del laboratorio.

DEDICATORIAS

Ésta tesis se la dedico con agradecimientos a personas que han sido importantes de alguna manera durante éste período en mi vida, las cuales me apoyaron en todo momento para no desfallecer y seguir adelante tanto en aspectos académicos como personales.

Agradezco infinitamente a mi mamá María Eugenia Avila, por todo el apoyo brindado, por ser uno de mis pilares, por escucharme y por todas sus palabras de aliento para continuar a lo largo de éste camino.

Agradezco a mis hermanas Marychuy, Nelly y Mirella por ser un ejemplo para mí y por ser un apoyo siempre.

También agradezco a mis amigos de toda la vida Lea, Brenda, Nancy, Aurora, Georgina, Sandy, Bibiana y Alejandro por estar para apoyarme cuando más lo he necesitado y por hacer que esto sea mejor.

A lo largo de éste doctorado conocí a personas maravillosas que sí bien comenzamos siendo compañeros hemos forjado lazos de amistad. Agradezco enormemente a Dalia, Jenny, Jussara, Sandra Chánez, Karla Castro, Dafné, Juan Carlos y Armando por su apoyo, las risas y por todos sus consejos fuera y dentro del laboratorio.

¡Gracias infinitas!

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ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
B-ALL	B-precursor cell acute lymphoblastic leukemia
BBB	Blood-brain-barrier
B-CLL	B-cell chronic lymphoid leukemia
BM	Bone marrow
CAM-DR	Cell adhesion-mediated drug resistance
CAR	CXCL12-abundant reticular
CaR	Ca^{2+} influx sensed by calcium receptor
CNS	Central nervous system
CSC	Cancer stem cells
CSF	Cerebral spinal fluid
CXCL12	C-X-C motif chemokine 12 or SDF1
CXCR4	C-X-C chemokine receptor type 4 (CXCR-4 or CD184
Cx-43	Connexin 43
DAG	Diacylglycerol
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FLT-3	FMS-like tyrosine kinase-3
G-CSF	Granulocyte colony stimulating factor
GPCR	G-protein coupled receptor
HIF1-α	Hypoxia-inducible factor 1α
HNSCC	Head and neck squamous cell carcinoma
HPC	Hematopoietic progenitor cells
HS1	Hematopoietic cell-specific lyn substrate

HUVEC	Human umbilical vein endothelial cells
IgVH	Immunoglobulin heavy-chain variable region
IKZ	Ikaroz
IL	Initiating cell
IP3	Phosphatidylinositol 3
JAMs	Junctional adhesion molecules
LSC	Long-term leukemic stem
LT-HSC	Long-term hematopoieic stem cells
MLL	Mixed lineage leukemia
MMP-2	Matrix metalloprotease-2
MPB	Mobilized peripheral blood
MRD	Minimal residual disease
NPF II	Nucleation promoting factor II
PDGFR	Platelet-derived growth factor receptor
PDX	Patient-derived leukemia xenografts
PF4	Patelet factor 4
PIK-2	Proline-rich kinase-2
PSGL-1	P-selectin glycoprotein ligand-1
REH-DX	REH derived leukemia xenograft
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SCF	Stromal cell factor
SDF1	Stromal derived-factor 1
SH3	Src-homology-3
TEM	Transendothelial migration

THPO	Thrombopoietin
UCB	Umbilical cord blood
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor

ABSTRACT

Cancer is a major cause of death in children worldwide, with acute leukemias being the most frequent childhood malignancy. Relapse, treatment failure and organ infiltration worsen the prognosis, thus warranting a better understanding of the implicated mechanisms. Cortactin and HS1 are actin-binding molecules involved in cell adhesion and migration by regulating actin cytoskeleton remodeling. High expression of cortactin is associated with invasiveness of cancer cells in a number of solid tumors; and recently high levels of cortactin have also been correlated to poor prognosis in adult B-cell chronic lymphoid leukemia.

In this work, we have investigated the expression profile of cortactin in B-cell populations in acute lymphoblastic B-cell childhood leukemia and the potential impact on infiltration and disease prognosis. We found higher amounts of cortactin in the leukemic B-cell lines RS4:11 and REH, and in lymphoid precursors from bone marrow (BM) and cerebrospinal fluid (CSF) of B-ALL patients when compared to normal B lineage cells. Cortactin expression was highest in primary leukemic pro-B and pre-B precursor cell populations. Of note, these cells only expressed the 60 kDa SV2 cortactin isoform. Transendothelial migration assays revealed that cortactin was significantly higher expressed in transmigrated cells in response of CXCL12 (or SDF-1) when compared to non-transmigrated cells suggesting that cortactin may support infiltration of extramedullary organs. Clinical data showed that the highest cortactin levels were related to drug-resistant high-risk groups and BM relapse. Moreover, cortactin^{hi}-ALL progenitors showed low proliferation rates and were capable of colonizing hypoxic hematopoietic niches suggesting that cortactin is a key element in the positioning of primitive cells within specialized niches. *In vivo* assays, using immunodeficient mice, showed that leukemic cells that infiltrated to extramedullary sites express higher cortactin levels. These findings emphasize the importance of cortactin for bone marrow relapse in B-ALL and extramedullary infiltration. Thus, its detection could be useful as a diagnostic tool to identify high-risk patients and optimal treatment strategies.

1. RESUMEN

El cáncer es la principal causa de muerte en la población infantil a nivel mundial, siendo las leucemias agudas las de mayor frecuencia. Los casos de recaídas, la resistencia al tratamiento y la infiltración a órganos agravan el pronóstico, por lo que es necesario garantizar un mejor entendimiento de los mecanismos implicados.

Cortactina y HS1 son moléculas de unión a actina involucradas en la adhesión celular y migración participando en la regulación de la remodelación del citoesqueleto de actina. La alta expresión de cortactina ésta asociada con la invasividad de células cancerígenas en varios tumores sólidos, recientemente también se ha correlacionado con el mal pronóstico en adultos con leucemia linfocítica crónica de células B. En éste trabajo, hemos investigado el perfil de expresión de cortactina en la población de precursores de células B y el potencial impacto sobre la infiltración y la progresión de la enfermedad. Encontramos altos niveles de cortactina en las líneas celulares de leucemia linfoblástica aguda RS4:11 y REH, así como en los precursores linfoides de médula ósea y líquido cefalorraquídeo de pacientes con LLA-B en comparación con su contraparte normal, detectando la más alta expresión de la proteína en los precursores pro-B y Pre B de células primarias, además que estas células expresaron únicamente la variante SV2 de cortactina de 60 kDa.

Los ensayos de transmigración revelaron que la expresión de cortactina fue significativamente alta en células que transmigraron en respuesta a CXCL12, cuando las comparamos con las células que no transmigraron, sugiriendo que cortactina podría participar en la infiltración de órganos extramedulares. Los datos clínicos mostraron que los niveles más altos de cortactina se relacionaron con la clasificación clínica de grupos de alto riesgo por resistencia a la quimioterapia y por recaída a médula ósea. Por otro lado, los progenitores en LLA cortactina^{hi} mostraron rangos de baja proliferación y fueron capaces de colonizar nichos hipóxicos hematopoyéticos, sugiriendo que cortactina es un elemento clave en el posicionamiento de células primitivas dentro de nichos especializados. Ensayos *in vivo* en ratones inmunodeficientes, mostraron que las células leucémicas que infiltraron en sitios extramedulares muestran la mayor expresión de cortactina. Estos hallazgos enfatizan la importancia de cortactina en la recaída a médula ósea en LLA-B, e infiltración extramedular por lo que su utilidad podría estar implicada como herramienta de diagnóstico para identificar pacientes de riesgo alto y proponer estrategias optimas de tratamiento.

INTRODUCTION

Childhood cancer is considered a global health priority (Gupta, Sutradhar, Guttmann, Sung, & Pole, 2014; Magrath et al., 2013; Steliarova-foucher et al., 2017). Even though more efficient therapeutic agents have been developed that increased the overall survival rates over the last decades, leukemia remains a leading cause of morbidity and mortality in children worldwide, with incidence rates of 140.6 per million new cases per year in children of 0–14 years (Steliarova-foucher et al., 2017). Among leukemias, B-precursor cell acute lymphoblastic leukemia (B-ALL) represents 73 to 85 % of total cases (Pérez-Saldivar et al., 2011). Current efficient therapeutic agents have increased overall survival rates up to 80%. However, disease relapse with organ infiltration correlates with poor prognosis due to treatment failure and remains a main obstacle for curing the disease (Bhojwani & Pui, 2013). Thus, more accurate diagnosis tools to identify high-risk groups for diseases relapse are warranted. Although relapse in B-ALL is most common and clinically more complicated in bone marrow (BM), extramedullary sites such as central nervous system (CNS) or testis can also be compromised by infiltration of leukemic cells (Bhojwani & Pui, 2013; Gómez et al., 2015; Malempati, Gaynon, Sather, La, & Stork, 2007). The precise environmental cues and molecular mechanisms driving both ALL relapse and CNS infiltration during relapse remain elusive. However, factors that promote cell adhesion, transendothelial migration (TEM) and homing could be essential for microenvironment-related infiltration and relapse (Gossai & Gordon, 2017; Infante & Ridley, 2013). Moreover, the participation of actin binding proteins involved in adhesion, such as cortactin and HS1, has been recently reported in several cancers as factors triggering cell migration and metastasis formation that are related to poor prognosis (Hill et al., 2006; Y. N. Kim et al., 2012; MacGrath & Koleske, 2012).

2.1 BACKGROUND

By virtue of their tightly regulated multi-lineage differentiation potentials, hematopoietic stem/progenitor cells generate the whole blood system throughout the postnatal life, driven to a large extent by lineage-specific transcription factors. In the setting of malignant hematological disorders including acute lymphoblastic leukemias (ALL), a number of intrinsic and extrinsic cues influence the hematopoietic differentiation pathway and cooperate to make aberrant cell fate decisions concomitant to cell transformation (Vilchis et al., Springer 2016). The cellular origin and dynamics of these

disorders is a fundamental matter in question. In keeping with the hierarchical model of tumor evolution, a conspicuous and unique long-term leukemic stem/initiating cell (LSC/LIC) population is most likely the foundation of acute leukemias. Moreover, B-cell differentiation stages in acute lymphoblastic leukemia functioning as leukemia-initiating cells (LIC) are apparently endowed with some primitive stem cell properties, and might be responsible for long-term maintenance of tumor growth within bone marrow (BM) and for disease relapse following remission. Furthermore, LIC reveal the ability to create irregular BM microenvironments that may result in pro-inflammatory scenarios with permissive roles by allowing leukemic cell development at the expense of normal hematopoiesis. By unraveling the identity and location of relapse clones, we may learn about the pathobiology of primitive cancer initiating cells, responsible for disease progression, minimal residual disease (MRD), extramedullary infiltration and relapse, in the context of abnormal BM microenvironments.

2.2 Biology of acute lymphoblastic leukemia

The uncontrolled production of lymphoid hematopoietic precursors within BM is a major feature of ALL (Dorantes-Acosta & Pelayo, 2012). This pathology is the most common cause of childhood cancers worldwide and accounts for 23% of malignancies and for 85% of the leukemia cases, whereas acute myeloid leukemia (AML) constitutes 15% of them (Pérez-Saldivar et al., 2011; Xie, Davies, Xiang, Robison, & Ross, 2003). Of note, a slight but gradual increase in the incidence of ALL appears to be highest in Latin America (McNeil, Coté, Clegg, & Mauer, 2002; Pérez-Saldivar et al., 2011; Steliarova-foucher et al., 2017; Xu et al., 2013), where also superior rates of high risk patients are apparent.

According to lineage and maturation stages of leukemic cells, 80 to 85% of ALL cases have a B-cell immunophenotype, whereas only 15% show a T-cell immunophenotype. Congenital leukemia represents only 3% of leukemic cases, and mixed lineage leukemias only 2%. Strikingly, disease-free survival is improved by a proper management based on stratification by risk groups and identification of relapse factors (Izraeli, 2010; Juarez-Velazquez et al., 2014). Currently, the most useful prognostic indicators are age, white blood cell count, immunophenotype, minimal residual disease detection and therapy responses. The molecular mechanisms driving relapse in any of the leukemia entities are still poorly understood. In addition, cell infiltration remains an obstacle for curing ALL patients. The central nervous system (CNS) is the most

frequently affected extramedullary site (30-40%), and a number of apparent risk factors related to CNS relapse include the cellular immunophenotype, high-risk cytogenetic abnormalities, hyperleukocytosis and leukemic cells present in CNS or in traumatic lumbar puncture at diagnosis (Xu et al., 2013). Most parameters considered for high-risk identification remain insufficient for establishing an early stratification, highlighting the complexity of the disease and the need of new biomarkers or cellular profiles to better predict outcomes. The combination of genomics and clonal studies with xenotransplantation approaches has revealed unsuspected genetic diversity supporting multiclonal co-evolution in the context of tumor microenvironments (Notta et al., 2011; Purizaca, Meza, & Pelayo, 2012). Recurring chromosome abnormalities are detected in 80 percent of ALL cases, with aneuploidy, chromosome translocations, inversions, or deletions being often associated with risk of relapse (Greaves & Wiemels, 2003; Pui & Robison, 2008). ETV/RUNX1 and BCR/ABL1 fusions are universally related to good or bad prognosis, respectively (Lilljebjörn et al., 2012; Zuna et al., 2011), while the mixed lineage leukemia (MLL) gene is involved in more than 50 fusions mostly connected to cell transformation and adverse outcome, largely due to cellular drug resistance (Meijerink, den Boer, & Pieters, 2009). Remarkably, genetic alterations of master regulators of the early hematopoietic differentiation, including Ikaros (IKZF1), and downstream lymphoid development pathway PAX5, EBF1, E2A, CRLF2 and LEF1 are hallmarks of ALL (Mullighan & Willman, 2011) (McManus et al., 2011, Kawamata et al., 2012). Moreover, crucial growth factor receptors, like FMS-like tyrosine kinase-3 (FLT3) and platelet-derived growth factor receptor (PDGFR) (Gu et al., 2011; Takahashi, 2011) are disregulated. A number of aberrations are apparent from the earliest stages of the hematopoietic differentiation program underlining the relevance of primitive cells in the ALL pathobiology.

2.3 The hematopoietic microenvironment as regulator of normal and leukemic cells

Lineage fate decisions are known to result from the continuing intercommunication between developing stem/progenitor cells and the surrounding microenvironment, constituted by a complex network of mesenchymal cells, osteoblasts, endothelial cells, fibroblasts, adipocytes, innate and adaptive immune cells, and their products including extracellular matrix, cytokines, chemokines and growth factors that form niches (Dick, 2008; Dorantes-Acosta & Pelayo, 2012; Purizaca et al., 2012), Vilchis Springer 2016).

Such specialized structures participate in stem cell maintenance, self-renewal, adhesion, expansion, differentiation, and mobility. Disruption of crucial microenvironmental signals can lead to stem cell depletion, altered hematopoiesis and malignancy (Guerrouahen, Al-Hijji, & Tabrizi, 2011; Hoffman & Calvi, 2014). Because of the biological similarities between LIC and HSC, it has been proposed that malignant precursor cells accordingly need specialized niches to survive and progress (Scadden, 2014). A comprehensive model for stem/progenitor cell population dynamics in the context of a regulating (inductive, repressive or permissive) microenvironment is not yet available, but recent advances in the development of tridimensional culture systems may favor normal and leukemic stem cell research, as well as the identification of therapeutic targets and drug candidates (Balandrán et al., 2017, Balandrán Porrúa 2017).

At least three distinct niches within the BM architecture are dedicated to support hematopoiesis in mammals (Figure 1): the endosteal niche, mainly composed of osteoblasts lining the bone surface, the vascular niche, formed by endothelial cells, and the reticular niche, where the chemokine/chemokine receptor CXCL12/CXCR4 axis has a pivotal role in the regulation of early lymphopoiesis (Purizaca et al., 2012) (Vilchis Springer 2016).

The endosteal niche is the major hypoxia zone in BM, formed by osteoblasts that regulate HSCs by a variety of mechanisms, including the production of granulocyte colony stimulating factor (G-CSF), Notch, Wnt, CXCR4 (or CD184) ligand CXCL12, osteopontin, N-cadherin, angiopoietin-1 (Ang-1), Tie-2 ligand, Thrombopoietin (THPO) and stem cell factor (SCF), the ligand of c-Kit on HSC (Lee, Decker, Lee, & Ding, 2017; Purizaca et al., 2012). The vascular niche is a network of blood vessels and sinusoids formed by endothelial cells that constitutes the structure for HSC and precursor cells to get out of or get into the BM (Suárez-Álvarez, López-Vázquez, & López-Larrea, 2012). These cells express adhesion molecules such as E-selectin, P-selectin, VCAM-1, ICAM-1, PECAM-1 and VE-cadherin that interact with ligands such as ESL-1, PSGL-1, VLA-4 and LFA-1, respectively, on hematopoietic cells. Together, they maintain HSC and HPC attached to the niche and regulate their self-renewal, homing and mobility. Indeed, vascular location of HSC and HPC allows their quick release, an important event in leukemia (Smith & Calvi, 2013; Suárez-Álvarez et al., 2012). Some studies have demonstrated that pre-B ALL cells preferentially reside in association with microvascular domains, where leukemic cells decrease CXCL12

domains to displace normal stem and progenitors cells (Colmone & Sipkins, 2008; Doan & Chute, 2012; Sipkins et al., 2005). The vascular niche also plays a critical role during stress after irradiation or myeloablation (Lee et al., 2017). Finally, the reticular niche is composed of CAR (CXCL12-abundant reticular) and nestin^{+} cells. In this niche, primitive hematopoietic cells, especially B-lymphoid precursors, interact with reticular cells through their membrane proteins VLA-4, IL-7R, Tie-2, c-Kit and CXCR4 and their corresponding ligands on reticular cells VCAM-1, IL-7, Ang-1, SCF and CXCL12, respectively, as well as collagen, fibronectin, laminin and proteoglycans of the ECM (Purizaca et al., 2012).

Based on experimental data, the following mechanisms have been proposed to function as 'cooperating' microenvironment-related factors in leukemogenesis: a competition of tumor cells for normal HSC niches, the manipulation of normal environments led by tumor cells, and disruption of HSC-niche communication. Any of these scenarios would favor tumor progression at the expense of homeostatic hematopoietic differentiation (Raaijmakers, 2011; Vilchis-Ordoñez et al., 2015). Increasing evidence indicates the prevalence of functional defects in both, soluble and cellular microenvironmental elements that may escort oncogenesis. However, it is still unclear whether ALL microenvironmental abnormalities appear as a consequence of leukemic activity or they constitute intrinsic pre-leukemic lesions. Seminal work by the group of Dr. Sipkins has demonstrated that tumor cells create aberrant microenvironments by disrupting HSC niches (Colmone et al., 2008). Recent findings underline the active role of leukemic cells and their products in the modulation of the BM microenvironment (Conforti et al., 2013; Vicente López et al., 2014; Vilchis-Ordoñez et al., 2015)

2.4 Bone marrow hypoxia and the HSC fates

The hypoxic condition of the BM has a major impact on HSC homing and biological functions, providing an adequate microenvironment to either, positively select seminal quiescent long-term hematopoieic stem cells (LT-HSC) or to induce/maintain quiescence and long term reconstitution abilities in the most primitive HSC subset, whereas an activated or inflammatory condition in which high reactive oxygen species (ROS) prevail, HSC contents diminish as well as their self-renewal potential (Jang & Sharkis, 2007; Lee et al., 2017). Accordingly, the notion that LICs or LSC reside in normal hypoxic HSC niches is gaining high interest and is explored in a number of

leukemia models indicating that hypoxia promotes secretion of crucial factors to maintain the HSC pool, including CXCL12, vascular endothelial growth factor (VEGF) and IL-6 (Deynoux, Sunter, Hérault, & Mazurier, 2016).

The major regulator of hypoxic conditions and CXCL12 production in BM is the transcription factor HIF-1α (hypoxia-inducible factor 1α), whose activation is related to poor prognosis in leukemia due to its participation in angiogenesis, leukemic metabolism, blast proliferation, homing, mobilization, cell motility and LSC self-renewal (Deynoux et al., 2016; Schito, Rey, & Konopleva, 2017). HIF-1α regulates CXCL12 and CXCR4 expression on hematopoietic cells in the context of hypoxic microenvironments and may play a role in cell trafficking. Inhibition of HIF-1α in CLL has effects on cell homing and retention, and diminishes CXCR4, VLA-4 and CXCL12 levels, thus impairing *in vitro* cell migration and bone marrow colonization (Schito et al., 2017; Valsecchi et al., 2016). By contrast, a substantial CXCL12 decrease in ALL BM stromal cells seems to contribute to leukemic progression in the late stages of B-cell development, a mechanism that relates to their HIF1α and connexin 43 (Cx-43) expression levels suggesting important implications of CXCL12-dependent niches in homing and retention of malignant cells (Balandrán et al., 2017).

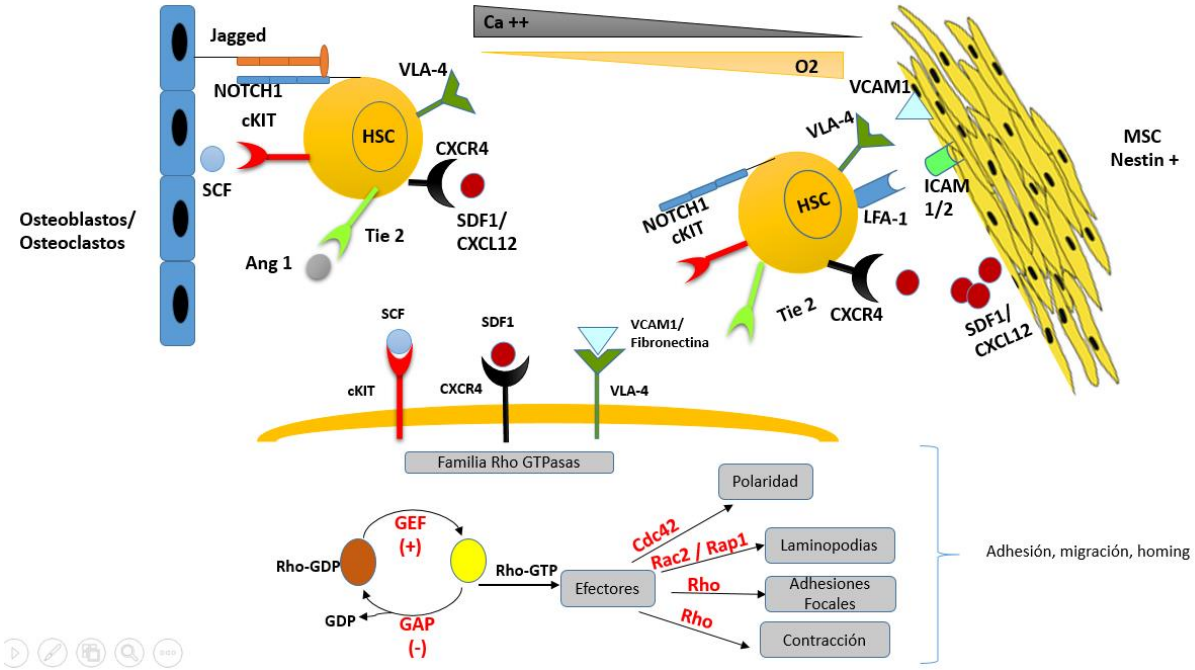

Figure 1. Biology of hematopoietic cells binding to bone marrow niches. General representation of the regulation and retention of hematopoietic progenitor cells (HPC) in the bone marrow microenviroment by molecules controlling adhesion, homing and migration within the different niches.

2.5 Leukemic Relapse

Relapse is the major challenge in leukemia. Although most ALL cases respond positively to chemotherapy, there is a minor population (near 20%) that develops drug resistance or does relapse with poor prognosis. A number of current investigations are focused to reveal whether distinct relapse sub-clones pre-exist before treatment, if secondary mutations are acquired as a consequence of therapy, or if relapse is a re-appearance of dormant cells which were unaffected by chemotherapy and may reside and survive in protective niches for long periods of time (Clevers, 2011; Ebinger et al., 2016; Kunz et al., 2015). Some evidences indicate that unique cells responsible for relapse are present since the initiation stage of the disease, as part of a minor subpopulation that expands during treatment (Mullighan et al., 2008) Relapse leukemia cells might then originate from a pool of LIC sharing characteristics with normal HSC and with dormant characteristics and the ability of creating a leukemic niche to escape from treatment that target cycling cells (Chiarini et al., 2016).

BM is the most common site of relapse, and leukemic cells should respond to critical signals from the surrounding microenvironment to promote quiescence, survival and treatment resistance. It has been identified that the ALL subpopulations pro-B and pre-B have distinct anatomical locations buried beneath mesenchymal cells, which could be the most quiescent and resistant area (Moses et al., 2016). Quiescence and proliferation outcomes are mediated by intercommunication between BM stromal cells and LIC through adhesion molecules. In T-ALL, the interaction of LIC with stromal cells by LFA-1/ICAM-1 is a necessary event, although not sufficient, to sustain T-ALL survival, while the axis VLA-4 integrin/VCAM-1 retains leukemic cells within the BM. High expression of VLA-4 has been detected in B-ALL relapse patients and associated with poor outcome (Shalapour et al., 2011).

Although BM relapse in B-ALL is most common and clinically more complicated, extramedullary tissues such as the CNS or testis can also be compromised by infiltration of leukemic cells (Bhojwani & Pui, 2013; Gómez et al., 2015; Malempati et al., 2007). The precise environmental cues and molecular mechanisms driving both ALL relapse and CNS infiltration during relapse remain elusive. However, a migrating primitive stage of differentiation of the infiltrating cells and factors that promote cell adhesion, transendothelial migration (TEM) and homing have been considered (Gossai & Gordon, 2017; Infante & Ridley, 2013).

Accordingly, the hypothesis that metastases originate from a primitive surviving cell has been experimentally proven for some cancer types. However, hematological tumors may also show homing to microdomains within BM and to extramedullary compartments resulting in residual disease and consequently relapse episodes. Normal HSC are known to be constantly trafficking within BM and between BM and peripheral tissues via the blood-lymph-thoracic duct-blood system (Massberg et al., 2007; Merchand-Reyes, Pelayo, Pavón, Pestell, & Velasco-Velázquez, 2014) , Mazo et al., 2011 Vilchis Springer 2016), implying the active participation of chemokines and adhesion molecules. Again, while intrinsic properties of malignant cells contribute to formation of abnormal niches (Colmone & Sipkins, 2008; Raaijmakers, 2011), LSC maintenance might result from microenvironmental factors, CXCL12/CXCR4 or integrins, that normally support self-renewal of healthy HSC (Purizaca et al., 2012; Sipkins et al., 2005), suggesting a tight connection between CXCR4, LSC and infiltration. Recent investigations show that CCR1, CCR2 and CCR5 play a role in NFkB-activated LSC cell retention and that IL1a and some genes targeted by NFkB such as VCAM1, ICAM1, CyclinD1 and Myc are upregulated (Bigildeev, Shipounova, Svinareva, & Drize, 2011; Guzman et al., 2001). How leukemic cells disrupt the blood-brain-barrier (BBB) and enter the central nervous system is not well understood, but disruption of transmembrane molecules like claudin-5 and occludin by matrix metalloprotease-2 (MMP-2) and MMP-9 has been identified as key for loss of BBB integrity (Feng et al., 2011).

For any of these phenomena, cell movement is controlled by the actin cytoskeleton via formation of protrusive structures such as lamellipodia and invadopodia and activation and clustering of adhesion molecules such as integrins to allow interaction with its corresponding ligands on stromal cells in the BM microenvironment or with vascular endothelial cells of extramedullary sites.

2.6 Mechanisms of homing and transendothelial migration of HSC and HPC

Homing is a process by which cells that exited the BM, return through sinusoids by a process termed transendothelial migration (TEM). Although mature hematopoietic cells are physiologically mobilized from bone marrow to the periphery, immature cells are also found in the circulation at low frequencies and it is not clear why they escape from the BM, although it has been proposed that it could happen to maintain homeostasis within BM (Heazlewood, Oteiza, Cao, & Nilsson, 2014).

The mechanism of homing of circulating hematopoietic cells, including HSPCs, is poorly understood, but it has been proposed that TEM occurs in a similar fashion to that of other leukocytes that transmigrate to sites of inflammation. Homing follows mainly three adhesive steps: Tethering and rolling, firm adhesion and transmigration across the endothelial barrier. (Heazlewood et al., 2014). However, although crawling after firm adhesion is a critical step during extravasation of normal leukocytes, it has not been described in homing to BM.

BM vessels express P- and E-selectin and vascular adhesion molecule-1 (VCAM-1) that are constitutively expressed without an inflammatory stimulus unlike in other endothelial barriers outside BM. These molecules allow the thethering and rolling of HSPCs via the selectin ligand PSGL-1 (P-selectin glycoprotein ligand). Additionally, selectins interact with glycosylated CD44 that is another molecule expressed on HSPCs that participates in rolling (Figure 2). Next, low affinity LFA-1 (αLβ2) and VLA-4 (α4β1 integrin) present on HSPC are activated via a signal cascade induced by CXCL12 binding to its receptor CXCR4. The resulting conformational change in the integrins enables them to interact with their endothelial ligands ICAM-1 and VCAM-1, respectively, leading to firm adhesion. In contrast to other settings, in the BM, the chemokine CXCL12 is the principal chemoattract for hematopoietic cells, which promotes BM homing and retention mainly via its G-protein coupled receptor (GPCR) CXCR4 on HSPCs (Ding & Morrison, 2013; Mazo, Massberg, & von Andrian, 2011).

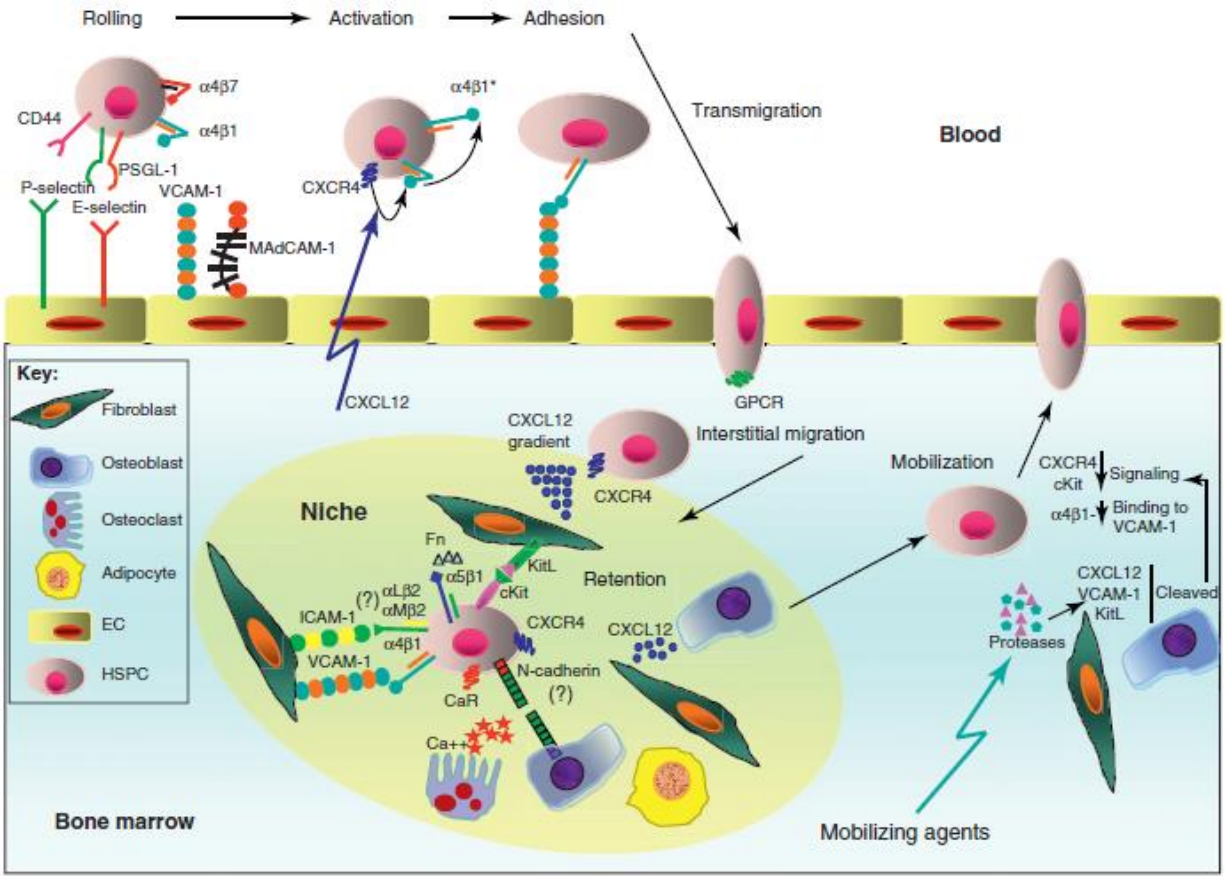

Figure 2. HSPC homing to the BM. Tethering and rolling on bone marrow endothelial cells is mediated by interaction of E- and P- selectins with their ligands on HSPCs, CD44 and PSGL-1. Activation of LFA-1, VLA-4 and CXCR4 on hematopoietic cells in response to chemokine sensing leads to interaction with ICAM-1, VCAM-1 and CXCL12, respectively, to promote firm adhesion and subsequent trans-endothelial migration. Molecules involved in niche retention of HPSCs within the BM are also represented. (Massberg & von Andrian, 2011)

During transedothelial migration of leukocytes, the binding of chemokines to GPCR induces downstream signaling including PLC activation, increase in intracellular Ca^{2+} levels, activation of the small GTPases Rap1 to eventually enhance integrin affinity via recruitment of the adaptor proteins kindling-3 and talin-1 that interact with the β-subunit of integrins (Nitzsche et al., 2017). Actin polymerization promotes lamellopodia formation, necessary to extend the contact with endothelial cells, crawling on the endothelial surface and transmigratory cup formation to guide leukocytes to the spot of transmigration (Figure 3).

Leukocyte crawling toward chemotactic gradients is dependent of Mac-1 activation on leukocyte that interacts with ICAM-1 on endothelial cells; and although this step has not been described yet for HPSC, it does not mean that it does not occur (Schnoor, Alcaide, Voisin, & Van Buul, 2015). A docking structure termed transmigratory cup is formed to surround adherent leukocytes that is enriched in LFA-1/ICAM-1 and VLA-

4/VCAM-1 clusters (Figure 3), that are formed via recruitment of adaptors molecules such as cortactin, and GTPases such as Rac1 and RhoG leading to actin remodeling driving protrusion formation that build the transmigratory cups (Schnoor et al., 2015). Additionally cortactin is necessary for regulating vascular permeability through Rap1 because endothelial cells deficient for cortactin have decrease Rap1 levels and show increased permeability (Schnoor et al., 2011). These data highlight the importance of cortactin for both endothelial barrier integrity and leukocyte recruitment.

After ICAM-1 and VCAM-1 clustering on endothelial surface, VE-cadherin, the major component of endothelial adherens junctions is internalized to pave the way for transmigrating leukocytes. By contrast, other junctional proteins such as JAMs (junctional adhesion molecules), PECAM-1 or CD99 serve as counterreceptors for transmigrating leukocytes thus facilitating their passage through endothelial junctions, a process termed paracellular migration (Muller, 2016). Transcellular migration across a single endothelial body also occurs but at a low frequency (<10%). Finally, leukocytes breach the endothelial basal membrane by proteolytic degradation of its components laminins and collagen type IV (Nitzsche et al., 2017).

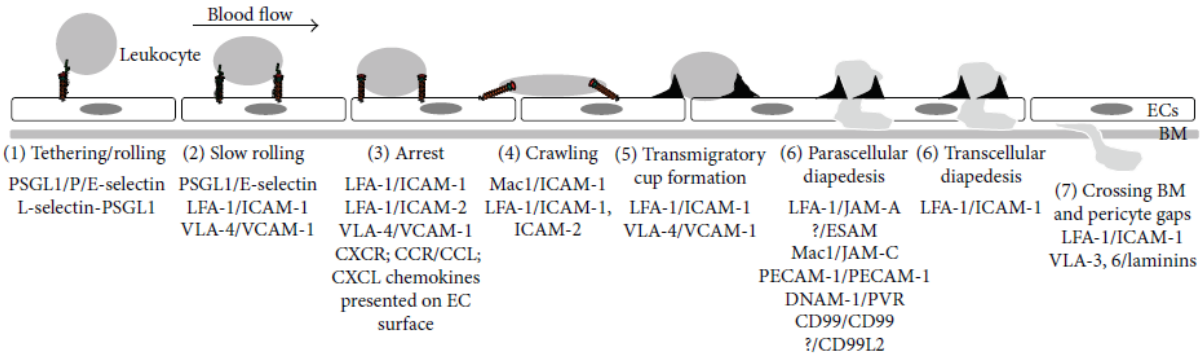

Figure 3. General scheme of transendothelial migration of leukocytes. Principal steps of transendothelial migration are representated and the receptor-ligand pair involve are listed below each step. Actin cytoskeleton remodeling in both, leukocyte and endothelial cells is required to complete TEM (Schnoor et al., 2015).

Once completed TEM, HSPCs inside the BM are retained mainly by VLA-4 interaction with VCAM-1 on stromal cells via the CXCR4/CXCL12 axis. By contrast, the β2 integrins LFA-1 and Mac-1 do not seem to participate in retention. Additional molecules that participate in retention are c-kit that induces activation of β1 integrins and also binds to its ligand stromal cell factor (SCF); N-cadherin which is present on quiescent HSPCs and osteoblasts, as well as Ca^{2+} influx sensed by calcium receptors (CaR) on hematopoietic cells (Mazo et al., 2011) (Figure 2).

In general, leukocytes cross the endothelium in low numbers into lymph nodes and tissues during immune surveillance under normal conditions. Under inflammatory conditions, the rapid activation of adhesion receptors allows efficient recruitment of leukocytes. Leukemic cells also need to perform TEM to infiltrate the BM or extramedullary sites (Infante & Ridley, 2013), and it is tempting to speculate that inflammatory stimuli trigger this process.

2.7 Infiltrating leukemias

Leukemic cells have a preference for bone marrow or in some cases for certain organs, such as CNS, and although the mechanisms of extramedullary dissemination of leukemic cells is not completely understood, they include the same factors that drive TEM of normal leukocytes (Gossai & Gordon, 2017).

The hierarchial theory of cancer development sustains that cancer stem cells (CSC) support the initiation, maintenance, and clinical outcome of tumors, and may also constitute the subpopulation of tumor cells responsible for local invasion and the development of metastatic tumors (Kakarala & Wicha, 2008). Indirect support of the participation of CSC in metastasis comes from studying hematopoietic stem cell (HSC) migration demonstrating a constant exchange of migratory primitive cells within the bone marrow (BM) and between BM and peripheral tissues under physiological conditions. They travel from extramedullary tissues into the lymph, and from the lymph via the thoracic duct back into the blood (Massberg et al., 2007; Mazo et al., 2011; Welner & Kincade, 2007). While adhesion molecules such as N-cadherin, integrins, and CD44 are involved in cell retention within BM, the chemokine CXCL12 and its receptor CXCR4 are crucial for BM colonization and migration to periphery. On the other hand, exiting into peripheral organs via lymphatics is dependent on gradients of the sphingolipid sphingosine-1-phosphate (S1P) (Massberg et al., 2007). Many of these factors known to govern HSC migration also play an important role in infiltrative leukemias that display not only homing to the BM but also infiltration into extramedullary tissues. When leukemic cells acquire the capacity to invade extramedullary tissues, they form tumor masses referred to as chloromas. Interestingly, some myeloid leukemias are capable to disseminate into many organs, but they do not infiltrate well the central nervous system (CNS), whereas ALL cells often infiltrate the CNS and the testis. In ALL models, PECAM-1 expression enhances transendothelial migration capacity (Gossai & Gordon, 2017). Myeloid leukemia

initiating cells produce VEGF and its receptors VEGFR-1 and VEGFR-2 and disruption of VEGFR-2 signaling is known to inhibit tumor growth and metastasis in a murine model of human promyelocytic leukemia. Thus, an autocrine loop generated by VEGF/VEGFR-2 may constitute a crucial axis participating in leukemic invasion. Additionally, invasion of the blood-brain-barrier by leukemic cells requires high expression of MMP-2 and MMP-9 to disrupt the intercellular junctions between adjacent brain microvascular endothelial cells (Feng et al., 2011)

2.8 CXCR4/CXCL12 axis in the migration and retention of hematopoietic cells

The chemokine CXCL12 is the principal chemoattractant for hematopoietic cells that promotes BM homing and retention including hematopoietic stem, progenitors and precursor cells, in normal or malignant conditions and its disruption promotes their mobilization to peripheral blood. CXCL12 is expressed mainly by perivascular stromal cells, and at lower levels by endothelial cells, osteoblasts and some hematopoietic cells of the BM. Deletion of CXCL12 from perivascular cells depletes HSC and restricts mobilization of progenitors into the circulation (Ding & Morrison, 2013). The importance of the CXCL12/CXCR4 axis in hematopoietic cells for normal hematopoiesis is evident because CXCR4 or CXCL12 knock out mice are lethal due to impaired bone marrow granulopoiesis and lack of B lymphopoiesis (Juarez et al., 2009; Nagasawa et al., 1996). Indeed, quimeric studies have demonstrated that $CXCR4^{-/-}$ HSPCs display defects not only in retention within the BM but also in adult hematopoiesis and mantainance of quiescence of the HSC pool (Ratajczak, Kim, Janowska-Wieczorek, & Ratajczak, 2012; Sugiyama, Kohara, Noda, & Nagasawa, 2006). CXCR4 signaling controls B lineage cell motility within the BM parenchyma that is highly perfused by the blood flow, and depends on interactions of α4β1 integrin with VCAM-1. CXCR4 deficiency promotes the exit from BM to the periphery, and CXCR4 downregulation in immature B cells promotes cell depolarization and reduces its amoeboid shape to reduce cell movement in peri-sinusoidal niches and facilitate export into sinusoids (Beck, Gomes, Cyster, & Pereira, 2014).

CXCL12 contributes to leukemic infiltration to bone marrow, and spreading to extramedullary organs, such as liver, spleen, lymph nodes and central nervous system related with a high expression of CXCR4 by ALL cells.

After CXCL12 stimulus, in normal and leukemic cells, CXCR4 is internalized and promotes an increase of the cytoplasmic calcium store via PLC activation and

formation of diacylglycerol (DAG) and phosphatidylinositol 3 (IP3). Then, the small GTPase Rac 1 is activeated and co-localizes with CXCR4 within membrane lipid rafts that is necessary for cell migration (De Lourdes Perim, Amarante, Guembarovski, De Oliveira, & Watanabe, 2015). CXCR4 activation in response to CXCL12 also promotes STAT 3 activation via phosphorylation by JAK2, thus activating downstream pathways such as MAPK and PI3K-Akt, and PKC, leading to activation of VLA-4 via phosphorylation of paxilin, focal adhesion kinase (FAK), proline-rich kinase-2 (Pyk-2), p130Cas, Crk, and Nck and subsequent adhesion (De Lourdes Perim et al., 2015). Of note, in fibroblasts and T-ALL cells internalization of CXCR4 in response to CXCL12 is mediated by the acting binding protein cortactin that co-localizes with the receptor at the plasma membrane and in early endosomes (Luo et al., 2006; Passaro et al., 2015). This finding further suggests the importance of cortactin of CXCR4/CXCR12-controlled B-ALL cell biology.

2.9 Cortactin, an actin-binding protein involved in cell adhesion, migration and leukemia

Cell movement, adhesion and transmigration are processes that require dynamic assembly and disassembly of the actin filament network in response to a chemoattractant gradient. Actin filaments can be assembled, elongated or disassembled, thus regulating protrusion formation and cell contraction. Actin dynamics are in large part orchestrated by actin-binding proteins (ABP) (García-Ponce, Citalán-Madrid, Velázquez-Avila, Vargas-Robles, & Schnoor, 2015; Kirkbride, Sung, Sinha, & Weaver, 2011).

Cortactin is an ABP classified as type II nucleation promoting factor (NPF II) that plays important roles in cell polarity, adhesion and migration by controlling actin cytoskeleton remodeling, activation levels of GTPases, and clustering of adhesion molecules such as ICAM-1 on endothelial cells. (García-Ponce, et al., 2015; Schnoor et al., 2011).

Cortactin is upregulated in several solids cancers and triggers cell migration and metastasis formation, and very recently, cortactin has been demonstrated to be expressed in B-cells of adult B-CLL patients where it was associated with the expression of the known adult B-CLL risk factors ZAP70 and CD38 (Gattazzo et al., 2014).

Cortactin has a homologue in hematopoietic cells, HS1 (hematopoietic cell-specific lyn substrate) that is widely studied in hematopoietic cells and has been reported to play

an important role in the progression of adult B-cell chronic lymphoid leukemia (B-CLL) where it correlates with poor prognosis (Frezzato et al., 2012; Scielzo et al., 2005, 2010). Cortactin shares the same structural domain with HS1, containing a N-terminal acidic domain that interacts directly with the Arp2/3 complex, followed by six and a half actin-binding tandem repeat regions (three and a half actin-binding tandem repeat regions in the case of HS1), a helical region of unknown function, a proline-rich domain with several phosphorylation sites, and finally, a C-terminus src-homology-3 (SH3) domain through which cortactin and HS1 are able to bind to a multitude of other proteins (Martinez-Quiles, 2003) (Figure 4).

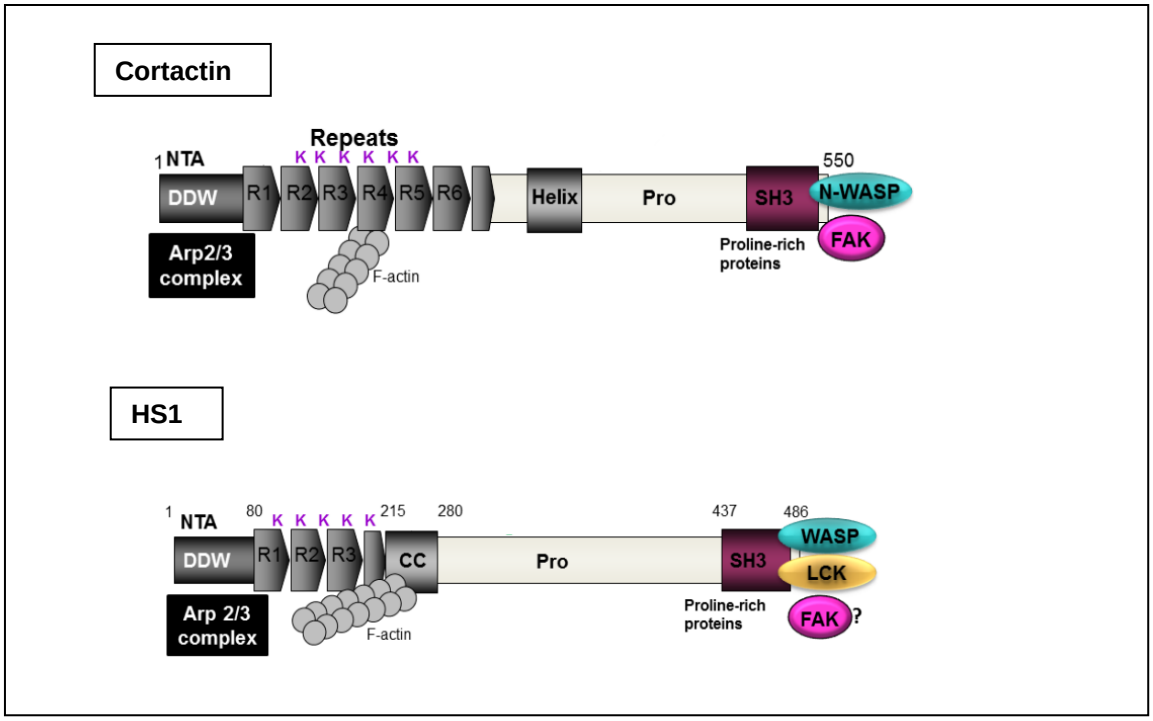

Figure 4. Cortactin and HS1 structure. Domain organization and principal molecules that interact with them (Modified from Martinez-Quiles, OA Immunology, 2013).

Interestingly, Rossum and colleagues reported two different alternative splicing variants of cortactin that differ from WT cortactin in the number of actin-binding repeats (van Rossum et al., 2003). These variants are termed SV1 and SV2, with SV1 containing the actin-binding repeats 1 to 5, and SV2 containing only the repeats 1 to 4, generating proteins of 70 kDa, and 60 kDa, respectively, while the WT cortactin protein has a molecular weight of 80 kDa. All three cortactin variants are able to activate the Arp 2/3 complex in the same way, but *in vitro* they bind F-actin with a different affinity depending on the number of repeats, with WT cortactin having the highest F-actin affinity, and SV2 the least. All splice variants contain repeat 4 that has

been demonstrated to be critical for F-actin binding (van Rossum et al., 2003; Weed et al., 2000).

Cortactin interacts with the Arp2/3 complex, another ABP classified as principal NPF that mediates the assembly of new branched actin filaments on existing filaments predominantly at cortical cell areas. This interaction contributes to Arp2/3 activity and stabilization of actin branches by preventing debranching/depolymerization of the actin network (Helgeson & Nolen, 2013). Cortactin shows a subcellular localization at sites of dynamic actin assembly contributing to the formation of actin-rich motility protrusions such as lamellipodia and invadopodia. Although cortactin is a weak activator of the Arp2/3 complex, mutations in its N-terminal domain prevent assembly of actin in invadopodia, vesicle trafficking, and lamellipodial protrusion required for migration suggesting that cortactin contributes to Arp2/3 complex recruitment to actin filaments and actin branching (Helgeson & Nolen, 2013; Kirkbride et al., 2011). However, this notion was challenged by a study demonstrating that lamellipodia form normally in cortactin-deficient murine embryonic fibroblasts (Lai et al, 2009). Thus, currently it is thought that cortactin is rather required to stabilize *de novo* formed actin branches.

On the other hand, phosphorylated cortactin contributes to the activation of GTPases such as Cdc42 and Rac1. For example, cortactin triggers PDGF-induced Rac1 activation, stabilizes Rac-induced lamellipodia and membrane ruffles, and controls the activity of basal cdc42 levels in cortactin-deficient murine embryonic fibroblasts, decreases (Lai et al., 2009). In endothelial cells, cortactin is essential for transendothelial migration of leukocytes by controlling RhoG-mediated ICAM-1 clustering around adherent neutrophils thus allowing for firm adhesion and extravasation (Schnoor et al., 2011). Cortactin gets phosphorylated by Src tyrosine kinases on the tyrosine residues Y421 and Y470 and these post-translational modifications are important for promoting integrin-mediated cell adhesion and spreading by regulating SH3 domain-dependent interactions with many proteins such as N-WASP and focal adhesion kinase (FAK) (Helgeson & Nolen, 2013; Kirkbride et al., 2011; Wang, Liu, & Liao, 2011). In cytokine-activated endothelial monolayers, phosphorylation of cortactin by Scr tyrosin kinases is required for ICAM-1 clustering on the endothelial surface and transmigration (Yang et al., 2006). Moreover, cortactin phosphorylation by Src and Arg tyrosine kinases on Y421 and Y482 occurs in response to epithelial growth factor (Kelley, Hayes, Ammer, Martin, & Weed, 2011),

CXCL12 and fibroblast growth factor in fibroblasts (Luo et al., 2006). These phosphorylations promote recruitment of the Nck1 adaptor protein, N-WASP and cofilin to control leading edge protrusion in invadopodia by increasing Arp2/3-mediated actin filament polymerization required for increasing the invasive capability of tumor cells (Magalhaes et al., 2011) (Figure 5). Thus, cortactin is used as invadopodia marker. Other studies have demonstrated that downregulation of cortactin decrease the number of invadopodia and actin-rich podosome rings. In several cancer types, it has been demonstrated that Abl kinases are activated downstream of Src kinase and phosphorylate cortactin in response to CXCL12 binding to its receptor CXCR4 to promote cell invasion and matrix degradation (Smith-Pearson, Greuber, Yogalingam, & Pendergast, 2010).

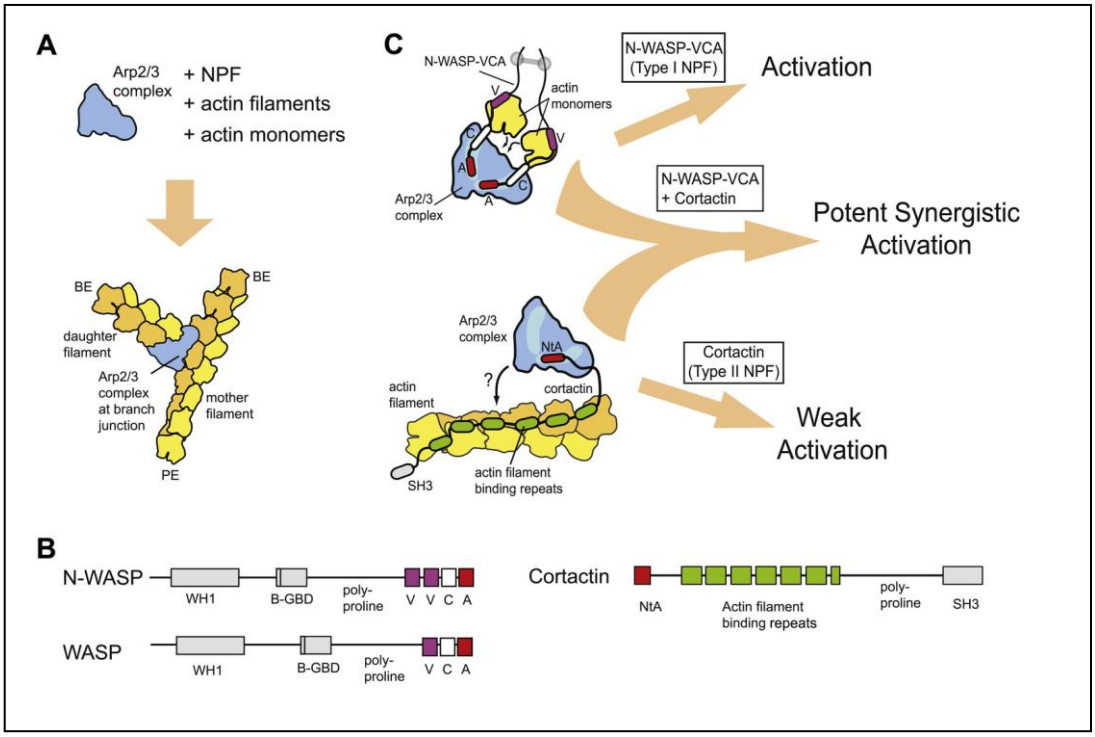

Figure 5. Actin nucleation, nucleation promoting factors (NPF) and branching. A) Branching nucleation requires the presence of the Arp2/3 complex, NPF, actin monomers and filaments to form the first Y-shaped actin branch. BE: Barbed end of actin filaments, PE: Pointed end of actin filaments. B) Structure of the type I NPFs (WASP/N-WASP) and type II NPF (cortactin). WH1: WASP Homology 1; B-GBD: Basic region and GTPase binding domain; VCA: Verprolin homology Central Acidic domains; NtA: N-terminal acidic region; SH3: Src homology 3. C) Activation of the Arp2/3 complex by NPF I or NPF II (Helson and Nolen. eLife, 2013).

Cortactin is upregulated in several cancers to trigger cell migration and metastasis formation (Hill et al., 2006; Y. N. Kim et al., 2012; MacGrath & Koleske, 2012). In head

and neck squamous cell carcinoma (HNSCC), oral squamous cell carcinoma, lung squamous cell carcinoma, gliosarcoma, breast cancer, colorectal cancer and melanoma, cortactin is overexpressed as a consequence of 11q13.3 chromosomal amplification resulting in an increase of gene copy numbers. Due to the relation of cortactin overexpression with cancer aggressive and metastasis, it is considered as a marker of invasiveness (Kirkbride et al., 2011; MacGrath & Koleske, 2012). High cortactin levels in HNSCC induce the formation of invadopodia and correlate with an aggressiveness phenotype (Clark, Whigham, Yarbrough, & Weaver, 2007). In breast cancer, Hill et al. found that interaction of CD44 with hyaluronic acid (HA) promotes the transcription of cortactin, and triggers metastasis formation in the BM by breast cancer cells (Hill et al., 2006).

Recently, overexpression of cortactin has been related to poor prognosis in adult B-cell chronic lymphoid leukemia (B-CLL), where high cortactin levels were accompanied by high levels of the known risk factors ZAP70 and CD38 (Gattazzo et al., 2014). In addition, cortactin participates in the internalization and trafficking of CXCR4, the receptor for CXCL12 (Luo et al., 2006; Passaro et al., 2015). Of note, CXCL12 is constitutively produced within BM as well as in the CNS and activates homing, adhesion and TEM of pro-B, pre-B and immature B cells mediated by integrins such as VLA-4 and LFA-1 (de Gorter et al., 2007; Spiegel et al., 2008). Indeed in T-ALL cells, Passaro and colleagues found that cortactin enhances membrane expression of CXCR4 in LIC by calcineurin, an calcium-dependent serine-threonine phosphatase that is activated after accumulation of calcium in the cytoplasm, and activatation of calmodulin to induce transcription factors involve in migration and adhesion allowing engraftment of leukemic cells in the bone marrow, which could be responsible for relapse cases (Passaro et al., 2015a; Sugiyama & Nagasawa, 2015).

A complication of leukemias is the infiltration of BM or other organs by leukemic cells which is responsible for the high mortality in relapses cases. TEM is a prerequisite for infiltration. Given the important roles of cortactin during TEM, we speculated that this protein plays an important role during B-ALL relapse and infiltration.

1. JUSTIFICATION

ALL is the most common childhood cancer disease in Mexico and worldwide. A principal problem is relapse to the BM or to extramedullary organs such as the CNS, testis, liver or lung. Infiltration of these organs represents a major complication with poor prognosis. It has been proposed that adhesion, migration and homing of LIC are implicated in disease relapse, but how these processes are driven remains elusive.

Cortactis is an ABP involved in cell adhesion and migration by regulating actin dynamics. High cortactin expression levels have been related to invasion and metastasis formation of many solid tumors and recently high cortactin levels have also been reported in leukemic B cells and related to poor prognosis of B-CLL. However, it is not known whether this is also true for ALL. Thus, differential expression of cortactin in LIC may be relevant for disease relapse and infiltration capabilities in ALL.

2. HYPOTHESIS

Cortactin promotes the transmigratory capacity of leukemic precursor B cells, extramedullary infiltration, BM colonization ability and B-ALL relapse

3. GENERAL OBJETIVE

To determine the role of cortactin for transendothelial migration of B-ALL cells and a possible correlation of cortactin expression with disease prognosis

4. PARTICULAR OBJETIVES

- To investigate expression levels of cortactin and its homologue HS1 as well as their subcellular localization in B-ALL cells

- To determine cortactin expression levels in BM-derived B cell progenitor and precursor populations from ALL patients and analyze a putative correlation with clinic parameters

- To determinate the transmigratory capacity of primary leukemic B ALL cells and pre-B leukemic cell lines

- To study the relation of cortactin expression levels with the ability of B ALL precursor cells to colonize BM

- To determinate the relation of cortactin expression levels and the extramedullary infiltration capacity

5. MATERIAL AND METHODS

7.1 MATERIAL

7.1.1 Culture medium

Medium	**Company**
RPMI 1640	Gibco, ThermoFischer. Ref 11875093
Advanced DMEM	Gibco, Thermo Fisher. Ref 12491015
Endothelial cell growth medium EGM-2	Lonza. Ref CC-2935

7.1.2 Antibodies

Antibody	**Company**
Anti-human CD34 Pacific Blue	Biolegend. Ref 343512
Anti-human CD19 Allophycocyanin	Biolegend. Ref 302212
Anti-human CD19 Phycoerytrin	Biolegend. Ref 302208
Anti-human CD184 PE-Cyanin 7	Biolegend. Ref 306514
Anti-human CD34 PE-Cyanin 5	BD. Ref 348057
Anti-human CD45 Phycoerytrin	Biolegend 304008
Anti-human HS1 D83A8	Cell signaling. Ref 3890
Anti-cortactin-Alexa488 clone 289H10	Kindly provided by Dr. Klemens Rottner, TU Braunschweig, Germany
Anti-human CXCR4	Abcam. Ref 124828
Goat anti-rabbit Alexa 488	Invitrogen, ThermoFischer. Ref A-11034
Donkey anti-rabbit Alexa 568	Invitrogen, ThermoFischer. Ref A-10042
Goat anti-mouse Alexa 488	Invitrogen, ThermoFischer. Ref A-11001
Goat anti-mouse IgG-HRP	Santa Cruz. Ref SC-2005
Goat anti-rabbit IgG-HRP	Santa Cruz. Ref SC-2004

7.1.3 Reagents

Chemical	Company
Recombinant Human TNFα	Preprotech. Ref 300-01A
Recombinant Human SDF-1α (CXCL12)	Preprotech. Ref 300-28A
Phalloidin Alexa Fluor 647	Molecular probes, ThermoFisher Ref A22287
CellTrance Far Red Cell Proliferation Kit	ThermoFischer. Ref C34564
CellTrance Violet Cell Proliferation Kit	ThermoFisher. Ref C34557
Fetal bonive serum	Biowest. Ref. S1810-100
Penicillin-Streptomycin (10,000 U/mL)	Gibco, ThermoFisher. Ref 15140122
Ficoll Histopaque GE Heathcare	Sigma-Aldrich. Ref GE17-1440-03
CD34 MicroBead Kit UltraPure human	MACS Miltenyi Biotec. Ref 130-100-453
TRIzol reagent	ThermoFisher Scientific. Ref 15596026
Chloroform	Sigma-Aldrich. Ref C2432
Isopropyl alcohol	Sigma-Aldrich. Ref 67-63-0
Ethanol BioUltra	Sigma-Aldrich. Ref 64-17-5
UltraPure DNase/RNase-Free Distilled Water	ThermoFisher Scientific. Ref 109977023
SuperScript II Reverse Transcriptase Kit	Invitrogen. Ref 18064-022
Platinum Taq DNA Polymerase Kit	Invitrogen. Ref 10966026
FastStart TaqMan Probe Master Kit	Roche. Ref 04673417001
Cytofix/Cytoperm Kit	BD. Ref 554714
Attachment Factor 1X	Gibco, ThermoFisher. Ref S006100
EDTA	Sigma-Aldrich. Ref E6758-500G
Tween 20	Sigma –Aldrich. Ref P2287-100ML
Triton X-100	Sigma-Aldrich. Ref 93443-100ML
Vectashield with DAPI	Vector Laboratories. Ref H-12000
Bovine Serum Albumin	Sigma-Aldrich. Ref A9418-100G
SuperSignal WestPico	ThermoFischer Scientific. Ref 34087

SuperSignal WestFemto	ThermoFischer Scientific. Ref 34095
Nitrocellulose membrane, pore 0.45 µm	Bio rad. Ref 1620115
6.5 mm transwell with 5 µm pore polycarbonate membrane insert	Corning. Ref 3421
Poly-Prep slides	Sigma Aldrich. Ref P0425-72EA

7.1.4 Buffers

PBS 1X	138 mM NaCl 3 mM KCl 8.1 mM Na2HPO4 1.5 mM KH2PO4
PBS-T	PBS 1X 0.05% Tween20
Immunofluorescence Blocking buffer	PBS-T 5% BSA
Mg^{2+} lysis buffer 1X	25 mM HEPES pH 7.5 150 mM NaCl 10 mM $MgCl_2$ 1 mM EDTA 1 % NP-40 2 % glycerol Complete Protease inhibitor cocktail (ROCHE)
SDS-PAGE Buffer	25 mM Tris 192 mM glycine 0.1% SDS pH 8.3
Transfer Buffer	20% methanol 25 mM Tris 192 mM glycine

	pH 8.3
TBS 1X Buffer	150 mM NaCl 10 mM Tris pH8.0
TBS-T	TBS 1X 0.1% Tween20
Blocking Buffer	TBS-T 5% Skim milk

7.2 METHODS

7.2.1 Patients

BM aspiration and cerebrospinal fluid (CSF) samples were collected according to international and institutional guidelines from children and adolescents younger than 18 years that were newly diagnosed with B-ALL before starting any treatment, or upon relapse. All samples were collected after written informed consent from parents. Patients were recruited and followed in the Hospital Infantil de Mexico “Federico Gomez” (Mexico City) and the IMIEM Hospital para el Niño (Toluca, Mexico) according to the National Treatment Protocol. All procedures were approved by the Ethics, Research and Biosafety Committees of the hospitals and by the Bioethics committee of CINVESTAV. Clinical and laboratory data were collected from patient records and institutional laboratory registers, and correlated with our data on cortactin.

7.2.2 Inclusion criteria

Patients younger than 18 years

Patients newly diagnosed with ALL without treatment

Patients newly diagnosed with ALL relapse

Normal control: Umbilical cord blood (UCB) cells from healthy consenting individuals

7.2.3 Exclusion criteria

Patients whose parents did not agree to participate

Samples stored at room temperature or at 4°C for more than 24 hours

Samples from patients in remission or under treatment

Small samples (less than 40 milliliters) of normal control UCB yielding less than 300,000 $CD19^{+}$ cells after sorting

7.2.4 Cell culture

The ALL lines REH (Pre-B leukemic cells, with the translocation 12:21, from peripheral blood of a relapse case, ATCC CRL-8286) (Matsuo & Drexler, 1998) and RS4:11 (Pre-B leukemic cells, with the translocation 4:11, from bone marrow ATCC CRL-1873) (Stong, Korsmeyer, Parkin, Arthur, & Kersey, 1985) were cultured in RPMI 1640 (Gibco) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HS-5 stromal cells (ATCC CRL-11882) and OP9 stromal cells

(ATCC CRL-2749) were cultured in DMEM (Gibco) medium supplemented with 10% FBS and 1% penicillin/streptomycin.

7.2.5 Isolation and propagation of HUVEC

To obtain human umbilical vein endothelial cells (HUVEC), umbilical cord was washed with water to remove excess of blood. Under sterile conditions, in the upper end a canule with a syringe containing 20 mL PBS and 1% antibiotic solution was inserted into the vein and the content was flushed. The syringe was removed and both ends were sealed with hemostatic clamps. Then, 0.25% trypsin-EDTA was added into the vein using a syringe, until the vein was filled and the cord was incubated at 37°C for 10 min in a water bath. Every 2 minutes the cord was gently massaged to facilitate the digestive process. Subsequently, the clamp of the lower end was removed and the content was recovered and centrifuged at 1500 rpm for 5 minutes. Finally, the cell pellet was resuspend in EBM-2 medium (Lonza) and plated placed in a 60 mm cell-bind dish (Corning).

To propagate and maintain HUVEC culture, the medium was aspirated and cells were washed with PBS three times. Then 1 mL of 0.25% trypsin-EDTA was added and cells were incubated at 37°C for 5min, then 3 mL of medium was added for trypsin inactivation, and the cell suspension was plated in three dishes.

7.2.6 Isolation of mononuclear cells

Mononuclear cells from bone marrow specimens of pediatric patients were obtained from lumbar puncture. Mononuclear cells from UCB were used as controls. Cells were separated by Ficoll-Paque (GE Healthcare) density gradient centrifugation at 200xg for 30 min without deceleration. The mononuclear cell ring was recovered with a Pasteur pipette and washed with PBS. Cells were counted after trypan blue staining (dilution 1:2) to quantify the number of viable cells in a Neubauer chamber, applying the following formula: (number of cells in four quadrants divided by 4) X dilution factor X 1000 X volume of cells in suspension.

In some cases, the $CD34^+$ fraction was enriched using the human CD34 MicroBead Kit UltraPure human (MiltenyiBiotec) according to the manufacturer's instructions.

7.2.7 RNA isolation

Total RNA was isolated from cell lines and CD19+ populations obtained by cell sorting from UCB. $1X10^6$ cells were suspended in 200 µl of TRIzol (Invitrogen Thermofischer) that was prewarmed to 37°C. The suspension was vortexed for 15 s for 1 min to lyse the cells. Then 40 µl of chloroform was added and the suspension was gently mixed by inversion before incubation for 5 min at room temperature and centrifugation at 12,000xg for 15 min at 4°C. All following steps were carried out at 4°C. Subsequently, the aqueous phase was removed and 500 µl of isopropanol were added for each milliliter of TRIzol used. Then the samples were centrifuged at 12,000xg for 10 min at 4°C. The supernatant was removed and the pellet was washed with 200 µl of 70% ethanol, and centrifuged at 7500xg for 5 min at 4°C. The Supernatant was removed and the pellet dried at room temperature, and resuspended in molecular biology grade water DEPC (Gibco).

7.2.8 cDNA synthesis

1.5 µg of total RNA was used for all cDNA syntheses. RNA was mixed with 1500 ng of oligo dT and 1mM dNTP and filled to a final volume of 12 µL with molecular biology grade water, followed by incubation at 65°C for 5 min and 1 min at 4°C. Next, 5X First strand buffer (Invitrogen) were added to a final concetration of 1X, 0.01 M of DTT (Invitrogen) was added, and the reaction solution brought to a final volume of 19 µL. The mixture was warmed to 42°C for 2 min, 400 U of reverse transcriptase SuperScript II (Invitrogen) were added and incubated at 42°C for 50 min. To inactivate the enzyme, the suspension was incubated at 70°C for 5 min. Integrity of cDNA was tested by amplification of GAPDH and 18S by PCR followed by gel electrophoresis. Concentration and purity were quantified by spectrophotometry reading the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) using a nanodrop device. All samples were used with a ratio 260/280 of at least 1.8.

7.2.9 End-point PCR

The PCR reaction was prepared using the following end concentrations: 1X PCR buffer, 2.5mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 20 ng cDNA and 0.05 U Taq polymerase enzyme (Invitrogen) in a final volume of 20 µl with molecular biology grade water. Amplifications were performed in a thermocycler (Applied Biosystems) using the following conditions: Denaturalization at 94°C for 2

min; followed by 35 cycles of 94°C for 10 s, 65°C for 20 s, 72°C for 5 min followed by a final extension at 72°C for 5 min. PCR amplicons were analyzed in a 2% agarose gel for 30 min at 100 V.

7.2.10 Quantitative RT-PCR

Expression of cortactin, HS1 and molecules involved in adhesion and migration were analyzed by real time RT-PCR using Taqman probes and primers described in table 1. β2- microglobulin and β-actin served as housekeeping genes. cDNA derived from REH cells and $CD34^{-}CD19^{+}$ populations from UCB as controls were diluted 1:10 corresponding to 7.5 ng/µl per reaction. PCR reaction mixtures were prepared as follows: 5µL of master mix FastStart TaqMan Probe Master 2X (Roche); 0.25 µLTaqman probe 20X (Roche); 0.25 µL forward and reverse primers at 5 µM; 2.5 µL cDNA dilution 1:10, in a final volume of 10 µl with molecular biology grade water. Quantitative PCR reactions were carried out in a Light Cycler 480 (Roche) using the following conditions: Pre-incubation at 95°C for 10min; 45 cycles of amplification at 95°C for 10s, 60°C for 30s and 72°C for 10s; 40°C for 30s; and continuous cooling at 4°C. Relative gene expression was calculated using the LyghtCycler 480 SW1.5 software and the $\Delta\Delta C_T$ method, that consists in a direct comparation of C_T values. The ΔC_T value of REH cells and $CD19^{+}$ cells from UBC was determined by subtracting the C_T of the housekeeping gene from the C_T of target gen in a given sample. Next, the $\Delta\Delta C_T$ for each target gene was determinated by subtracting the ΔC_T of $CD19^{+}$ control cells derived from UBC from the ΔC_T of REH cells. Normalized expression was calculated as $2^{-\Delta\Delta CT}$.

Table 1. Primer sequences and Taqman probes tested (numbers refer to universal Probe-Library of Roche).

Gene	Taqman Probe	Primer sequences
Cortactin	2	5´ ggtgtgcagacagacagacaa 3’
		5´ gtctttttgggattcatgcag 3'
HS1	12	5’ cccaagagtcctctctatcctg 3’
		5’ ggtggcagagaggtgttcat 3’
CXCR4	4	5’ accctgtttccgtgaagaaa 3’
		5’ cccacaatgccagttaagaag 3’
VLA4	2	5’ gcgtggtacaacttgactgc 3’
		5’ tcctcttccgctctgctg 3’
Rap1	2	5’ aagtgccagcattccagact 3’
		5’ gggaacttgtgcaaaccaat 3’
CDC42	12	5’ atgttccccatctggtgct 3’
		5’ ggcaactttcaaaaaggagtatgt
Rac2	3	5’ gacagcaagccagtgaacct 3’
		5’ cggagacggtcgtagtcct 3’
β2-MICRO-GLOBULIN	42	5’ tcaggaaatttgactttccattc 3’
		5’ ttctggcctggaggctatc 3’
β-ACTIN	64	5’ ccagaggcgtacagggatag 3’
		5’ ccaaccgcgagaagatga 3'

7.2.11 Flow cytometry analysis and sorting

Mononuclear cells were stained with anti-human CD34 Pacific Blue (1:100; Biolegend), and anti-human CD19 allophycocyanin (1:100; Biolegend) or anti-human CD19 phycoerytrin (1:100; Biolegend) in PBS with 3% FBS for 20 minutes at room temperature to identify $CD34^+$ progenitors, $CD34^+CD19^+$ pro-B and $CD34^-CD19^+$ pre-B cells. To investigate CXCR4 expression, the anti-human CD184 PE-cyanin 7 (1:100; Biolegend) antibody was used. Cortactin and HS1 expression was evaluated after permeabilization and fixation with cytofix/cytoperm (BD Bioscence) for 20 minutes at 4°C. Then cells were incubated for 30 minutes at room temperature with a monoclonal Alexa 488-labelled anti-cortactin antibody (1:50 dilution; clone 289H10; kindly provided

by Dr. Klemens Rottner, TU Braunschweig, Germany), which recognizes all cortactin isoforms; or with a monoclonal anti-HS1 (D83A8; 1:200; Cell Signaling Technologies) and a secondary goat anti-rabbit Alexa 488-labelled antibody (1:1000). Cells were analyzed using a FACSCanto BD flow cytometer and FlowJo v10.0 software. For cell sorting, mononuclear cells from BM of ALL patients or UCB were stained with anti-human CD34 PE-cyanin 5 (1:100 Becton-Dickinson) and anti-human CD19 allophycocyanin (1:100; Biolegend) as described above and sorted using a BD FACS Aria II flow cytometer.

Cell purity was measured by flow cytometry using a BD FASCanto II. Progenitor cells were identified as $CD34^+$, pro-B blasts as $CD34^+CD19^+$ and pre-B cells as $CD34^-CD19^+$. Mononuclear cells from UCB were used as non-malignant progenitor and B-cell precursor control cells.

7.2.12 Immunofluorescence microscopy

$1X10^6$ cells from cell lines or less from patient samples (depending on the number of cells recovered from tissue samples) were attached to a microscopic glass slide treated with poly-L-lysine (Poly-Prep slides,Sigma-Aldrich) by cytospin centrifugation at 1700 rpm for 10 min. Then cells were fixed with 3% paraformaldehyde (PFA) for 20 min at room temperature. Cerebrospinal fluids (CSF) from ALL patients were collected immediately after first lumbar puncture, subjected to cytospin and fixed with 3% (PFA) for 20 min at RT. Then cells were washed with PBS three times and permeabilized with PBS containing 1% Triton X-100. Preparations were blocked in PBS containing 5% BSA and 0.05% Tween20 for 1 h at room temperature followed by incubation with primary antibodies: anti-cortactin 289H10 undiluted hybridome supernatant, or alexa488-labelled anti-cortactin 289H10 (1:50), anti-HS1 D83A8 (1:200), and anti-CXCR4 (1:200) overnight at 4°C. After extensive washing, cells were incubated with species-specific Alexa-Fluor-labelled secondary antibodies and alexa647-labelled phalloidin for 1 h at RT. Then, slides were washed two times with PBS and three times with bidestilled water (Millipore) to remove salts. Preparations were mounted in Vecta-Shield containing DAPI. Samples were analyzed using a confocal laser scanning microscope (Leica TCS SP8). Image analysis was performed using Imaris x64 7.4.2 software.

7.2.13 Western blotting

Cells were lysed in 25mM HEPES pH7.5, 150mM NaCl, 10mM MgCl2, 1mM EDTA, 1% NP-40, 2% glycerol and complete protease inhibitor cocktail. Equal protein amounts were separated by 8% SDS-PAGE, transferred to PVDF membranes (Millipore) and blocked with 5% skim milk in TBS with 0.1% Tween20. Membranes were incubated with primary anti-cortactin 289H10 hybridome supernatant (1:10), anti-HS1 D83A8 (1:4000) or anti-γ tubulin (1:4000, loading control) at 4°C over night. Then membranes were washed three times with TBS containing 0.1% Tween20 and incubated with species-specific peroxidase-conjugated secondary antibody (1:10,000 Santa Cruz) for 1 h at RT. Then membranes were washed three times with TBS-T and developed using SuperSignal West Pico or Femto Chemiluminescent substrates (Thermo Scientific). Chemiluminescence signals were recorded on a Chemidoc imaging system (Bio Rad).

7.2.14 Transendothelial migration assay

$8x10^4$ HUVEC were grown to confluence on 5 µm pore transwell filters (Corning) pre-treated with attachment factor (Gibco, Thermo Scientific) for 30 min at 37°C. As a control to monitor monolayer formation, HUVEC were seeded in 96 well plates at the same density. Once the monolayer was formed (usually 48 h), $1.5x10^4$ REH, RS4:11 or primary mononuclear cells resuspended in 200 µl EGM-2 medium were placed on top of the endothelial monolayer. 100 ng/ml of CXCL12 (Peprotech) was added to the bottom well as chemoattractant. In some cases, HUVEC were pre-activated with 50 nM TNF-α for 16 h. Cells were allowed to transmigrate for 4 hours. Transmigrated cells from the bottom chamber were counted using a Neubauer chamber. Cells from the top and bottom chambers were stained with anti-cortactin to determine their expression levels by flow cytometry. For competition assays, REH and RS4:11 cells were incubated 20 minutes at 37° C with Celltrace far red and Celltrace violet (Thermo-Fisher), respectively, using a 1:10,000 dilution. Subsequently, cells were washed with medium to inactivate the dyes, centrifuged at 1,500 rpm for 5 min, resuspended in 200 µl EGM-culture medium, mixed in a 1:1 cell ratio, and subjected to transmigration as described above. Transmigration efficiency and cortactin expression was determined using a BD FACSCanto flow cytometer and FlowJo v10.0 software.

7.2.15 B-ALL-stromal-cell co-culture

Glass coverslips were pre-treated with attachment factor (Gibco, Thermo Scientific) in 24 well plates for 30 min at 37°C. HS-5 human stromal cells were then plated at a density of $1x10^5$ cells per well in DMEM medium containing 10% FBS. After 24h, $1x10^6$ REH cells were stained with celltrace violet (Invitrogen-Thermofischer) at a 1:1000 dilution in PBS for 15 min at 37°C and cells were washed with medium to inactivate the dye. $3x10^5$ cells were placed on top of the stromal cell monolayer for 2 h at 37°C and 5% CO_2. Co-cultures were fixed with 3% PFA for 20 minutes at room temperature and subjected to IF staining with anti-cortactin and anti-CXCR4 antibodies as described above.

7.2.16. Tridimensional co-culture system

The mesenchymal stromal cells OP9 were cultivated in DMEM medium supplemented with 10% FBS. To form spheroids in non-adhesion wells, a suspension of stromal cells were seeded in 96 well plates filled with 1% agarose to avoid adhesion to the plastic surface and incubated at 37°C, 5% CO_2 allowing for cellular aggregation and formation of spheroids.

B-ALL cell lines or leukemic precursor cells from relapsed patients were co-cultured with a spheroid for 24 hours. After this period, hematopoietic cells outside of spheroids were collected from supernatants, and spheroids with hematopoietic cells inside were collected separately. To disaggregate spheroids, they were extensively washed with PBS-EDTA an enzymatic digestion using trypsin-EDTA was performed. The cell suspensions of inner and outer cells were then stained for CD45 (hematopoietic marker) and cortactin was analyzed by flow cytometry as analyzed above. In competition assays, hematopoietic cells were pre-labeled using the fluorescent dyes CellTraceViolet or CellTrace Far Red, respectively, as described above.

7.2.17 Cell cycle status assays

REH and RS4:11 cells within spheroids and those that did not enter spheroids after co-cultivation with stromal cells were stained with anti-CD45, then fixed and permeabilized with cytofix/cytoperm for 20 min at 4°C. Cell suspensions were washed with perm wash buffer and centrifuged at 1,500 rpm for 5 min. Cells were then stained with anti-Ki67 FITC (ThermoFisher Scientific) at a 1:50 dilution for 1 h

at room temperature, followed by washing with perm wash buffer and staining with DAPI (ThermoFisher Scientific) 20µM at a 1:60 dilution for 30 min at room temperature. Stained cells were analyzed by flow cytometry..

7.2.18 Xenografts

All animal studies were approved by the IACUC of CINVESTAV. $3x10^6$ REH cells were injected via the tail vein into non-irradiated male NSG mice (Jackson Laboratories, ME, USA). Mice were kept in the animal facility of CINVESTAV and leukemia progression was monitored weekly by flow cytometry of peripheral blood. Once disease was established, mice were euthanized and BM, liver, spleen, brain, lung and testis were harvested, digested and analyzed by flow cytometry.

7.2.19 Statistics

Prism V3.02 (GraphPad) software was used to perform statistical data analysis. A multi-variant statistical analysis was performed to determine the relevance of cortactin expression levels for primary CNS infiltration or BM relapse in childhood leukemia. Differences within groups were established by Student's t test when two groups were compared, non-parametric one or two-way ANOVA test, when more than two groups were compared, with one or two variables, respectively, considering significant probability values of $p<0.05$. U Mann-Whitney test with α of 5% to define statistical significance was applied.

6. RESULTS

8.1 Determination of the expression of molecules involved in regulating migration and adhesion in pre-B ALL cells

First, we analyzed the mRNA expression levels of the ABP cortactin and HS1 in the pre-B cell line REH that was isolated and established from a B-ALL relapse patient. Both HS1 and cortactin were expressed to a significantly higher amount in REH cells when compared to non-malignant $CD19^+$ cells from UCB (Figure 6). While HS1 expression was almost three times higher compared to control cells, cortactin expression was increased 14-fold in REH cells. Also, we determined the expression levels of the GTPases Rap 1, Cdc42 and Rac2, known to be involved in actin remodeling and integrin activation during transmigration, and found only significantly higher expression of Rap1 in REH cells. Moreover, the chemokine receptor CXCR4 and the integrin VLA-4, known to be activated via the CXCR4/CXCL12 axis, were not significantly altered in REH cells in comparison to CD19+ cells fom UCB.

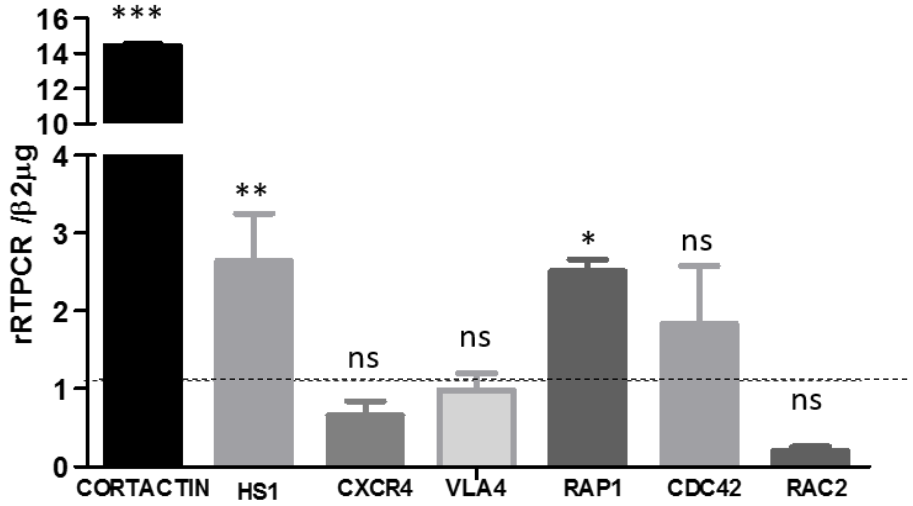

Figure 6. qRT-PCR to analyze gene expression of molecules involved in migration and adhesion. qRT-PCR was performed with cDNA synthesized from REH mRNA or umbilical cord blood (UCB)-derived $CD19^+$ cells as normal control. Data were normalized to the housekeeping gene β-2-microglobulin. Relative expression was determined by the ΔΔCT method (n=2). One-way ANOVA with Bonferroni post-test for p value calculations were performed for multiple comparisons. *p<0.05, **p<0.01, ****p<0.0001.

8.2 Hematopoietic linage cells express the 60 kDa cortactin SV2 variant

Interestingly, western blot analysis of whole cell lysates revealed that only the 60 kDa SV2 isoform of cortactin was present in REH cells and the neutrophil-like cell line

HL60, in contrast to the epithelial cell line CaCo-2 that is known to express only the 80 kDa WT isoform of cortactin (Figure 7). The 60 kDa isoform results from alternative splicing and lacks the last 2.5 repeats within the actin-binding region suggesting that this isoform has altered affinities for filamentous actin compared to the WT isoform (van Rossum et al., 2003). However, the real functional relevance of the different cortactin isoforms remains elusive.

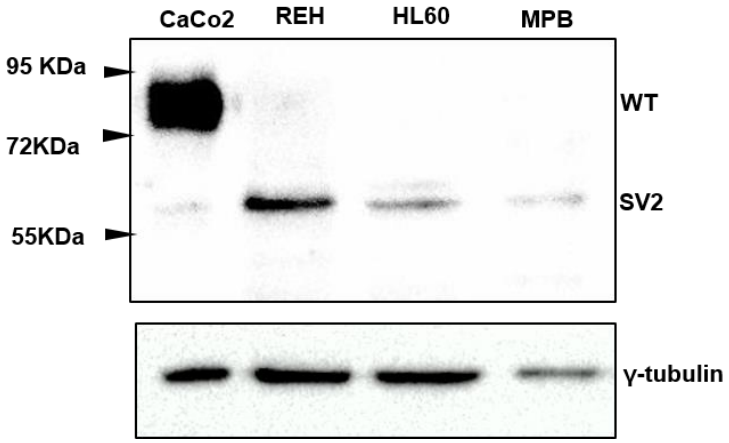

Figure 7. Expression of cortactin isoforms. Cortactin expression was determined by Western Blot using lysates from the cell lines REH, HL60 and mononuclear cells from mobilized peripheral blood (MPB) as normal control. These cell lines only express the SV2 isoform at 60 kDa. CaCo-2 epithelial cells served as positive control for wild-type cortactin (80 kDa) expression (n=2). Tubulin served as loading control (lower panel).

8.3 Cortactin and HS1 co-localize in the cytosol and at the edge of B-ALL cells

To identify the localization of cortactin and HS1, we performed IF staining in REH cells. Confocal images revealed that both proteins mostly co-localize in the cytosol and at the cell periphery in proximity to the plasma membrane (Figure 8).

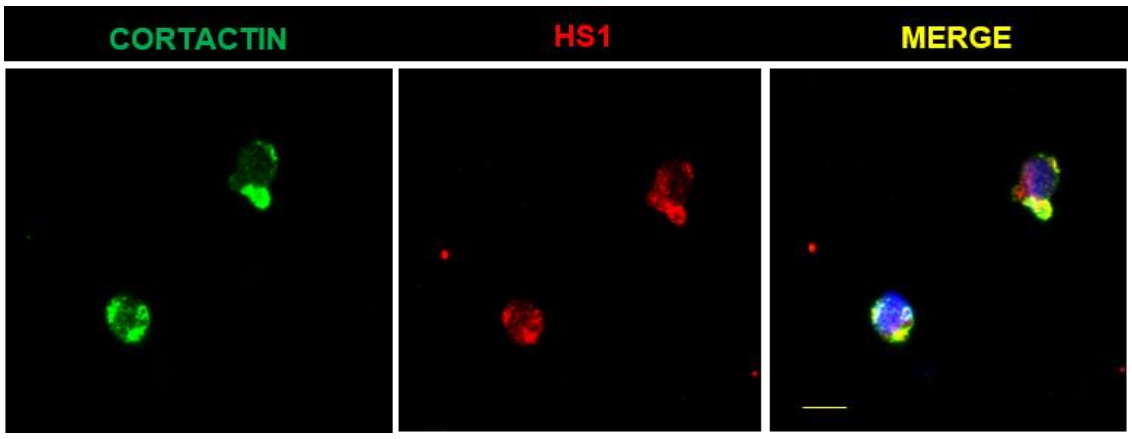

Figure 8. Immunufluoresce staining of cortactin and HS1 in REH cells. Adherent REH cells were fixed and stained for cortactin (green), HS1 (red) and DAPI (nuclei, blue). Partial co-localization of cortactin and HS1 in the cytosol and the cell periphery can be observed Bar=20µm (n=3).

8.4 Cortactin and HS1 protein levels are higher in REH cells compared to control and RS4:11 cells and correlate with their aggressivness

Flow cytometry analysis confirmed the high levels of cortactin (Figure 9A) and HS1 (Figure 9B) in REH cells compared to UCB-derived $CD19^+$ cells. Of note, the preB-ALL cell line RS4:11 express significantly less cortactin and HS1 compared to REH cells (Figures 9A and 9B). Higher expression levels of these two proteins in REH cells correlates with a more aggressive phenotype of REH cells, as they were isolated from a relapsed patient with poor prognosis (Table 2).

Table 2. Characteristics of the pre-B ALL cell lines REH and RS4:11.

Characteristics	**REH cell line**	**RS4:11 cell line**
Immunophenotype	Pre-B	Pre-B
Source	Periphery blood	Bone Marrow
Chromosomal aberration	12:21 (good prognosis)	4:11 (poor prognosis)
Relapse	Relapse to bone marrow	Relapse to bone marrow

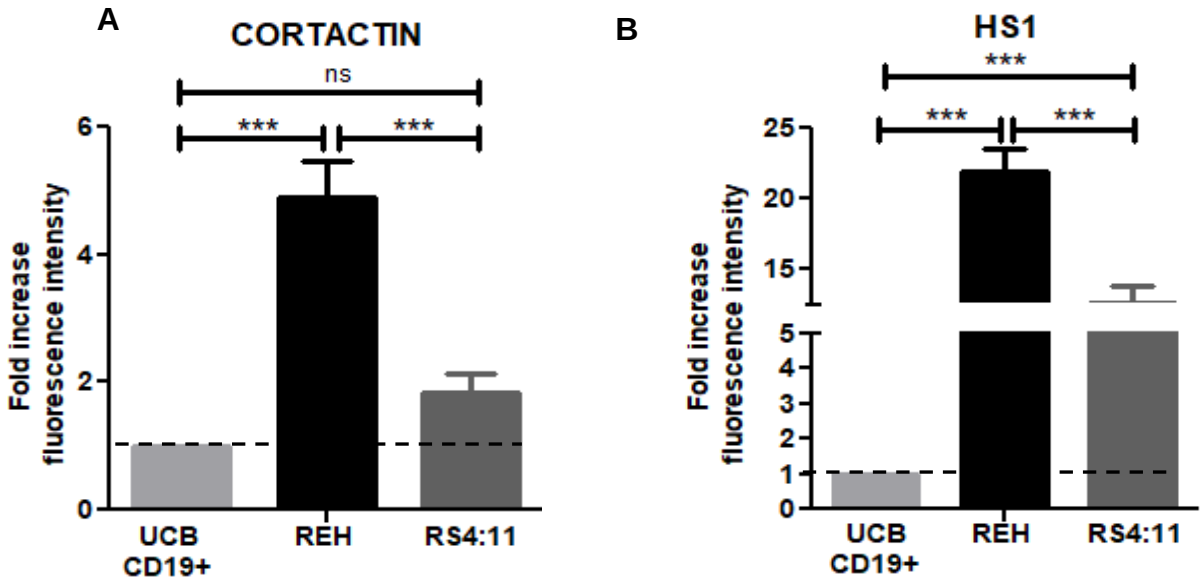

Figure 9. Flow cytometry analysis of cortactin (A) and HS1 (B) protein levels in REH and RS4:11 cell lines. Flow cytometry data are displayed as fold increase fluorescence intensity normalized to UCB-derived $CD19^+$ cells (set to 1) used as controls (n=3). One-way ANOVA with Bonferroni post-test for p value calculations were performed for multiple comparisons. ***p<0.05, ns= not significant

8.5 Cortactin and HS1 co-localize in the cytosol and at the edges in B-ALL cells at sites of F-actin enrichment

Since cortactin and HS1 are homologous ABP, we analyzed their localization with respect to the actin cytoskeleton. Confocal IF images revealed that both proteins indeed co-localize with actin, preferentially in the periphery of both REH and RS4:11 cells (Figure 10), suggesting that they are involved in actin-dependent cellular processes such as adhesion and migration.

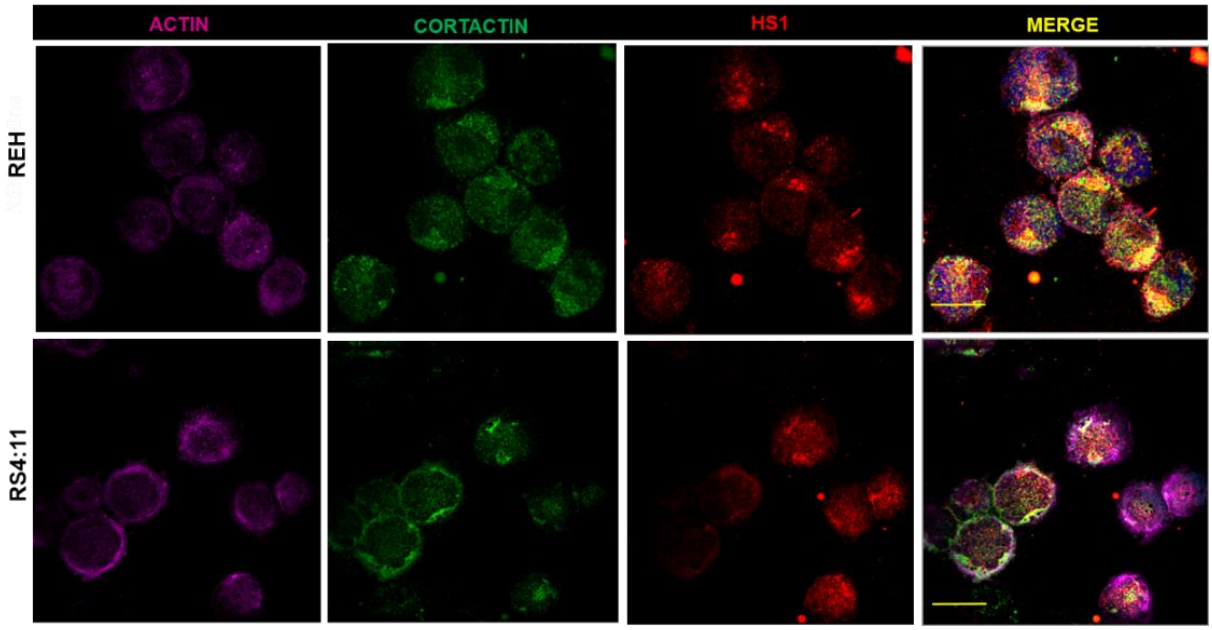

Figure 10. Immunofluorescence stainings of cortactin, HS1 and actin in REH and RS4:11 cell lines. REH and RS4:11 cells were fixed and stained for cortactin (green), HS1 (red), actin (phalloidin-Alexa647) and DAPI (nuclei, blue). Images show cortactin and HS1 presence at the cell periphery in co-localization with cortical actin Bar=20µm (n=3).

8.6 Cortactin levels determine the transmigratory capacity of B-ALL cells, and cortactin expression levels distinguish at least two types of B cell precursor cells in ALL

Given the the known involvement of cortactin in leukocyte extravasation (Schnoor et al., 2011), we wondered if high cortactin levels would promote transendothelial migration of B-ALL cells. Therefore, we performed transendothelial migration assays *in vitro* and found that 20% of applied REH cells transmigrated in response to a CXCL12 chemokine gradient (Figure 11). Without CXCL12, REH cells did not transmigrate. Of note, CXCL12-induced transmigration was independent of endothelial activation by TNFα (Figure 11). To analyze if this CXCL12-induced transmigration of B-ALL cells is cortactin-dependent, we first did a competitive transmigration assay using a 1:1 cell suspension of fluorescently-labeled REH (cortactin-high) and RS4:11 (cortactin-low) (compare Figure 9). REH cells transmigrated significantly more than RS4:11 cells (Figure 12A), and transmigrated REH cells had indeed significantly higher cortactin levels (Figure 12B). Then we wanted to know if cortactin levels within the same cell line differed in transmigrated vs non-transmigrated B-ALL cells. Analysis of cortactin expression in cells recovered after transmigration from the bottom chambers of the transwell filters versus cells that stayed on top of the endothelial monolayer (non-transmigrated) revealed that transmigrated cells had significantly higher levels of cortactin, which was true for both REH (Figure 13A) and RS4:11 (Figure 13B).

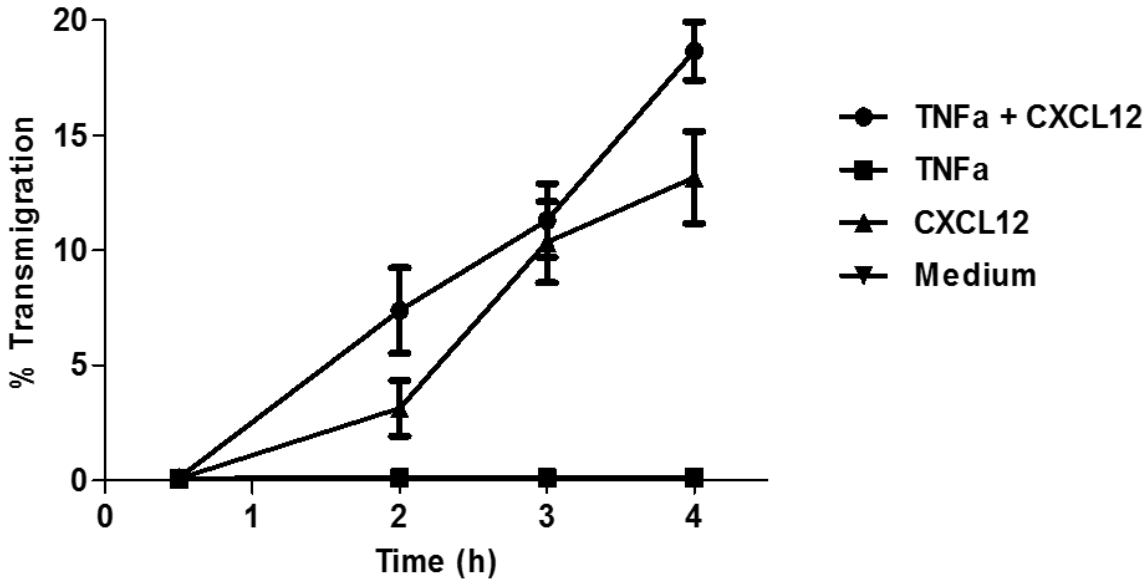

Figure 11. Transendothelial migration assays of REH cells transmigrating across a monolayer of HUVEC. REH cells transmigrate in a CXCL12-dependent fashion but do not require endothelial pre-activation by TNFα. Percentage of transmigrated cells after 4 h was calculated from the proportion of cells counted from the bottom (transmigrated cells) and cells from the top (non-transmigrated cells) (n=2).

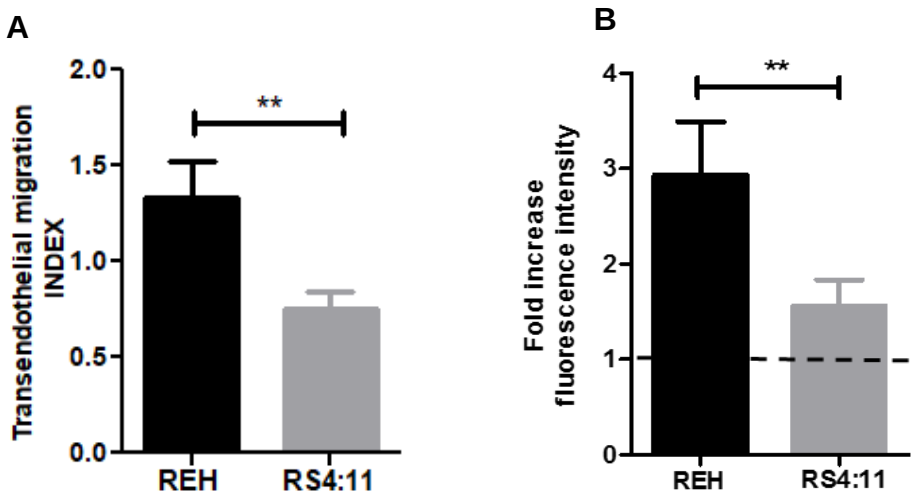

Figure 12. Transendothelial migration is related to cortactin levels. A) Transendothelial migration competition assays were performed for 4 h with both REH cells (labelled with Celltrance far red) and RS4:11 cells (labelled with Celltrance violet) in a 1:1 ratio. The transendothelial migration index was calculated considering the cell frequency of each cell line in the bottom (n=3). **B)** Difference in fold increase of cortactin levels in REH and RS4:11 cells from the bottom of the transwell filters after transmigration was determinated by flow cytometry (n=3). Differences in fold increases of cortactin levels in REH and RS4:11 cells from the bottom of the transwell filters after transmigration was determined by flow cytometry (n=3). Unpaired, two-tailed Student's t-test was used to determine statistically significant differences between experimental groups. Error bars in figures represent SD **p<0.05

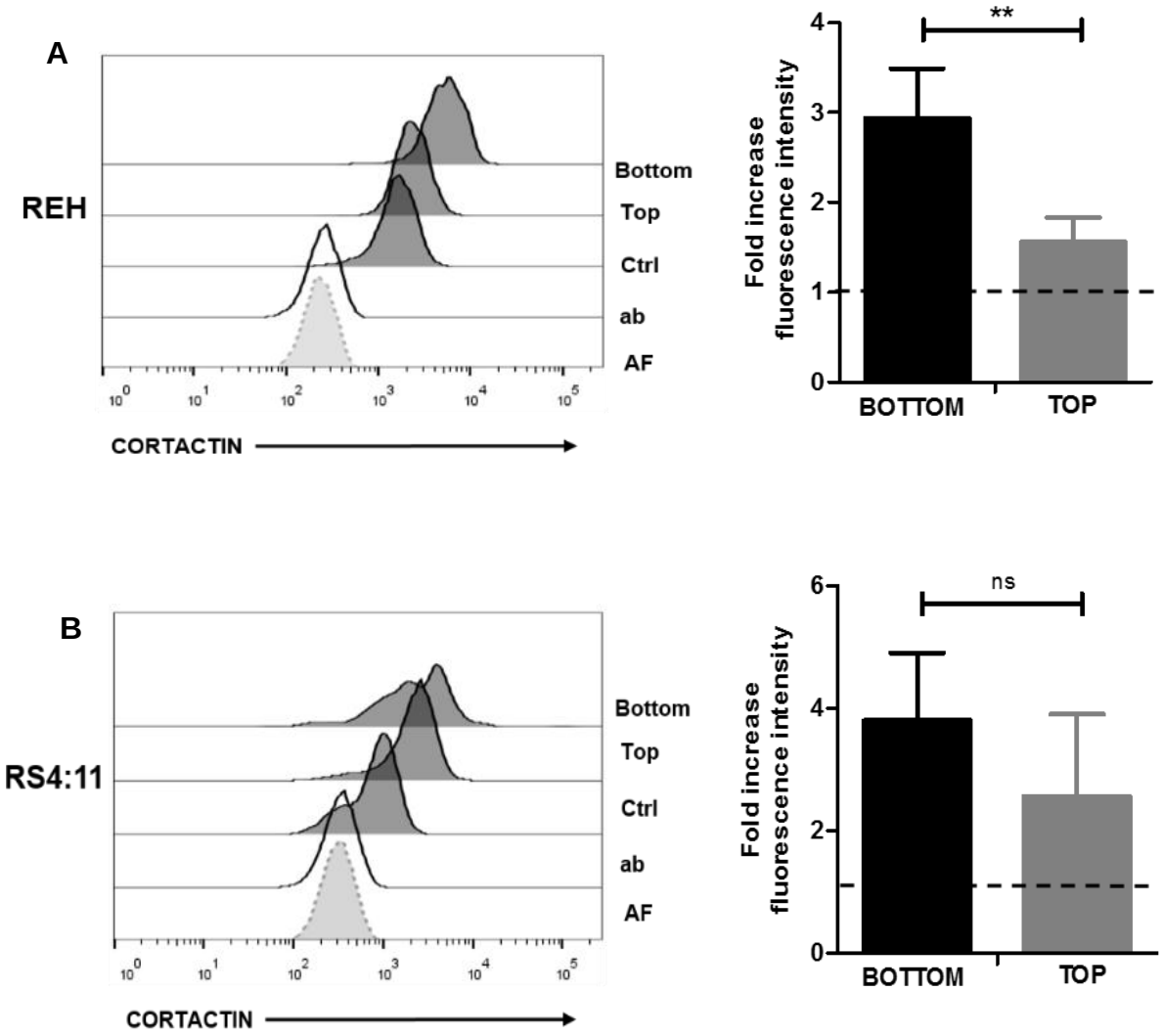

Figure 13. High expression of cortactin is related to a transmigratory advantage. Cortactin levels of transmigrated cells (bottom) and non-transmigrated cells that remained on top in REH (A) and RS4:11 cell lines (B), respectively, were determined by flow cytometry after 4 h. Data are display as fold increase of the mean fluorescence intensity normalized to the levels in cells that were not subjected to transmigration (ctrl) (n=3). One-way ANOVA with Bonferroni post-test for p value calculation were performed for multiple comparisons. **p<0.05, ns= not significant.

Leukocyte transmigration depends on selectins, integrins and chemokine receptors (Schnoor, 2015). However, while we observed substantial surface expression of the VLA-4 subunits CD49d and CD29, expression of L-selectin, PSGL1, Mac-1 and LFA-1 was rather low and not increased by CXCL12 (Fig 14). Both cells had significant surface amounts of CXCR4, which decreased after CXCL12 treatment, probably due to internalization (Nevins & Marchese, 2018).

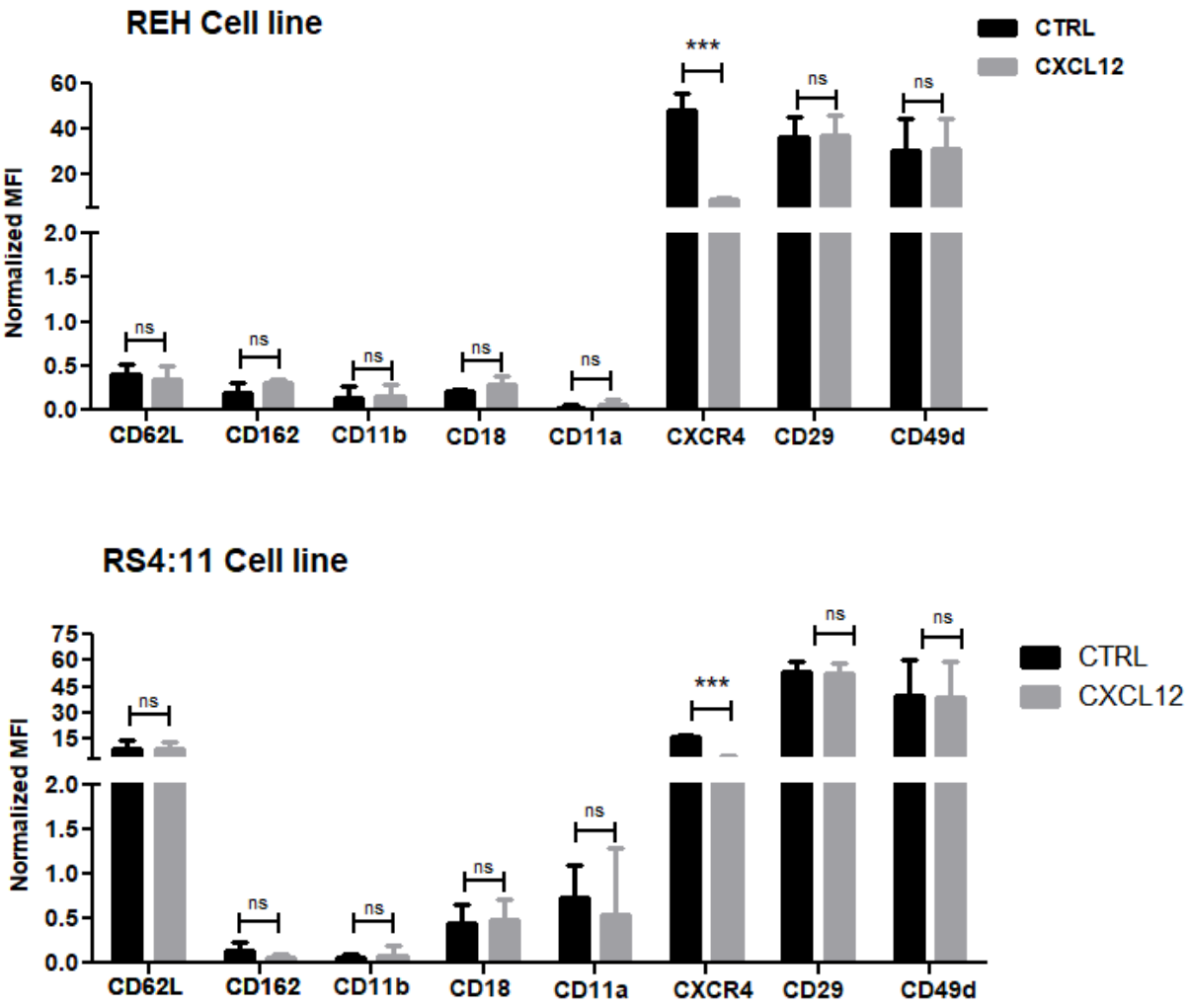

Figure 14. Expression of surface receptors in B-ALL cell lines. Expression of F-Selectin (CD6L), CD162 (PSGL1), Ma-1(CD11b/CD18), LFA-1 (CD11a/CD18), VLA-4 (CD49d/CD29) and CXCR4 were investigated in REH and RS4:11 cells with or without 100ng/ml CXCL12 for 4 h. Data are displayed as normalized mean fluorescence intensity (n=3) ns=non-significant, ***p<0.001

We speculated that relapsed B-ALL cells also have an increased transmigratory capacity. To answer this, we subjected cells from 3 relapse patients to transendothelial migration assays. Transmigrated P19 progenitor cells had significantly higher cortactin levels compared to control cells, whereas cortactin levels were not significantly different between transmigrated and non-transmigrated pro-B and pre-B cells (Figure 15). No significant differences were detected analyzing cell populations from P20. By contrast, all cell populations (progenitors, pro-B and pre-B) isolated from P21 showed significant higher cortactin levels in transmigrated cells compared to non-transmigrated cells (Figure 15). These data clearly demonstrate the

importance of cortactin for transendothelial migration of primary B-ALL cells and show for the first time the correlation of cortactin expression levels with B-ALL relapse.

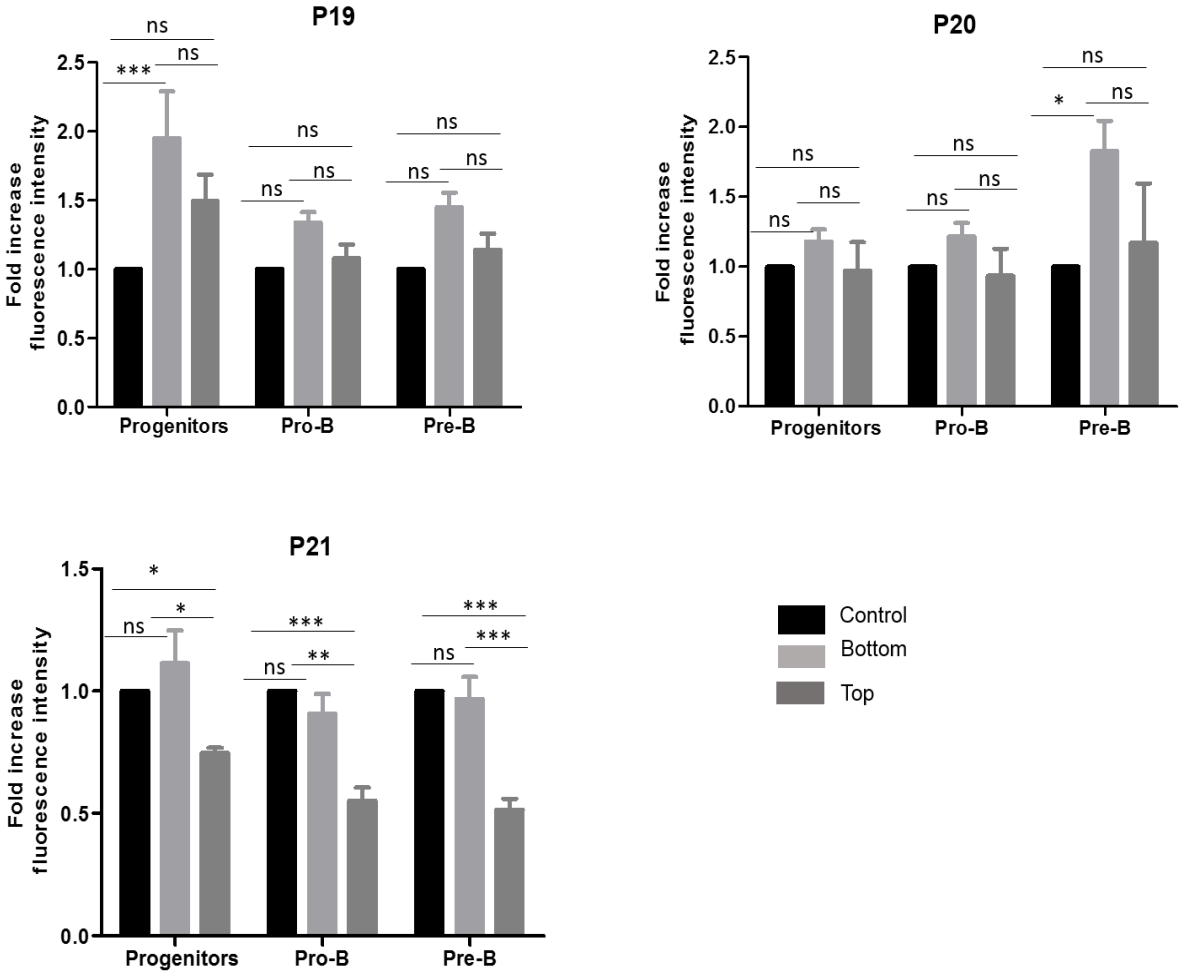

Figure 15. Transmigratory capacity of primary B-ALL cells from relapsed patients. Transendothelial migration assays of mononuclear cells from three B-ALL relapse patients for four h in the presence of CXCL12. Transmigrated cells (bottom) and non-transmigrated cells (top) were stained with anti-CD34 and anti-CD19 to distinguish progenitors, pro B and pre B cells, before permeabilization for cortactin staining and analysis by flow cytometry. Data were normalized to cortactin expression of cells not subjected to transmigration (set as 1). Triplicate of each patient are depicted. Two-way ANOVA with Bonferroni post-test for p value calculations were performed for multiple comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

8.7 Cortactin is expressed in primary B-ALL cell progenitors and precursors

Next, we analyzed primary B-ALL cell populations for cortactin expression to identify whether certain progenitor populations are related with cortactin expression. Bone marrow cells from B-ALL patients were sorted after staining with CD34 and CD19 to define the primitive populations progenitors (CD34+), pro B (CD34+CD19+) and pre B (CD34-CD19+). Figure 16 shows the mean fluorescence intensities for cortactin in

these cell populations from 18 patients. We observed heterogeneous cortactin expression among patients but there was no significant correlation of cortactin expression levels with a specific population (Figure 16 and Table 3) as determined by Mann Whitney U test. However, IF stainings suggested that cortactin levels decrease during differentiation with progenitors showing the strongest signal and pre B cells the weakest, whereas HS1 levels remained constant in these populations (Figure 17). Similar to the B-ALL cell lines, both proteins co-localized preferentially at cell edges.

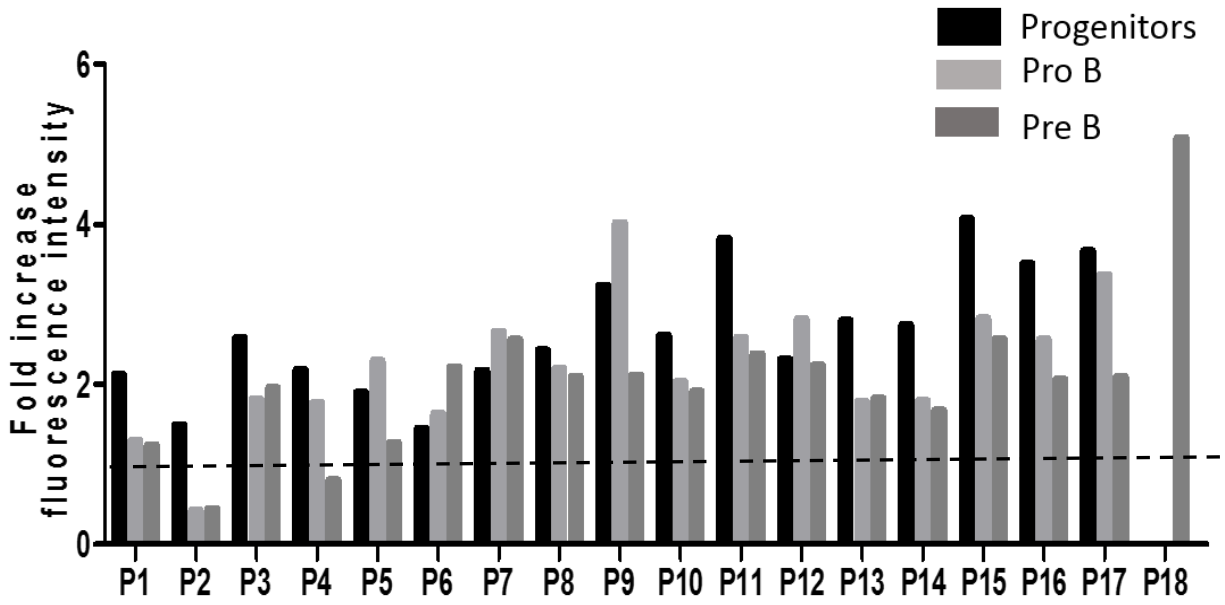

Figure 16. Cortactin is overexpressed in primary leukemic precursor B cells. High but heterogeneous cortactin levels were observed in B cell precursors from 18 B-ALL patients (P1-P18) as determined by flow cytometry. The different precursor populations present among bone marrow mononuclear cells (progenitors, pro-B and pre-B) were identified using antibodies against CD34 and CD19. Data are presented as fold increase of mean fluorescence intensity normalized to UCB mononuclear cells (set to 1).

8.8 Cortactin is expressed in blasts that infiltrated the central nervous system

Given the important roles of these ABP in cell adhesion and migration, we speculated that they could be related with disease relapse that often involves infiltration of the CNS. To test this idea, we collected cerebral spinal fluid from 26 B-ALL patients. 15% of these samples were positive for cellular infiltration, and 100% of infiltrated leukemic cells displayed strong cortactin expression that again colocalized with HS1 preferentially at the cell periphery (Figures 18 & 19).

8.9 High cortactin levels in primary leukemic precursor B cells are related to bone marrow relapse in children suffering from B-ALL

Next, we analyzed cortactin levels in B-ALL cells from patients with and without disease relapse to the BM. Interestingly, B-ALL cells from relapsed patients consistently and significantly expressed 3-fold increased cortactin levels (Figure 20). Separate analyses of different B progenitor populations from these patients showed that cortactin expression was higher in all populations of relapsed patients compared to the populations from non-relapsed patients (Figure 21).

8.10 High cortactin levels in leukemic cells from B-ALL patients are related with relapse to bone marrow, failure of steroid response, higher platelets numbers and clinical manifestations of infiltrative syndrome such as adenomegaly.

In order to correlate cortactin expression levels to clinical parameters, we analyzed patient files and performed statistical studies (Table 3 and Figure 22). We used non-parametric Mann-Whitney U test to categorize variables and analyzed if they could be related with cortactin levels, considering a p-value <0.05 as an existing correlation. Mean cortactin levels are displayed as increase folds) for each category (Table 3). We identified a correlation of high cortactin levels with relapse cases to bone marrow, failure of response to steroids and adenomegaly that is one of the clinical signs to diagnose infiltrative syndrome. However, we did not observe any relation with risk classification, hyperleukocytosis, immunophenotype or translocations that are important variables that define an early prognosis.

To analyze cortactin levels with variables that were categorized as numeric variables, we used Pearson's correlation test. We found that high cortactin levels correlated with high platelet numbers, but not with LDH (Lactate Dehydrogenase) levels as measure of tumoral lysis. Cortactin levels neither correlated with altered hemoglobin levels, nor leukocytes number nor the percentage of blasts in peripheral blood and bone marrow. Also we did not find a correlation with nutritional condition.

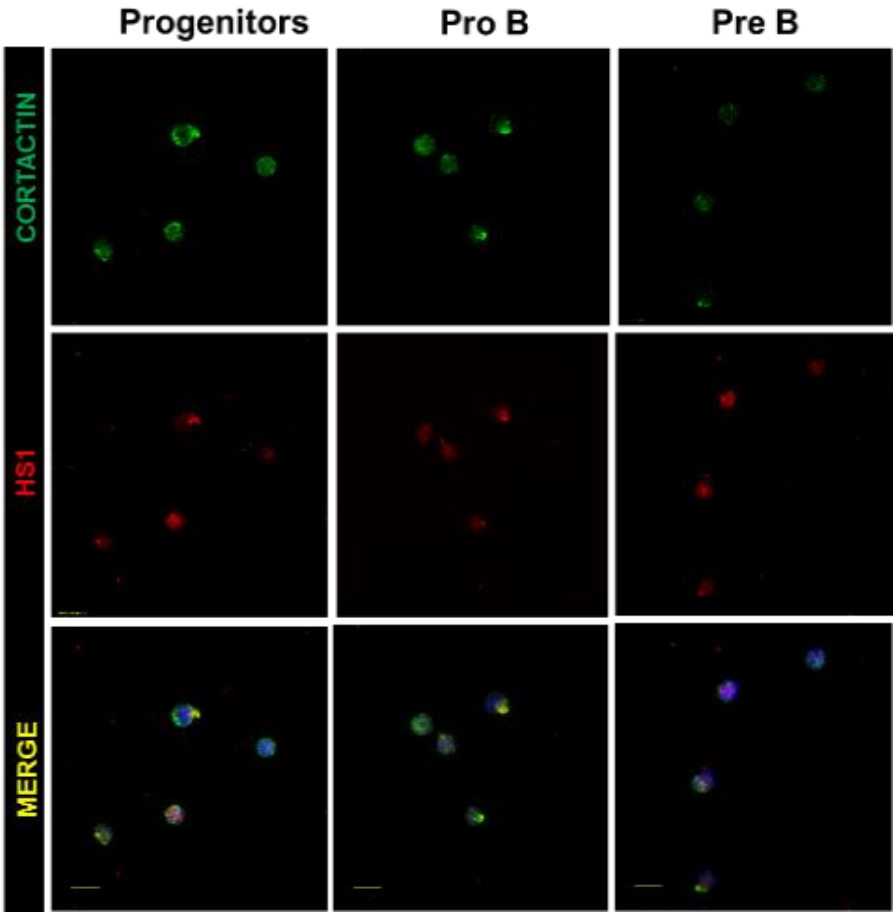

Figure 17. Immunofluorescence staining of CD34 and CD19-sorted mononuclear cells from B-ALL patients. Representative IF images show that cortactin (green) expression gets lost during maturation, whereas HS1 is expressed at similar levels in all populations Bar=20µm (n=3).

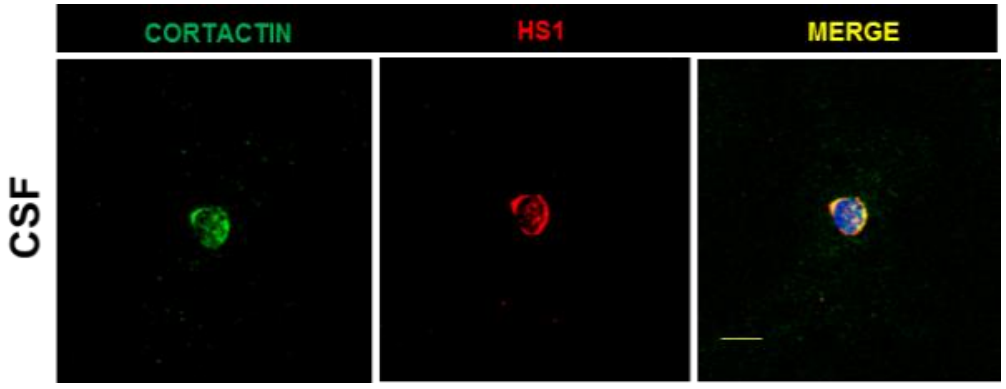

Figure 18. Immunofluorescence staining of a representative cell found in the cerebrospinal fluids (CSF) of relapsed B-ALL patient. All cells analyzed from CSF were positive for cortactin expression that co-localizes with HS1 Bar=20µm.

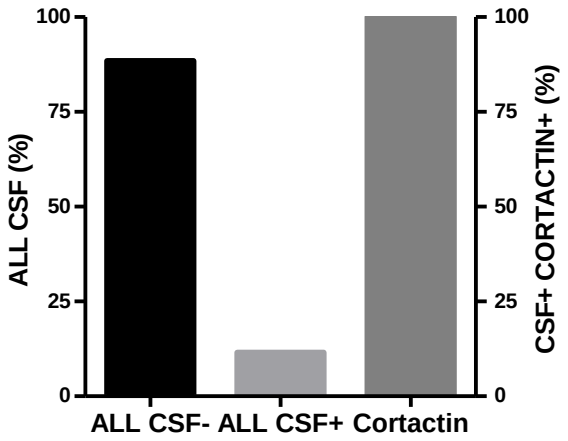

Figure 19. Cortactin expression of cerebrospinal fluids (CSF) from B-ALL patients positive for infiltration. CSF were obtained from remnant lumbar puncture samples (n=27) and 15% of all samples were positive for infiltrated leukemic cells that all expressed cortactin as revealed by IF (compare representative images of Fig. 13).

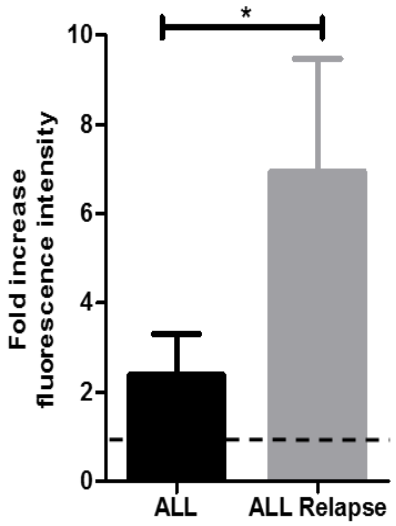

Figure 20. Cells from relapsed patients consistently expressed higher levels of cortactin. Cortactin expression was measured by flow cytometry in progenitor and precursor B cells from bone marrow of children diagnosed with either B-ALL (n=18) or B-ALL with bone marrow relapse (n=5). Cells from relapsed patients consistently expressed higher levels of cortactin. Unpaired, two-tailed Student's t-test was used to determine statistically significant differences between experimental groups. Error bars in figures represent standard desviation SD **p<0.01

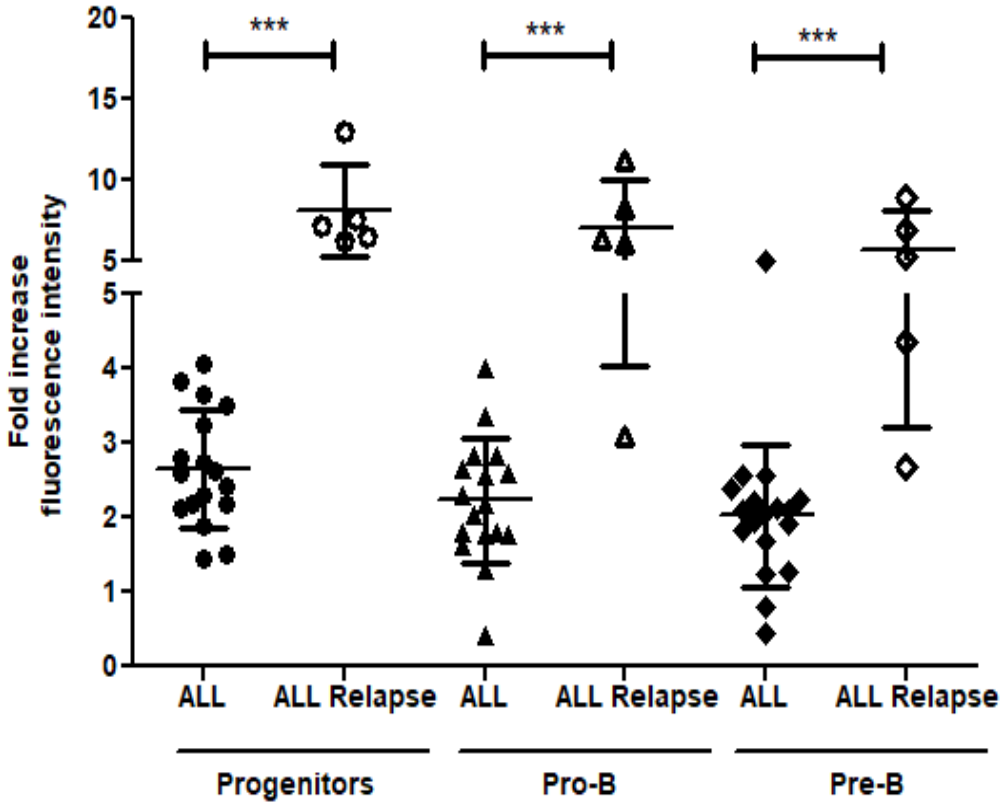

Figure 21. Cortactin expression levels in primitive populations of bone marrow from ALL patients. Flow cytometry analysis of cortactin levels in precursor, pro-B and pre-B cells from the same individual patients with either B-ALL or B-ALL with relapse. One-way ANOVA with Bonferroni post-test for p value calculation were performed for multiple comparisons. ***p<0.05.

Table 3. Comparison between cortactin expression levels and clinical parameters of patients (p values determined by Mann-Whitney U test).

VARIABLE	P value		MEAN (FOLD INCREASE EXPRESSION)
Sex	0.791	Female	3.04
		Male	3.97
Risk	0.382	High	3.90
		Standard	2.08
Hyperleukocytosis	1.000	Positive	3.18
		Negative	3.69
Immunophenotype	0.572	Pro B; Pro/Pre B	3.85
		Pre B	2.91
Translocation	0.556	Positive	3.51
		Negative	3.72
Response to steroids	**0.051**	**Positive**	**2.87**
		Negative	**6.57**
Infections	0.545	Positive	3.01
		Negative	3.69
Cytopenia	0.533	Positive	3.48
		Negative	3.75
Hepatomegaly	0.711	Positive	3.24
		Negative	3.97
Splenomegaly	0.903	Positive	3.68
		Negative	3.52
Adenomegaly	**0.013**	**Positive**	**4.17**
		Negative	**1.49**
Relapse	**0.005**	**Positive**	**6.89**
		Negative	**2.45**
Death	0.313	Positive	4.32
		Negative	3.69

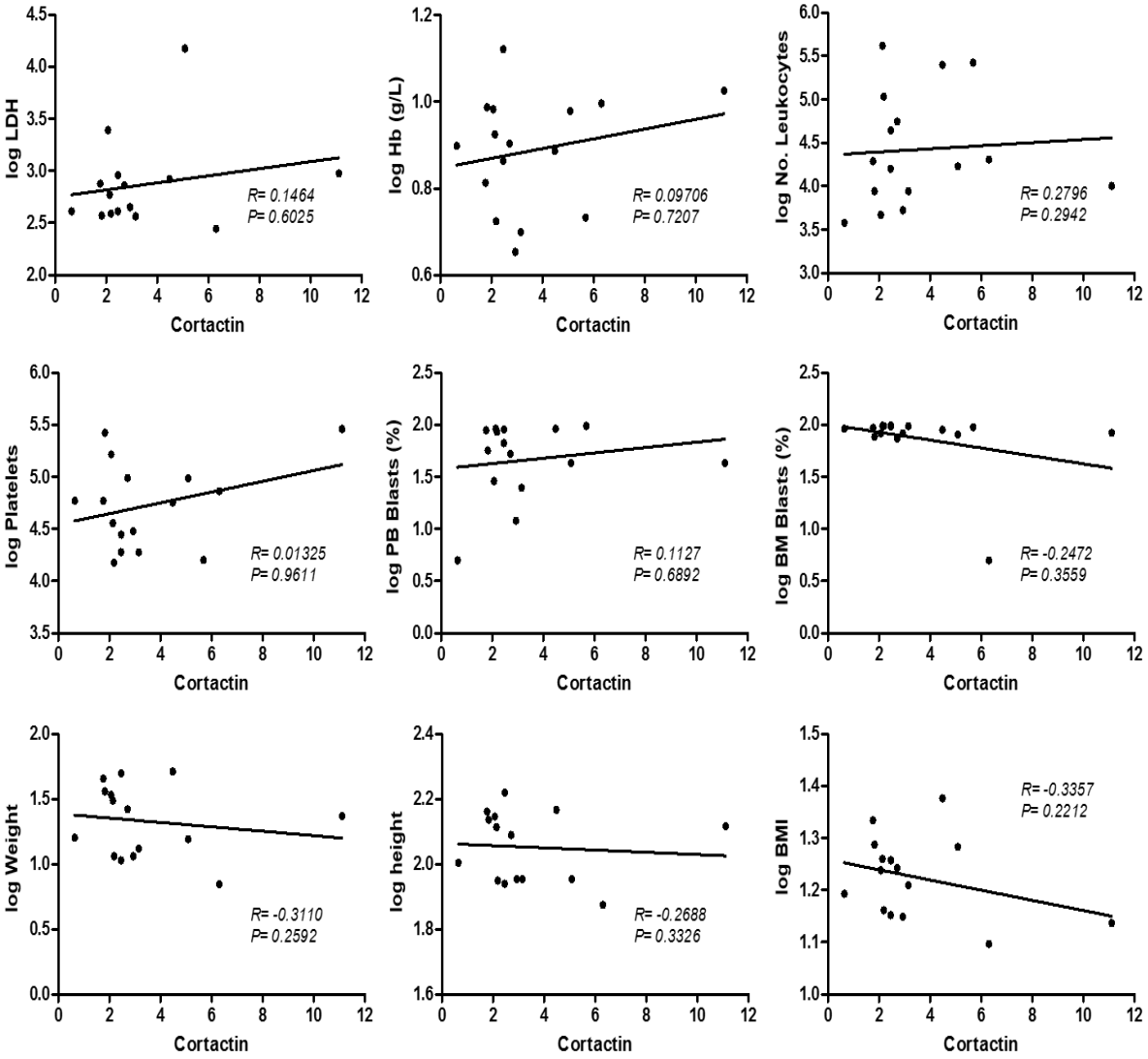

Figure 22. Correlation analysis of cortactin levels with patient data. Fold increase expression of cortactin in progenitor and precursor B cells from bone marrow of B-ALL patients (n=23) was determined by flow cytometry (normalized to the mean fluorescence intensities from UCB cells as controls), and then correlated to the indicated patient data. Y-axis in logarithmic scale.

8.11 Infiltrating and relapse cells from pediatric B-ALL patients show highest levels of cortactin.

The role of cortactin in *in vivo* migration and extramedullary infiltration was explored in REH- and patient-derived leukemia xenografts (REH-DX and PDX, respectively). Once the xenotransplanted NSG mice exhibited leukemic burden or disease signs, they were euthanized and BM, brain, testis, lung, liver and spleen processed and analyzed for infiltration by human $CD45^{+}$-hematopoietic cells (Fig. 23A and 23B). Both, REH-DX and PDX pre-clinical models demonstrated leukemic cell infiltration of brain,

testis and lung, organs known to be targets for leukemic infiltration (Malempati et al., 2007; Nucci, Nouér, & Anaissie, 2015). Importantly, infiltrated cells expressed cortactin, with the highest levels detected in B-ALL cells isolated from brain, testis and lung (Fig. 24A and 24B), strongly suggesting that only B-ALL cells with high cortactin levels are capable of inducing infiltrative B-ALL disease in the PDX model.

8.12 BM relapse in ALL is correlated with differential cortactin levels in primitive cells and their capability of colonizing bone marrow stem cell niches

Due to the fact that leukemia-initiating cells are established in normal niches and coexist with normal cells within the same niche, we wanted to know whether the ability of relapsed leukemic cells correlate with high cortactin levels in these cells. Applying an organoid-like tridimensional stromal cell co-culture system recently reported by us (Balandrán et al., 2017), we found that in all cell types tested, cortactin expression was highest in the cells that colonized the spheroids (Figure 25 A-D). Moreover, B-ALL cells from the relapsed patient P21 had a higher capacity of colonizing stromal cell spheroids compared to the non-relapsed patient P20, and the cell line REH, isolated from a relapsed patient, colonized spheroids in higher numbers compared to RS4:11 cells (Figure 25 E).

Keeping in mind the differences of cortactin levels in REH cells (cortactin-high) and RS4:11 cells (cortactin-low), we performed a competition colonization assay with both cell lines *in vitro* using spheroids of the stromal cell line OP9 transfected with GFP. REH cells were labelled with celltrance far red and RS4:11 cells with celltrance violet to distinguish them. Then they were co-incubated with the spheroids as described in Methods. As expected, we found a higher frequency of REH cells within the spheroids indicating that they have a clear advantage over RS4:11 cells to colonize the spheroids (Figure 26).

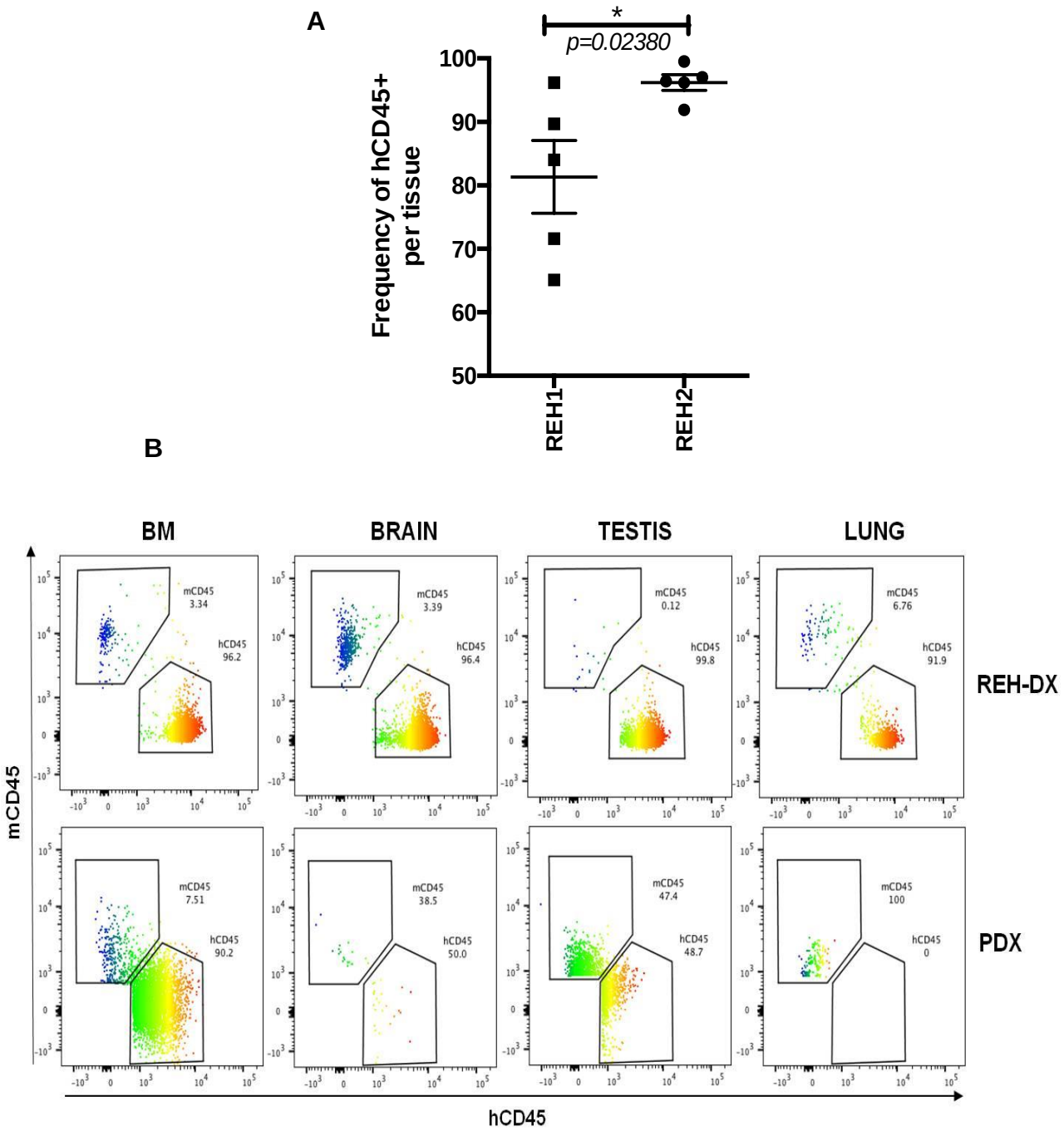

Figure 23. Xenotransplant of leukemic infiltrative cells. A) NSG mice were xeno-transplanted with REH or primary B-ALL cells. REH-derived xenografts (REH-DX) and patient-derived xenografts (PDX) were analyzed for infiltrated cells in BM, brain, testis and lungs by flow cytometry. Cell frequencies of human hematopoietic cells ($hCD45^+$) from total leukocyte counts ($mCD45^+$ and $hCD45^+$) were recorded 5 weeks (REH1) or 6 weeks (REH2) after transplantation. **E)** Representative plots of gated live, infiltrated human leukocytes are shown for REH-DX and PDX

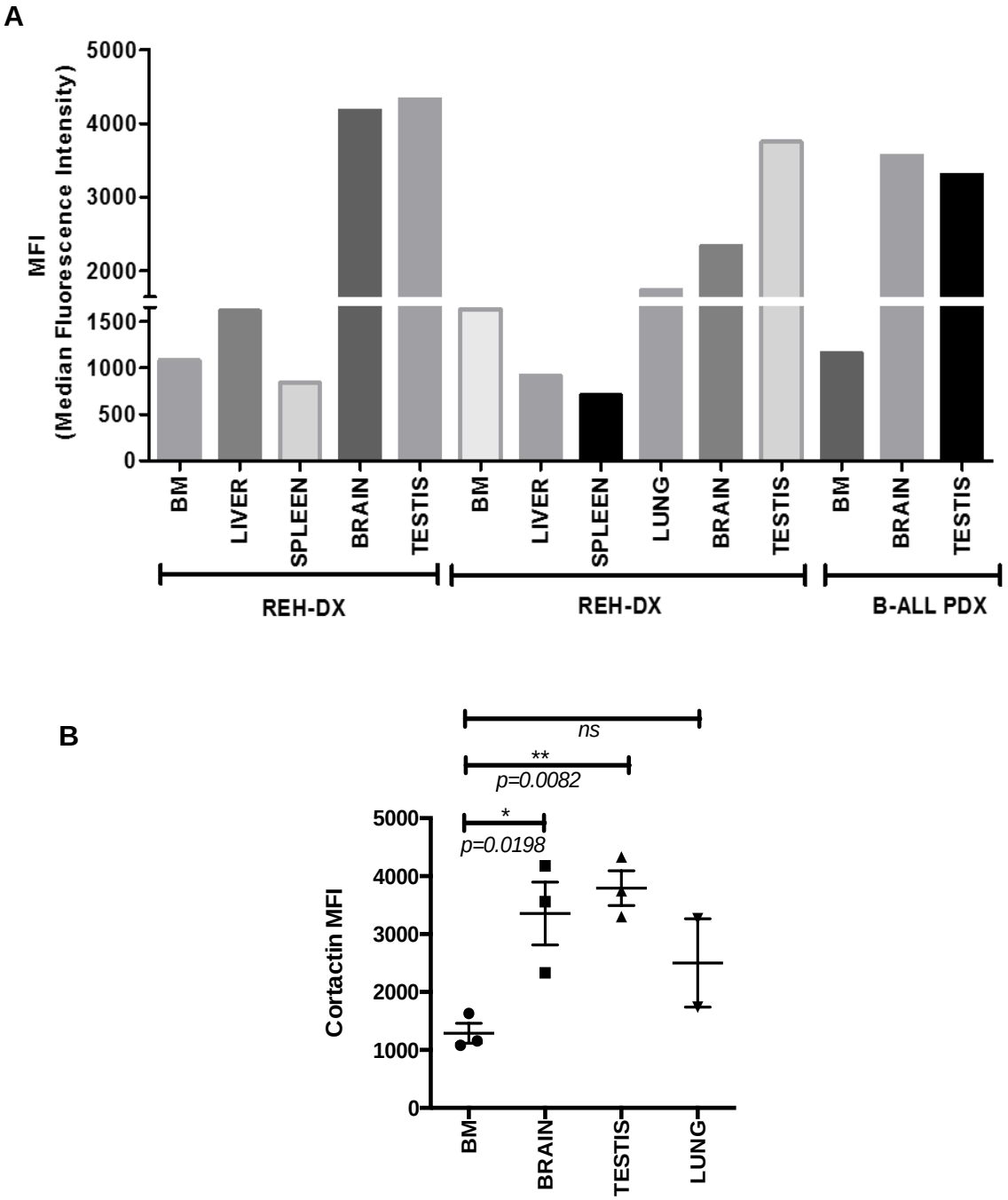

Figure 24. Cortactin level expression in leukemic infiltrative cells. **A)** Cortactin levels in hCD45^{+}-cells derived from BM, brain, testis and lungs analyzed by flow cytometry (n=3). **B)** Cortactin values of each xeno-transplant in the tested tissues.

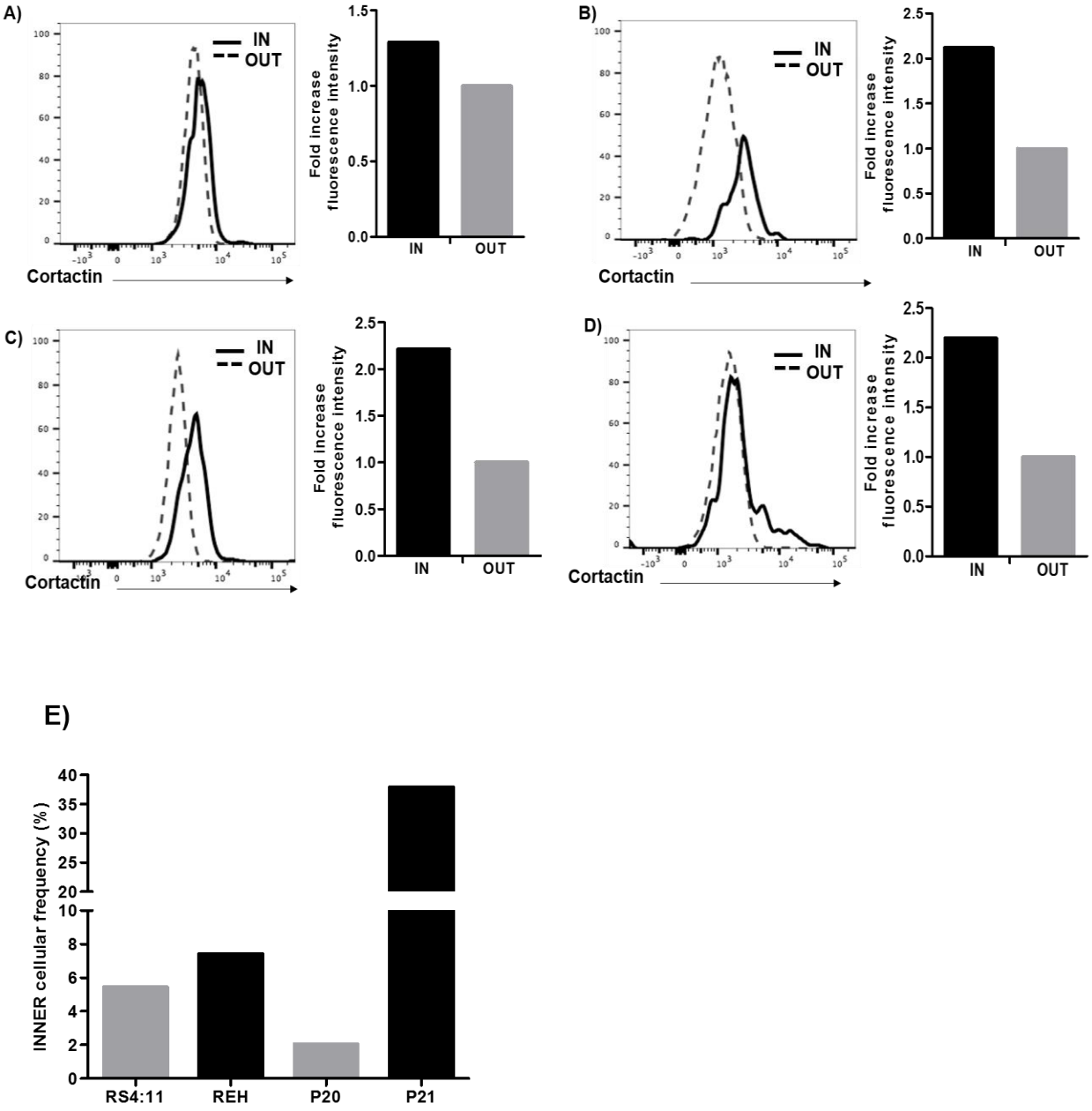

Figure 25. **Cortactin levels in B-ALL cells determine their potential to colonize bone marrow**. Stromal spheroids were co-cultured with RS4:11 and REH cells (**A** and **C**, respectively) or mononuclear cells from patients P20 and P21 (**B** and **D**, respectively). After 24 hours, stromal spheroids and the respective supernatants were analyzed for cells expressing CD45 to identify leukemic cells within spheroids (IN) and cells in suspension (OUT). Cortactin levels of IN and OUT cells were determined by flow cytometry. Data are shown as mean fluorescence intensity of $CD45^+$ cells. **E)** Comparison of the colonization potential of the different leukemic cells tested after 24 hours of co-culture. Data from a single experiment are shown.

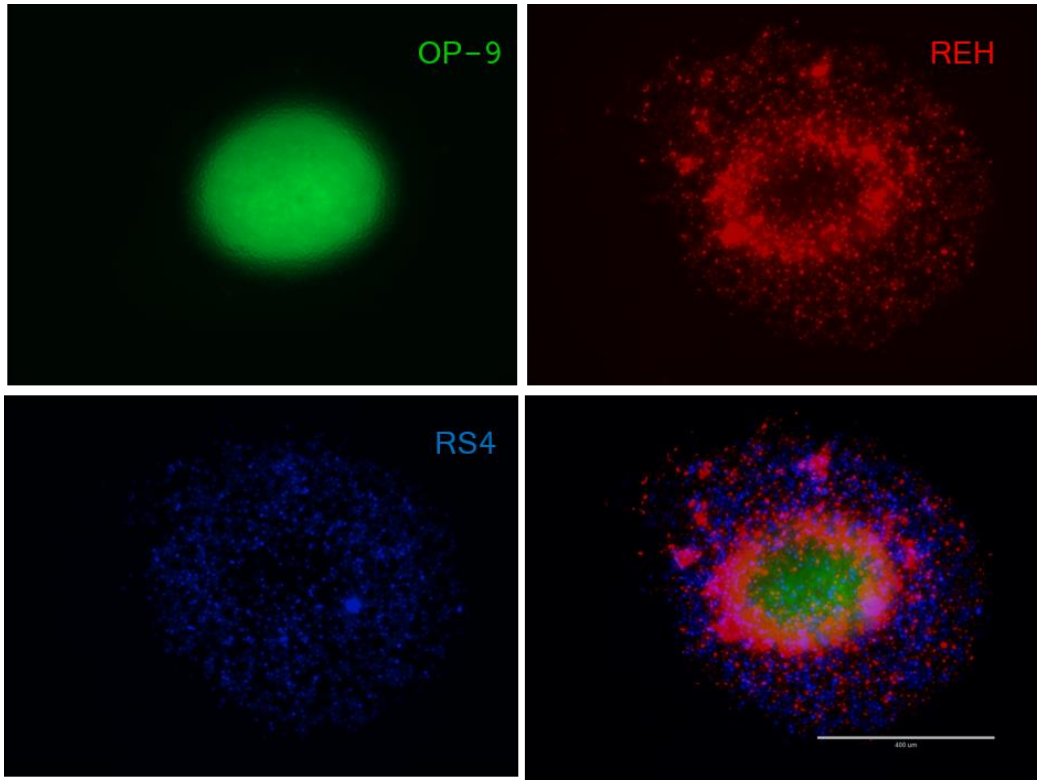

Figure 26. Representative images of competition colonization assays of the pre-B leukemic cells REH and RS4:11 in spheroids. Blue RS4:11 cells and red REH cells were put in co-culture with spheroids composed of the stromal cell line GFP-OP9 and after 24h cells were fixed and observed by confocal microscopy. The cortactin-high REH cell line has a clear advantage over RS4:11 cells to colonize spheroids. Bar=400 µm

Normal cells residing in hematopoietic stem cell niches within BM are mostly quiescent and intermittently enter the cell cycle. Thus, we investigated whether this is also true for leukemic precursors at disease relapse by using our organoid-like tridimensional stromal cells co-culture system (Kirkbride et al., 2011). This system provides more natural microenvironments like those constituting lymphoid BM reticular niches, including stromal interconnection and internal $CXCL12^{+}$-hypoxic zones. We indeed recovered significantly more G_0 cells from within the spheroids (Fig. 27A). However, the highest cortactin expression was recorded in the cycling cell population that entered the spheroids, whereas cortactin was lowest in the quiescent populations (Fig 27B) and derived xenograft of REH cells into NSG mice recipient model (REH-DX) confirmed that $cortactin^{high}$-leukemic cells in the BM were cycling *in vivo* (Fig. 27C and 27D). Thus, our study clearly shows that $cortactin^{high}$-B-ALL progenitors are mainly migrating and proliferating cells endowed with the capability of colonizing hypoxic hematopoietic niches and infiltrating extramedullary organs.

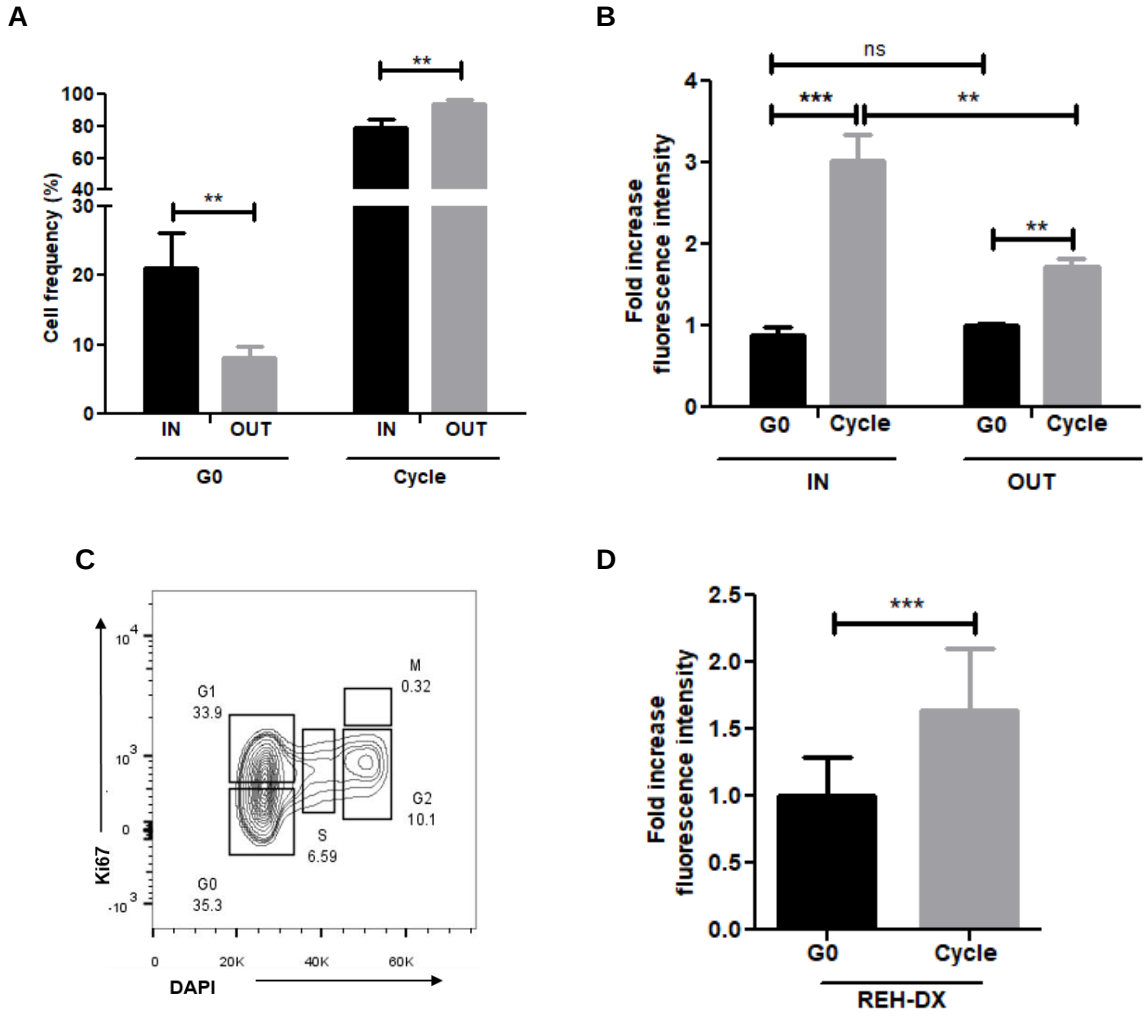

Figure 27 Cortactin^{high} B-ALL cells that home to BM niches are in cell-cycle. Stromal spheroids were co-cultured with REH cells for 24 h, followed by flow cytometry analyses to identify whether leukemic cells within spheroids (IN) or in suspension (OUT) are in cell cycle. **A)** Cell cycle status was determined by Ki67 and DAPI staining, and cell frequencies of quiescent (G0) and cycling (G1-S-G2-M) IN and OUT populations were calculated. **B)** Cortactin levels in quiescent (G0) and cycling (G1-S-G2-M) cells were investigated by flow cytometry and normalized to values of G0 cells (IN) in CD45$^+$-cells (n=3). **C)** NSG mice were xeno-transplanted with REH cells. Upon disease establishment, mice were sacrificed and REH-derived xenograft (REH-DX) cells collected from BM and stained with anti-mCD45, anti-hCD45, anti-hKi67 and DAPI. A representative plot of hCD45$^+$-cells is shown (n=3). **D)** Cortactin levels in G0 and cycling REH-DX BM cells were determined by flow cytometry and normalized to G0 cells (IN) (n=2). **p<0.01; ***p<0.001.

8.13 Cortactin participates in the establishment of B-ALL cells in CXCL12-dependent niches of the inner bone marrow

We reported before using a tridimensional stromal cell co-culture system that establishment of pre-leukemic cells in certain BM niches is CXCL12-depedent. Thus, we wondered whether cortactin participates in niche stabilization via CXCR4. We

observed by co-culture IF stainings discrete areas where CXCR4 and cortactin co-localized at cell contact areas of B-ALL with stromal cells (Figure 28A). Additionally, we detected a tendency towards higher CXCR4 expression at the plasma membrane of cortactin-high B-ALL cells from relapsed patients when compared to non-relapsed patients (Figure 28B).

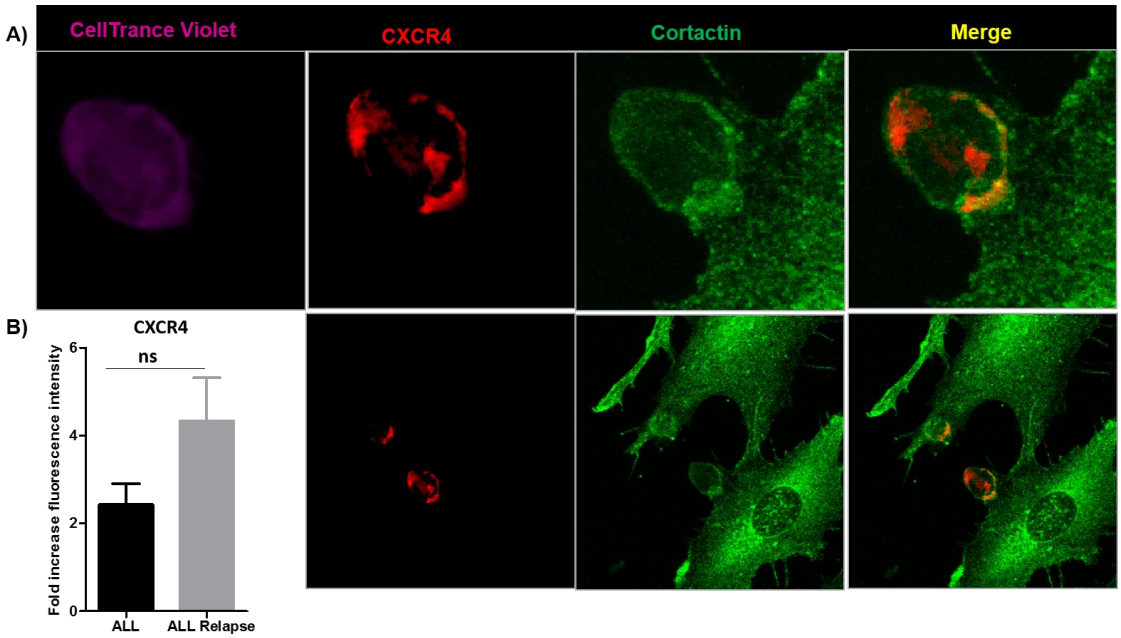

Figure 28. CXCR4 and cortactin participate in the establishment of leukemic cells in CXCL12 niches in the bone marrow. Co-culture systems of stromal cells and REH cells. REH cells were labelled using the vital dye cell trance violet before incubating them on a subconfluent monolayer of stromal cells for 2 hours. After fixation, cells were stained with anti-CXCR4 (red) and anti-cortactin (green) to determine areas of colocalization (n=2). **B)** Surface CXCR4 expression was analyzed by flow cytometry in progenitor and precursor B cells from bone marrow samples of children diagnosed with either B-ALL (n=18), or B-ALL with relapse to bone marrow (n=5). Data are normalized to CXCR4 levels in UCB-derived mononuclear cells (set to 1) and shown as fold increase of the mean fluorescence intensity. Unpaired, two-tailed Student's t-test was used to determine statistically significant differences between experimental groups. Error bars in figures represent SD. **p<0.01.

7. DISCUSSION

High expression levels of the ABP cortactin have been associated to poor prognosis in many solid cancers and in hematological malignancies like chronic lymphocytic leukemia. Our current investigation suggests that no significant differences in age, gender, WBC count at diagnosis and risk group between high and normal cortactin expressing individuals are obvious in childhood B-lineage acute lymphoblastic leukemia (ALL). However, patients with bone marrow (BM) relapse, patients with failure to steroids treatment, adenomegaly and high counts of platelets, displayed higher amounts of cortactin in their leukemic B-cell precursor cells.

Cortactin is an ABP highly relevant for cellular functions such as adhesion, migration and transmigration in most tissues and cell categories other than hematological. In contrast, the cortactin homologous protein HS1 has similar roles in both, myeloid and lymphoid lineage cells of the hematopoietic system, and has been clearly implicated in migration and invasion processes of malignant B cells in adult chronic lymphocytic leukemia (CLL) (Hacken et al., 2013; Yu et al., 2017). Both, cortactin and HS1 directly interact with F-actin via an F-actin domain and with the Arp 2/3 complex through their N-terminal domain to stabilize the actin branching process that is critical to allow formation of cell structures involved in migration such as lamellipodia or invadopodia (Kirkbride et al., 2011).

Strikingly, an aberrantly high cortactin expression was recently found in CD5+CD19+ cells from the adult B-lineage pathology chronic lymphoblastic leukemia (CLL), in comparison to the normal counterpart or even to CD5-CD19+ cells from the same patients. Indeed, high cortactin expression in CLL was related to unfavorable outcomes such as absence of somatic hypermutation in immunoglobulin heavy-chain variable region (IgVH), ZAP-70 high expression, CD38 high expression and karyotype 12+/11q-/17p- (Gattazzo et al., 2014).

In the current work, we demonstrate for the first time a graded overexpression and subcellular location of cortactin in pre-B leukemic cell lines from ALL. Cortactin was identified in the cytosol and preferentially at cell edges where HS1 and enriched F-actin structures were present suggesting that in pre-B ALL cells cortactin is implicated in actin cytoekeleton remodeling, and stabilizing actin-branching for the formation of lamellipodia, invadopodia or invadosomes as described in other cell

types (Helgeson & Nolen, 2013; Kirkbride et al., 2011; MacGrath & Koleske, 2012). Of note, we found that ALL pre-B cells express a 60 kDa cortactin variant, termed SV2 (Figure 6) that is a product of alternative splicing affecting the actin-binding repeats. While wild type cortactin has an actin-binding domain formed by 6.5 tandem repeat of 37 amino acid each resulting in a protein of 80 kDa, the actin-binding domain in variant SV1 consists only of repeats 1 to 5 (lacking exon 11) giving rise to a 70 kDa protein, and SV2 of repeats 1 to 4 (lacking exons 10 and 11), producing a 60 kDa protein (van Rossum et al., 2003). *In vitro* assays have shown that the affinity to bind F-actin is dependent on the number of repeats. Thus, WT cortactin is endowed with higher affinity, whereas SV2 affinity for F-actin is lower. Nevertheless, these variants conserve their capacity to interact with the Arp 2/3 complex and their subcellular location is apparently the same. Importantly, the fourth repeat is essential to participate in actin polymerization mediated by the Arp 2/3 complex (Weed et al., 2000).

When assessing cortactin levels in the two types of leukemic pre-B cells, we observed that the highest protein levels are obvious in the REH cell line when compared to RS4:11 cells (Figure 8). Despite their apparently similar phenotype and developmental stage, these two cell lines were derived from patients with distinct clinical conditions: the BM cell line RS4:11 cell is a poor-prognosis with BM relapse ALL characterized by the translocation 4:11, whereas the source of REH cells is peripheral blood from a patient diagnosed with the translocation 12:21, presumably considered as good prognosis, but with an episode of BM relapse (Table 2).

Of interest, high cortactin levels matched with CXCL12-dependent transmigratory capacity in REH cells, which outcompetes RS4:11 in a transendothelial migration assay with CXCL12. Independent experiments show that even in resting status, cortactin levels differ in the two cell lines, although in both cell lines the recorded expression is higher in transmigratory cells at the bottom compared to the cells on top of the endothelial monolayer. This was even more evident for relapsed REH cells than non-relapsed RS4:11 (Figure 12). This difference may result from two possible mechanisms: 1) a cell subset within the leukemic population that is not detectable until it is selected to transmigrate or 2) *de novo* synthesis or activation by environmental signals upon adhesion to endothelial cells and migration toward the CXCL12 gradient.

In a number of tumor cells, including T-ALL cells, cortactin activation seems to be first stimulated by CXCL12, promoting lamellipodia and invadopodia formation (de Bock & Cools, 2015; Passaro et al., 2015a; Pitt et al., 2015). According to recent findings from Luo and colleagues, cortactin regulates the dynamics of the GPCR CXCR4 in the presence of CXCL12. This response induces transient translocation of cortactin from endosomal compartments to the cell periphery, where it colocalizes with CXCR4 followed by internalization of the receptor and cortactin translocation into endosomes. Accordingly, cortactin depletion impaires CXCR4 recycling and results in a defect in migratory capacity in response to CXCL12 (Luo et al., 2006). On the other hand, CXCR4 traffic is calcineurin-dependent and regulated by the expression of cortactin in T-ALL. Its inhibition results in impaired actin dynamics in the endosomal compartment and defects in migration, homing to BM and abnormal positioning of T-ALL cells in hematopoietic niches resulting in defective interactions with stromal cells in the context of CXCR4/CXCL12 signaling (Passaro et al., 2015a).

As expected, B-precursor cells from primary ALL BM showed a generally high intracellular expression that did not distinguish patient groups at disease debut (Figure 16). Despite $CD34^+$ progenitor cells, $CD34^+CD19^+$ pro-B and $CD34^-CD19^+$ pre-B precursor cells from ALL BM all showing cortactin overexpression, no correlations with immunophenotypes were found by Mann Whitney U statistical test as shown in table 3. However, a tendency to greater abundance in primitive B-cell progenitors and pro-B subsets was identified. In keeping with the major roles of cortactin in migratory events, we then explored whether infiltrative leukemic cells to central nervous system (CNS) were cortactin-expressing cells. Cerebral spinal fluid specimens from infiltrative ALL cases were strikingly positive for cortactin.

Notably, when a relapse group was included in the study, our statistical analysis crucially showed a positive correlation of highest levels of cortactin with relapse to bone marrow, as suggested from our REH cell line data. Mann-Whitney U or Pearson's correlation tests indicated that nearly 10-fold increased expression of cortactin was detected in relapse BM cells with respect to their normal counterparts, while a nearly 8-fold increase was detected when compared to non-relapsed ALL patients (Figure 20). Furthermore, failure to steroids response, another critical aspect in the disease stratification, showed a negative relationship with cortactin levels (Table 3). In addition, adenomegaly, that is one of the signs of infiltrative

syndrome, and platelets numbers, were directly or indirectly related to cortactin expression within leukemic cells.

It is well known that the improvement in outcomes for childhood ALL is one of the greatest success stories of modern oncology, except for the terrible prognosis for patients who relapse with a drug resistant phenotype showing survival rates ranging from only 10% to just over 50% depending on important prognostic factors such as site of relapse, duration of first remission, and initial risk group (Wook Lee & Cho, K J Pediatr 2017). Relapse is the most important cause of treatment failure in ALL and novel treatment strategies like immunotherapy have proven to be highly promising, although the best way to treat relapse would be to prevent it (Pierro J, Expert Rev Anticancer Ther 2017; Wook Lee & Cho, K J Pediatr 2017).

Leukemia relapse has been related to survival of a unique leukemic initiating cell (LIC) with special biological attributes and resistance to drug therapy. A number of theories have been built on major LIC roles in relapse. First, therapy-resistant subclones may have quiescence characteristics and reemerge with intrinsic genetics different from that shown by similar clones at first diagnosis, until microenvironment stimuli allow their proliferation and promote disease progression after therapy (Bhojwani & Pui, 2013; Ebinger et al., 2016). Secondly, LIC might be conspicuous subclones that remain hidden and adhered in specific niches located in deep inner BM structures where therapy does not reach, assuring their survival (Duan et al., 2014; Ebinger et al., 2016). Chemotherapy resistance of LIC might be due to low cellular division or staying in the dormant phase that contribute to avoid their elimination. Indeed, survival could be promoted by interaction of leukemic cells with bone marrow stromal cells, a phenomenon named cell adhesion-mediated drug resistance (CAM-DR), where alpha 4 integrin is presumably the adhesion molecule responsible for homing to BM, retention of leukemic cells and chemoresistance (Shishido, Böning, & Kim, 2014). Xenotransplantation approaches and tridimensional models of tumor microenvironment in head and neck cancer have demonstrated that inhibition of β1 integrin enhances the sensitivity to radiotherapy and promotes dephosphorylation of focal adhesion kinase (FAK), resulting in dissociation of the FAK/cortactin protein complex that downregulates JNK signaling and induces cell rounding leading to radiosensitation (Eke et al., 2012).

Our observation of a correlation between high cortactin levels and high platelet counts is interesting, especially due to recent findings from Paul Frenette's Lab indicating the role of megakariocytes in hematopoietic niches and retention of HSC in a nonrandom localization by secretion of CXCL4 or PF4 (patelet factor 4) that regulates HSC cell cycle activity. Depletion of megakaryocytes results in loss of HSC quiescence (Bruns et al., 2014)

When only relapsed cases were investigated for transmigratory cell abilities, differential expression of cortactin in the same population determined such capacity, as previously observed with cell lines, where higher cortactin amounts in basal conditions presumably drive migration through endothelial monolayers. No obvious migration advantage for any of the progenitor or precursor populations was seen, supporting the idea that a phenotype-based hierarchy is lacking in ALL presentation or at re-emerging relapse clinical condition. However, a functional or metabolic/activational hierarchy may exist to determine the possibility to invade BM or extramedullary organs. As TEM is a prerequisite for infiltration and cortactin is required for proper TEM, high cortactin expression was also detected in infiltrated B-ALL cells from CSF specimens, and in primary B-ALL cells from xenografted lung, brain and testis.

Whether LIC, pre-programmed to re-emerge in relapse have also high affinity for normal hematopoietic stem cells (HSC) niches is unknown. Under homeostatic conditions, CXCL12 production by BM stromal cells is critical for lymphopoiesis development; and CXCL12 deficiency is lethal due to lack of B lymphopoiesis and myelopoiesis. CXCL12 constantly produced by bone marrow stromal and endothelial cells participate in migration, proliferation and differentiation of HSC through CXCR4 (Sison & Brown, 2011). In acute lymphoblastic leukemia of T cells, Pitt and colleagues demonstrated that CXCR4 signaling is critical for progression of the disease by permiting LIC stabilization in CXCL12-rich niches. Loss of CXCR4 signaling inhibits metastasis and leads to higher susceptibility of leukemic cells for apoptosis. Moreover, conditional deletion of CXCR4 in mouse T-ALL cells results in absence of infiltration to bone marrow and other extramedullary organs such as spleen and thymus (de Bock & Cools, 2015). Accordingly, high expression of CXCR4 by leukemic cells and activation of the CXCR4-CXCL12 axis is involved in poor prognosis of leukemia, possibly by an increased protection of malignant cells in the BM (Sison & Brown, 2011). LIC are potentially established in normal niches

and coexist with non-leukemic cells before the blast-driven microenvironmental remodeling diminishes CXCL12 and consequently alters normal hematopoiesis (Balandrán et al., 2017; J. A. Kim et al., 2015; Tabe & Konopleva, 2014). We have recently demonstrated in an organoid-like tridimensional stromal cell co-culture system, that under pro-inflammatory BM conditions, CXCL12 input is reduced and the very HSC hypoxic niches are diluted allowing gradual leukemogenesis and maintenance of tumor clones (Balandrán et al., 2017). After therapy and during remission, cellular contents and biological structures of the BM niches may resemble normality, as CXCL12 production should gradually return to normal values and normal hematopoiesis is re-established. Notably, our tridimensional co-culture system studies now indicate that in relapse, the capacity to colonize inner areas relates to highest cortactin levels in primary ALL B precursor cells, as well as in relapse-derived REH cell line, strongly suggesting that relapse cells are highly migrating and exhibit primitive characteristics such as affinity for inner hypoxic niches, low cycling and drug resistance.

We propose that high expression levels of the ABP cortactin provide probably relapsing $CXCR4^{+}$ ALL cells with the ability of migration and positioning themselves in protective microenvironmental structures that promote their survival after chemotherapy (Figure 28).

10 CONCLUSION

On the basis of cortactin expression levels, two major ALL cell populations can be distinguished with the highest amounts correlating with biological characteristics of leukemia initiating cells (LIC) and clinical aggressiveness such as transmigratory capacity, extramedullary infiltration, BM colonization ability, failure to response to steroids and relapse. Thus, cortactin expression levels might be a potential independent relapse risk marker for ALL children.

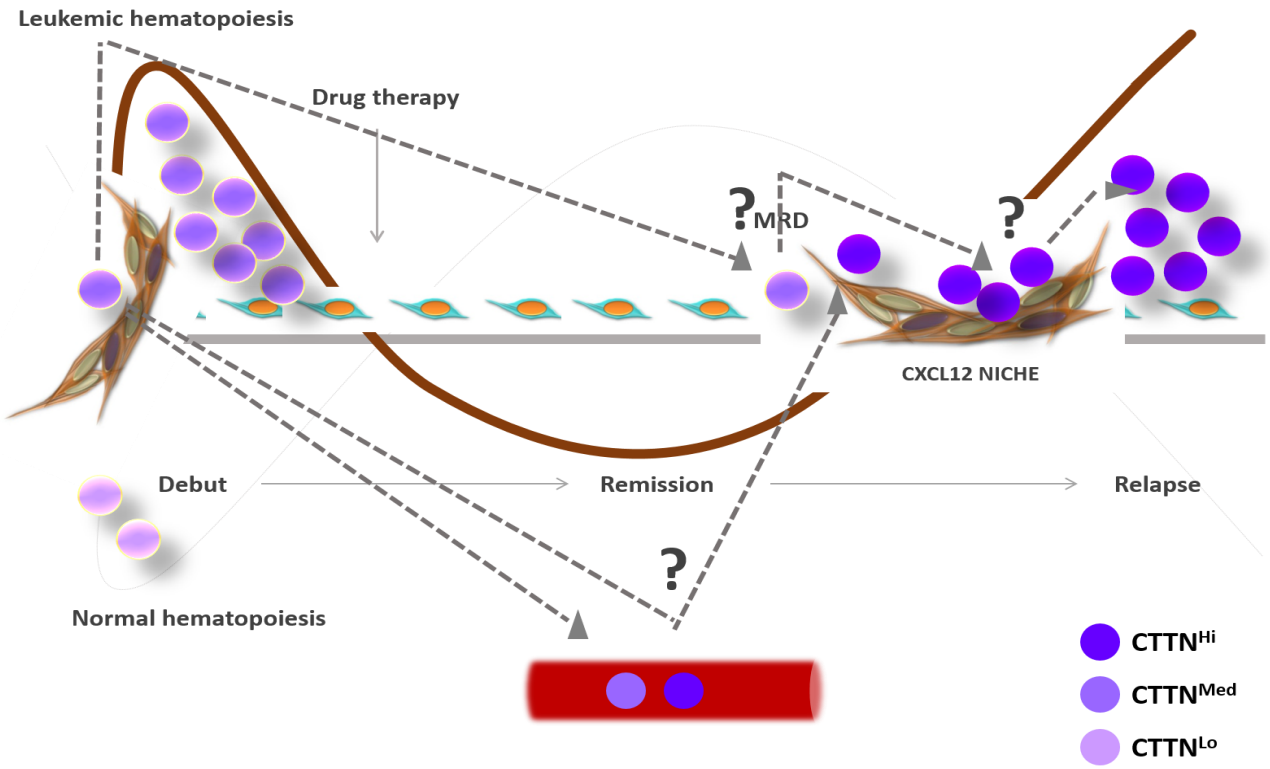

Figure 29. Model of potential implications of cortactin in repositioning cortactin-high expressing leukemic cells in protective niches during relapse
At leukemia presentation, malignant hematopoietic differentiation is prevalent, whereas normal hematopoiesis is crucially compromised. Over time and upon chemotherapy, cycling tumor cells are controlled and a gradual reconstitution of normal cells takes place. A number of cases relapse due to *de novo* growing of quiescent clones with chemoresistance and stemness properties, and endowed with the highest expression levels of cortactin. Cortactin in leukemic precursor cells might be involved in their CXCL12-dependent migratory abilities and their migration and survival in specific niches, from where leukemia-initiating cells (LIC) reemerge and resist to therapy leading to disease relapse. The population dynamics of cortactin^{lo}, cortactin^{med} and cortactin^{hi} subsets remains to be explored.

PERSPECTIVES

To analyze in a retrospective study with B-ALL patients cortactin levels at debut, during treatment and at relapse.

To observe transcriptome changes by RNA sequencing in bone marrow samples of B-ALL patients at debut and at relapse.

To determine resistance or sensitivity to chemotherapy treatments (vincristine and doxorubicin) of bone marrow samples of B-ALL patients, using 3D co-culture and to observe the effect of chemotherapy on cortactin levels.

To obtain a REH cortactin *knock-down* cell line and to analyze transendothelial migration *in vitro* and their BM colonization potential using 3D co-cultures.

To realize xenotransplants of REH cortactin *knock-down* and WT cells in a 1:1 ratio to observe their infiltrative capability into organs such as lung, brain and testis.

To analyze whether cortactin *knock-down* in REH cells has an effect on the expression and activation of VLA-4 and CXCR4 after treatment with CXCL12.

To identify possible interactions between cortactin and CXCR4 after treatment with CXCL12 using co-immunoprecipitation assays.

REFERENCES

Balandrán, J. C., Purizaca, J., Enciso, J., Dozal, D., Sandoval, A., Jiménez-Hernández, E., … Pelayo, R. (2017). Pro-inflammatory-related loss of CXCL12 niche promotes acute lymphoblastic leukemic progression at the expense of normal lymphopoiesis. *Frontiers in Immunology*, *7*(JAN), 1–14. https://doi.org/10.3389/fimmu.2016.00666

Beck, T. C., Gomes, A. C., Cyster, J. G., & Pereira, J. P. (2014). CXCR4 and a cell-extrinsic mechanism control immature B lymphocyte egress from bone marrow. *The Journal of Experimental Medicine*, *211*(13), 2567–81. https://doi.org/10.1084/jem.20140457

Bhojwani, D., & Pui, C.-H. (2013). Relapsed childhood acute lymphoblastic leukaemia. *The Lancet Oncology*, *14*(6), e205–e217. https://doi.org/10.1016/S1470-2045(12)70580-6

Bigildeev, A. E., Shipounova, I. N., Svinareva, D. A., & Drize, N. J. (2011). Leukemia cells invading the liver express liver chemokine receptors and possess characteristics of leukemia stem cells in mice with MPD-like myeloid leukemia. *Experimental Hematology*, *39*(2), 187–194. https://doi.org/10.1016/j.exphem.2010.11.005

Bruns, I., Lucas, D., Pinho, S., Ahmed, J., Lambert, M. P., Kunisaki, Y., … Frenette, P. S. (2014). Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nature Medicine*, *20*(11), 1315–1320. https://doi.org/10.1038/nm.3707

Chiarini, F., Lonetti, A., Evangelisti, C., Buontempo, F., Orsini, E., Evangelisti, C., … Martelli, A. M. (2016). Advances in understanding the acute lymphoblastic leukemia bone marrow microenvironment: From biology to therapeutic targeting. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*(3), 449–463. https://doi.org/10.1016/j.bbamcr.2015.08.015

Clark, E. S., Whigham, A. S., Yarbrough, W. G., & Weaver, A. M. (2007). Cortactin is an essential regulator of matrix metalloproteinase secretion and extracellular matrix degradation in invadopodia. *Cancer Research*, *67*(9), 4227–4235. https://doi.org/10.1158/0008-5472.CAN-06-3928

Clevers, H. (2011). The cancer stem cell: premises, promises and challenges. *Nature Medicine*, *17*(3), 313–319. https://doi.org/10.1038/nm.2304

Colmone, A., & Sipkins, D. A. (2008). Beyond angiogenesis: the role of

endothelium in the bone marrow vascular niche. *Translational Research*, *151*(1), 1–9. https://doi.org/10.1016/j.trsl.2007.09.003

Conforti, A., Biagini, S., Del Bufalo, F., Sirleto, P., Angioni, A., Starc, N., … Bernardo, M. E. (2013). Biological, functional and genetic characterization of bone marrow-derived mesenchymal stromal cells from pediatric patients affected by acute lymphoblastic leukemia. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0076989

de Bock, C. E., & Cools, J. (2015). T-ALL: Home Is where the CXCL12 Is. *Cancer Cell*, *27*(6), 745–746. https://doi.org/10.1016/j.ccell.2015.05.011

de Gorter, D. J. J., Beuling, E. A., Kersseboom, R., Middendorp, S., van Gils, J. M., Hendriks, R. W., … Spaargaren, M. (2007). Bruton's Tyrosine Kinase and Phospholipase Cγ2 Mediate Chemokine-Controlled B Cell Migration and Homing. *Immunity*, *26*(1), 93–104. https://doi.org/10.1016/j.immuni.2006.11.012

De Lourdes Perim, A., Amarante, M. K., Guembarovski, R. L., De Oliveira, C. E. C., & Watanabe, M. A. E. (2015). CXCL12/CXCR4 axis in the pathogenesis of acute lymphoblastic leukemia (ALL): A possible therapeutic target. *Cellular and Molecular Life Sciences*, *72*(9), 1715–1723. https://doi.org/10.1007/s00018-014-1830-x

Deynoux, M., Sunter, N., Hérault, O., & Mazurier, F. (2016). Hypoxia and Hypoxia-Inducible Factors in Leukemias. *Frontiers in Oncology*, *6*(February), 1–9. https://doi.org/10.3389/fonc.2016.00041

Dick, J. E. (2008). Stem cell concepts renew cancer research. *Blood*, *112*(13), 4793–4807. https://doi.org/10.1182/blood-2008-08-077941

Ding, L., & Morrison, S. J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*, *495*(7440), 231–5. https://doi.org/10.1038/nature11885

Doan, P. L., & Chute, J. P. (2012). The vascular niche: home for normal and malignant hematopoietic stem cells. *Leukemia*, *26*(1), 54–62. https://doi.org/10.1038/leu.2011.236

Dorantes-Acosta, E., & Pelayo, R. (2012). Lineage Switching in Acute Leukemias: A Consequence of Stem Cell Plasticity? *Bone Marrow Research*, *2012*, 1–18. https://doi.org/10.1155/2012/406796

Duan, C. W., Shi, J., Chen, J., Wang, B., Yu, Y. H., Qin, X., … Hong, D. L.

(2014). Leukemia propagating cells rebuild an evolving niche in response to therapy. *Cancer Cell*, *25*(6), 778–793. https://doi.org/10.1016/j.ccr.2014.04.015

Ebinger, S., Özdemir, E. Z., Ziegenhain, C., Tiedt, S., Castro Alves, C., Grunert, M., … Jeremias, I. (2016). Characterization of Rare, Dormant, and Therapy-Resistant Cells in Acute Lymphoblastic Leukemia. *Cancer Cell*, *30*(6), 849–862. https://doi.org/10.1016/j.ccell.2016.11.002

Eke, I., Deuse, Y., Hehlgans, S., Gurtner, K., Krause, M., Baumann, M., … Cordes, N. (2012). β1 Integrin / FAK / cortactin signaling is essential for human head and neck cancer resistance to radiotherapy. *Journal of Clinical Investigation*, *122*(4), 1529–1540. https://doi.org/10.1172/JCI61350DS1

Feng, S., Cen, J., Huang, Y., Shen, H., Yao, L., Wang, Y., & Chen, Z. (2011). Matrix metalloproteinase-2 and -9 secreted by leukemic cells increase the permeability of blood-brain barrier by disrupting tight junction proteins. *PLoS ONE*, *6*(8). https://doi.org/10.1371/journal.pone.0020599

Frezzato, F., Gattazzo, C., Martini, V., Trimarco, V., Teramo, A., Carraro, S., … Trentin, L. (2012). HS1, a lyn kinase substrate, is abnormally expressed in B-chronic lymphocytic leukemia and correlates with response to fludarabine-based regimen. *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0039902

García-ponce, A., Citalán-madrid, A. F., Velázquez-avila, M., Vargas-robles, H., & Schnoor, M. (2014). The role of actin-binding proteins in the control of endothelial barrier integrity Introduction : Vascular permeability and the, (11).

García-Ponce, A., Citalán-Madrid, A. F., Velázquez-Avila, M., Vargas-Robles, H., & Schnoor, M. (2015). The role of actin-binding proteins in the control of endothelial barrier integrity. *Thrombosis and Haemostasis*, *113*(1), 20–36. https://doi.org/10.1160/TH14-04-0298

Gattazzo, C., Martini, V., Frezzato, F., Trimarco, V., Tibaldi, E., Castelli, M., … Trentin, L. (2014). Cortactin, another player in the Lyn signaling pathway, is over-expressed and alternatively spliced in leukemic cells from patients with B-cell chronic lymphocytic leukemia. *Haematologica*, *99*(6), 1069–1077. https://doi.org/10.3324/haematol.2013.090183

Gómez, A. M., Martínez, C., González, M., Luque, A., Melen, G. J., Martínez, J., … Ramírez, M. (2015). Chemokines and relapses in childhood acute

lymphoblastic leukemia: A role in migration and in resistance to antileukemic drugs. *Blood Cells, Molecules, and Diseases*, *55*(3), 220–227. https://doi.org/10.1016/j.bcmd.2015.07.001

Gossai, N. P., & Gordon, P. M. (2017). The Role of the Central Nervous System Microenvironment in Pediatric Acute Lymphoblastic Leukemia. *Frontiers in Pediatrics*, *5*. https://doi.org/10.3389/fped.2017.00090

Greaves, M. F., & Wiemels, J. (2003). Origins of chromosome translocations in childhood leukaemia. *Nat Rev Cancer*, *3*(9), 639–649. https://doi.org/10.1038/nrc1164

Gu, T., Nardone, J., Wang, Y., Loriaux, M., Villén, J., Beausoleil, S., … Polakiewicz, R. D. (2011). Survey of Activated FLT3 Signaling in Leukemia. *PLoS ONE*, *6*(4), e19169. https://doi.org/10.1371/journal.pone.0019169

Guerrouahen, B. S., Al-Hijji, I., & Tabrizi, A. R. (2011). Osteoblastic and Vascular Endothelial Niches, Their Control on Normal Hematopoietic Stem Cells, and Their Consequences on the Development of Leukemia. *Stem Cells International*, *2011*, 1–8. https://doi.org/10.4061/2011/375857

Gupta, S., Sutradhar, R., Guttmann, A., Sung, L., & Pole, J. D. (2014). Socioeconomic status and event free survival in pediatric acute lymphoblastic leukemia : A population-based cohort study. *Leukemia Research*, *38*(12), 1407–1412. https://doi.org/10.1016/j.leukres.2014.08.017

Guzman, M. L., Neering, S. J., Upchurch, D., Grimes, B., Howard, D. S., Rizzieri, D. a, … Jordan, C. T. (2001). Nuclear factor- k B is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood*, *98*(8), 2301–2308. https://doi.org/10.1182/blood.V98.8.2301

Hacken, E., Scielzo, C., Bertilaccio, M. T. S., Scarf, L., Apollonio, B., & Barbaglio, F. (2013). Targeting the LYN / HS1 signaling axis in chronic lymphocytic leukemia. *Blood*, *121*(12), 2264–2273. https://doi.org/10.1182/blood-2012-09-457119.E.T.H.

Heazlewood, S. Y., Oteiza, A., Cao, H., & Nilsson, S. K. (2014). Analyzing hematopoietic stem cell homing, lodgment, and engraftment to better understand the bone marrow niche. *Annals of the New York Academy of Sciences*, *1310*(1), 119–128. https://doi.org/10.1111/nyas.12329

Helgeson, L. A., & Nolen, B. J. (2013). Mechanism of synergistic activation of Arp2/3 complex by cortactin and N-WASP. *eLife*, *2013*(2), 1–26.

https://doi.org/10.7554/eLife.00884

Hill, A., McFarlane, S., Mulligan, K., Gillespie, H., Draffin, J. E., Trimble, A., … Waugh, D. J. J. (2006). Cortactin underpins CD44-promoted invasion and adhesion of breast cancer cells to bone marrow endothelial cells. *Oncogene*, *25*(45), 6079–6091. https://doi.org/10.1038/sj.onc.1209628

Hoffman, C. M., & Calvi, L. M. (2014). Minireview: Complexity of Hematopoietic Stem Cell Regulation in the Bone Marrow Microenvironment. *Molecular Endocrinology*, *28*(10), 1592–1601. https://doi.org/10.1210/me.2014-1079

Infante, E., & Ridley, A. J. (2013). Roles of Rho GTPases in leucocyte and leukaemia cell transendothelial migration. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1629), 20130013–20130013. https://doi.org/10.1098/rstb.2013.0013

Izraeli, S. (2010). Application of genomics for risk stratification of childhood acute lymphoblastic leukaemia: From bench to bedside? *British Journal of Haematology*, *151*(2), 119–131. https://doi.org/10.1111/j.1365-2141.2010.08312.x

Jang, Y., & Sharkis, S. J. (2007). STEM CELLS IN HEMATOLOGY A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. *October*, *110*(8), 3056–3063. https://doi.org/10.1182/blood-2007-05-087759.An

Juarez-Velazquez, R., Reyes-Leon, A., Salas-Labadia, C., Rivera-Luna, R., Velasco-Hidalgo, L., Lopez-Hernandez, G., … Perez-Vera, P. (2014). Significance of CASP8AP2 and H2AFZ expression in survival and risk of relapse in children with acute lymphoblastic leukemia. *Leukemia & Lymphoma*, *55*(10), 2305–2311. https://doi.org/10.3109/10428194.2013.878458

Juarez, J. G., Thien, M., Dela Pena, A., Baraz, R., Bradstock, K. F., & Bendall, L. J. (2009). CXCR4 mediates the homing of B cell progenitor acute lymphoblastic leukaemia cells to the bone marrow via activation of p38MAPK. *British Journal of Haematology*, *145*(4), 491–499. https://doi.org/10.1111/j.1365-2141.2009.07648.x

Kakarala, M., & Wicha, M. (2008). Implications of the Cancer Stem-Cell Hypothesis for Breast Cancer Prevention and Therapy. *Journal of Clinical Oncology*, *26*(17), 2813–2820.

https://doi.org/10.1200/JCO.2008.16.3931.Implications

Kelley, L. C., Hayes, K. E., Ammer, A. G., Martin, K. H., & Weed, S. A. (2011). Revisiting the ERK/Src cortactin switch. *Communicative and Integrative Biology*, *4*(2), 205–207. https://doi.org/10.4161/cib.4.2.14420

Kim, J. A., Shim, J. S., Lee, G. Y., Yim, H. W., Kim, T. M., Kim, M., … Oh, I. H. (2015). Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myelogenous leukemia. *Cancer Research*, *75*(11), 2222–2231. https://doi.org/10.1158/0008-5472.CAN-14-3379

Kim, Y. N., Choi, J. E., Bae, J. S., Jang, K. Y., Chung, M. J., Moon, W. S., … Park, H. S. (2012). Expression of cortactin and focal adhesion kinase in colorectal adenocarcinoma: Correlation with clinicopathologic parameters and their prognostic implication. *Korean Journal of Pathology*, *46*(5), 454–462. https://doi.org/10.4132/KoreanJPathol.2012.46.5.454

Kirkbride, K. C., Sung, B. H., Sinha, S., & Weaver, A. M. (2011). Cortactin: a multifunctional regulator of cellular invasiveness. *Cell Adhesion & Migration*, *5*(2), 187–98. https://doi.org/10.4161/cam.5.2.14773

Kunz, J. B., Rausch, T., Bandapalli, O. R., Eilers, J., Pechanska, P., Schuessele, S., … Kulozik, A. E. (2015). Pediatric T-cell lymphoblastic leukemia evolves into relapse by clonal selection, acquisition of mutations and promoter hypomethylation. *Haematologica*, *100*(11), 1442–1450. https://doi.org/10.3324/haematol.2015.129692

Lai, F., Szczodrak, M., Oelkers, J. M., Ladwein, M., Acconcia, F., Benesch, S., … Rottner, K. (2009). Cortactin promotes migration and platelet-derived growth factor-induced actin reorganization by signaling to Rho-GTPases. *Molecular Biology of the Cell*, *20*(14), 3209–3223. https://doi.org/10.1091/mbc.E08

Latasiewicz, J., Artz, A., Jing, D., Blanco, M. P., Currie, S. M., Vel, M., … Vestweber, D. (2017). HS1 deficiency impairs neutrophil recruitment in vivo and activation of the small GTPases Rac1 and Rap1, *101*(May), 1–11. https://doi.org/10.1189/jlb.1A0416-195R

Lee, Y., Decker, M., Lee, H., & Ding, L. (2017). Extrinsic regulation of hematopoietic stem cells in development, homeostasis and diseases. *Wiley Interdisciplinary Reviews: Developmental Biology*, e279. https://doi.org/10.1002/wdev.279

Lilljebjörn, H., Rissler, M., Lassen, C., Heldrup, J., Behrendtz, M., Mitelman, F., … Fioretos, T. (2012). Whole-exome sequencing of pediatric acute lymphoblastic leukemia. *Leukemia*, *26*(7), 1602–7. https://doi.org/10.1038/leu.2011.333

Luo, C., Pan, H., Mines, M., Watson, K., Zhang, J., & Fan, G. H. (2006). CXCL12 induces tyrosine phosphorylation of cortactin, which plays a role in CXC chemokine receptor 4-mediated extracellular signal-regulated kinase activation and chemotaxis. *Journal of Biological Chemistry*, *281*(40), 30081–30093. https://doi.org/10.1074/jbc.M605837200

MacGrath, S. M., & Koleske, A. J. (2012). Cortactin in cell migration and cancer at a glance. *Journal of Cell Science*, *125*(7), 1621–1626. https://doi.org/10.1242/jcs.093781

Magalhaes, M. A. O., Larson, D. R., Mader, C. C., Bravo-Cordero, J. J., Gil-Henn, H., Oser, M., … Condeelis, J. (2011). Cortactin phosphorylation regulates cell invasion through a pH-dependent pathway. *Journal of Cell Biology*, *195*(5), 903–920. https://doi.org/10.1083/jcb.201103045

Magrath, I., Steliarova-foucher, E., Epelman, S., Ribeiro, R. C., Harif, M., Li, C., … Vi, M. (2013). Improving cancer care for children and young people 2 Paediatric cancer in low-income and middle-income countries. *Lancet Oncology*, *14*(3), e104–e116. https://doi.org/10.1016/S1470-2045(13)70008-1

Malempati, S., Gaynon, P. S., Sather, H., La, M. K., & Stork, L. C. (2007). Outcome After Relapse Among Children With Standard-Risk Acute Lymphoblastic Leukemia: Children's Oncology Group Study CCG-1952. *Journal of Clinical Oncology*, *25*(36), 5800–5807. https://doi.org/10.1200/JCO.2007.10.7508

Martinez-Quiles, N. (2003). Emerging roles of haematopoietic lineage cell-specific protein 1 in the immune system. *OA Immunology*, *1*(1), 1–7.

Massberg, S., Schaerli, P., Knezevic-Maramica, I., Köllnberger, M., Tubo, N., Moseman, E. A., … von Andrian, U. H. (2007). Immunosurveillance by Hematopoietic Progenitor Cells Trafficking through Blood, Lymph, and Peripheral Tissues. *Cell*, *131*(5), 994–1008. https://doi.org/10.1016/j.cell.2007.09.047

Matsuo, Y., & Drexler, H. G. (1998). Establishment and characterization of

human B cell precursor-leukemia cell lines. *Leukemia Research*, *22*(7), 567–79. https://doi.org/10.1016/S0145-2126(98)00050-2

Mazo, I. B., Massberg, S., & von Andrian, U. H. (2011). Hematopoietic stem and progenitor cell trafficking. *Trends in Immunology*, *32*(10), 493–503. https://doi.org/10.1016/j.it.2011.06.011

McNeil, D. E., Coté, T. R., Clegg, L., & Mauer, A. (2002). SEER update of incidence and trends in pediatric malignancies: Acute lymphoblastic leukemia. *Medical and Pediatric Oncology*, *39*(6), 554–557. https://doi.org/10.1002/mpo.10161

Meijerink, J. P. P., den Boer, M. L., & Pieters, R. (2009). New genetic abnormalities and treatment response in acute lymphoblastic leukemia. *Seminars in Hematology*, *46*(1), 16–23. https://doi.org/10.1053/j.seminhematol.2008.09.006

Merchand-Reyes, G., Pelayo, R., Pavón, L., Pestell, R. G., & Velasco-Velázquez, M. (2014). Role of Cancer Stem Cells in Metastasis. *Cancer Stem Cells*, 259–271. https://doi.org/10.1002/9781118356203.ch20

Moses, B. S., Slone, W. L., Thomas, P., Evans, R., Piktel, D., Angel, P. M., … Karen, H. (2016). HHS Public Access, *44*(1), 50–59. https://doi.org/10.1016/j.exphem.2015.09.003.Bone

Muller, W. A. (2016). Localized signals that regulate transendothelial migration. *Current Opinion in Immunology*, *38*, 24–29. https://doi.org/10.1016/j.coi.2015.10.006

Mullighan, C. G., Phillips, L. a, Su, X., Ma, J., Miller, C. B., Shurtleff, S. A., & Downing, J. R. (2008). Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science (New York, N.Y.)*, *322*(5906), 1377–80. https://doi.org/10.1126/science.1164266

Mullighan, C. G., & Willman, C. L. (2011). Advances in the Biology of Acute Lymphoblastic Leukemia-From Genomics to the Clinic. *Journal of Adolescent and Young Adult Oncology*, *1*(2), 77–86. https://doi.org/10.1089/jayao.2011.0012

Nagasawa, T., Nakajima, T., Tachibana, K., Iizasa, H., Bleul, C. C., Yoshie, O., … Kishimoto, T. (1996). Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry coreceptor

fusin. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(25), 14726–9. https://doi.org/10.1073/pnas.93.25.14726

Nevins, A. M., & Marchese, A. (2018). Detecting Cell Surface Expression of the G Protein-Coupled Receptor CXCR4. In K. R. Boheler & R. L. Gundry (Eds.), *The Surfaceome: Methods and Protocols* (pp. 151–164). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-7553-2_10

Nitzsche, F., Müller, C., Lukomska, B., Jolkkonen, J., Deten, A., & Boltze, J. (2017). The MSC adhesion cascade - insights into homing and transendothelial migration. *Stem Cells*, 1446–1460. https://doi.org/10.1002/stem.2614

Notta, F., Mullighan, C. G., Wang, J. C. Y., Poeppl, A., Doulatov, S., Phillips, L. A., … Dick, J. E. (2011). Evolution of human BCR–ABL1 lymphoblastic leukaemia-initiating cells. *Nature*, *469*(7330), 362–367. https://doi.org/10.1038/nature09733

Nucci, M., Nouér, S. A., & Anaissie, E. (2015). Distinguishing the Causes of Pulmonary Infiltrates in Patients With Acute Leukemia. *Clinical Lymphoma, Myeloma and Leukemia*, *15*(S), S98–S103. https://doi.org/10.1016/j.clml.2015.03.007

Passaro, D., Irigoyen, M., Catherinet, C., Gachet, S., Da Costa De Jesus, C., Lasgi, C., … Ghysdael, J. (2015a). CXCR4 Is Required for Leukemia-Initiating Cell Activity in T Cell Acute Lymphoblastic Leukemia. *Cancer Cell*, *27*(6), 769–779. https://doi.org/10.1016/j.ccell.2015.05.003

Passaro, D., Irigoyen, M., Catherinet, C., Gachet, S., Da Costa De Jesus, C., Lasgi, C., … Ghysdael, J. (2015b). CXCR4 Is Required for Leukemia-Initiating Cell Activity in T Cell Acute Lymphoblastic Leukemia. *Cancer Cell*, *27*(6), 769–779. https://doi.org/10.1016/j.ccell.2015.05.003

Pérez-Saldivar, M. L., Fajardo-Gutiérrez, A., Bernáldez-Ríos, R., Martínez-Avalos, A., Medina-Sanson, A., Espinosa-Hernández, L., … Mejia-Arangure, J. M. (2011). Childhood acute leukemias are frequent in Mexico City: descriptive epidemiology. *BMC Cancer*, *11*(1), 355. https://doi.org/10.1186/1471-2407-11-355

Pitt, L. A., Tikhonova, A. N., Hu, H., Trimarchi, T., King, B., Gong, Y., … Schwab, S. R. (2015). CXCL12-Producing Vascular Endothelial Niches Control Acute T Cell Leukemia Maintenance. *Cancer Cell*, *27*(6), 755–768.

https://doi.org/10.1016/j.ccell.2015.05.002

Pui, C.-H., & Robison, L. (2008). Acute lymphoblastic leukaemia. *Lancet*, *371*, 1030–1043. https://doi.org/10.1016/S0140-6736(12)62187-4

Purizaca, J., Meza, I., & Pelayo, R. (2012). Early Lymphoid Development and Microenvironmental Cues in B-cell Acute Lymphoblastic Leukemia. *Archives of Medical Research*, *43*(2), 89–101. https://doi.org/10.1016/j.arcmed.2012.03.005

Raaijmakers, M. H. G. P. (2011). Niche contributions to oncogenesis: Emerging concepts and implications for the hematopoietic system. *Haematologica*, *96*(7), 1041–1048. https://doi.org/10.3324/haematol.2010.028035

Ratajczak, M. Z., Kim, C., Janowska-Wieczorek, A., & Ratajczak, J. (2012). The Expanding Family of Bone Marrow Homing Factors for Hematopoietic Stem Cells: Stromal Derived Factor 1 Is Not the Only Player in the Game. *The Scientific World Journal*, *2012*, 1–11. https://doi.org/10.1100/2012/758512

Scadden, D. T. (2014). Nice neighborhood: Emerging concepts of the stem cell niche. *Cell*, *157*(1), 41–50. https://doi.org/10.1016/j.cell.2014.02.013

Schito, L., Rey, S., & Konopleva, M. (2017). Integration of hypoxic HIF-α signaling in blood cancers. *Oncogene*, (February), 1–10. https://doi.org/10.1038/onc.2017.119

Schnoor, M. (2015). Endothelial Actin-Binding Proteins and Actin Dynamics in Leukocyte Transendothelial Migration. *Journal of Immunology (Baltimore, Md. : 1950)*, *194*(8), 3535–3541. https://doi.org/10.4049/jimmunol.1403250

Schnoor, M., Alcaide, P., Voisin, M. B., & Van Buul, J. D. (2015). Crossing the Vascular Wall: Common and Unique Mechanisms Exploited by Different Leukocyte Subsets during Extravasation. *Mediators of Inflammation*, *2015*(Figure 1). https://doi.org/10.1155/2015/946509

Schnoor, M., Lai, F. P. L., Zarbock, A., Kläver, R., Polaschegg, C., Schulte, D., … Vestweber, D. (2011). Cortactin deficiency is associated with reduced neutrophil recruitment but increased vascular permeability in vivo, *208*(8), 1721–1735. https://doi.org/10.1084/jem.20101920

Scielzo, C., Bertilaccio, M. T. S., Simonetti, G., Dagklis, A., Ten Hacken, E., Fazi, C., … Caligaris-Cappio, F. (2010). HS1 has a central role in the trafficking and homing of leukemic B cells. *Blood*, *116*(18), 3537–3546. https://doi.org/10.1182/blood-2009-12-258814

Scielzo, C., Ghia, P., Conti, A., Bachi, A., Guida, G., Geuna, M., … Caligaris-cappio, F. (2005). HS1 protein is differentially expressed in chronic lymphocytic leukemia patient subsets with good or poor prognoses, *115*(6). https://doi.org/10.1172/JCI24276.1644

Shalapour, S., Hof, J., Kirschner-Schwabe, R., Bastian, L., Eckert, C., Prada, J., … Seeger, K. (2011). High VLA-4 expression is associated with adverse outcome and distinct gene expression changes in childhood B-cell precursor acute lymphoblastic leukemia at first relapse. *Haematologica*, *96*(11), 1627–1635. https://doi.org/10.3324/haematol.2011.047993

Shishido, S., BÃ¶nig, H., & Kim, Y.-M. (2014). Role of Integrin Alpha4 in Drug Resistance of Leukemia. *Frontiers in Oncology*, *4*(May), 1–10. https://doi.org/10.3389/fonc.2014.00099

Sipkins, D. A., Wei, X., Wu, J. W., Runnels, J. M., Côté, D., Means, T. K., … Lin, C. P. (2005). In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature*, *435*(7044), 969–973. https://doi.org/10.1038/nature03703

Sison, E. A. R., & Brown, P. (2011). The bone marrow microenvironment and leukemia: biology and therapeutic targeting. *Expert Review of Hematology*, *4*(3), 271–83. https://doi.org/10.1586/ehm.11.30

Smith-Pearson, P. S., Greuber, E. K., Yogalingam, G., & Pendergast, A. M. (2010). Abl kinases are required for invadopodia formation and chemokine-induced invasion. *Journal of Biological Chemistry*, *285*(51), 40201–40211. https://doi.org/10.1074/jbc.M110.147330

Smith, J. N. P., & Calvi, L. M. (2013). Concise review: Current concepts in bone marrow microenvironmental regulation of hematopoietic stem and progenitor cells. *Stem Cells (Dayton, Ohio)*, *31*(6), 1044–50. https://doi.org/10.1002/stem.1370

Spiegel, A., Kollet, O., Peled, A., Abel, L., Nagler, A., Bielorai, B., … Lapidot, T. (2008). result of altered CXCR4 expression and signaling Unique SDF-1 – induced activation of human precursor-B ALL cells as a result of altered CXCR4 expression and signaling, *103*(8), 2900–2907. https://doi.org/10.1182/blood-2003-06-1891

Steliarova-foucher, E., Colombet, M., Ries, L. A. G., Moreno, F., Dolya, A., Bray, F., … Shin, H. Y. (2017). Articles International incidence of childhood cancer

, 2001 – 10 : a population-based registry study, 719–731. https://doi.org/10.1016/S1470-2045(17)30186-9

Stong, R. C., Korsmeyer, S. J., Parkin, J. L., Arthur, D. C., & Kersey, J. H. (1985). Human acute leukemia cell line with the t(4;11) chromosomal rearrangement exhibits B lineage and monocytic characteristics. *Blood*, *65*, 21–31.

Suárez-Álvarez, B., López-Vázquez, A., & López-Larrea, C. (2012). Mobilization and homing of hematopoietic stem cells. *Advances in Experimental Medicine and Biology*, *741*, 152–170. https://doi.org/10.1007/978-1-4614-2098-9_11

Sugiyama, T., Kohara, H., Noda, M., & Nagasawa, T. (2006). Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*, *25*(6), 977–988. https://doi.org/10.1016/j.immuni.2006.10.016

Sugiyama, T., & Nagasawa, T. (2015). CXCL12 catches T-ALL at the entrance of the bone marrow. *Trends in Immunology*, *36*(9), 504–506. https://doi.org/10.1016/j.it.2015.08.001

Tabe, Y., & Konopleva, M. (2014). Advances in understanding the leukemia microenvironment. *British Journal Haematology*, *164*(6), 767–778. https://doi.org/10.1111/bjh.12725.ADVANCES

Takahashi, S. (2011). Downstream molecular pathways of FLT3 in the pathogenesis of acute myeloid leukemia: biology and therapeutic implications. *Journal of Hematology & Oncology*, *4*(1), 13. https://doi.org/10.1186/1756-8722-4-13

Valsecchi, R., Coltella, N., Belloni, D., Ponente, M., Hacken, E., Scielzo, C., … Bernardi, R. (2016). HIF-1 a regulates the interaction of chronic lymphocytic leukemia cells with the tumor microenvironment, *127*(16), 1987–1998. https://doi.org/10.1182/blood-2015-07-657056.The

van Rossum, A. G. S. H., de Graaf, J. H., Schuuring-Scholtes, E., Kluin, P. M., Fan, Y. -x., Zhan, X., … Schuuring, E. (2003). Alternative Splicing of the Actin Binding Domain of Human Cortactin Affects Cell Migration. *Journal of Biological Chemistry*, *278*(46), 45672–45679. https://doi.org/10.1074/jbc.M306688200

Vicente López, Á., Vázquez García, M. N., Melen, G. J., Entrena Martínez, A.,

Cubillo Moreno, I., García-Castro, J., … Zapata González, A. G. (2014). Mesenchymal stromal cells derived from the bone marrow of acute lymphoblastic leukemia patients show altered BMP4 production: Correlations with the course of disease. *PLoS ONE*, *9*(1), 1–11. https://doi.org/10.1371/journal.pone.0084496

Vilchis-Ordoñez, A., Contreras-Quiroz, A., Vadillo, E., Dorantes-Acosta, E., Reyes-López, A., Quintela-Nuñez Del Prado, H. M., … Pelayo, R. (2015). Bone marrow cells in acute lymphoblastic leukemia create a proinflammatory microenvironment influencing normal hematopoietic differentiation fates. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/386165

Wang, W., Liu, Y., & Liao, K. (2011). Tyrosine phosphorylation of cortactin by the FAK-Src complex at focal adhesions regulates cell motility. *BMC Cell Biology*, *12*(1), 49. https://doi.org/10.1186/1471-2121-12-49

Weed, S. A., Karginov, A. V., Schafer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A., & Parsons, J. T. (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *Journal of Cell Biology*, *151*(1), 29–40. https://doi.org/10.1083/jcb.151.1.29

Welner, R. S., & Kincade, P. W. (2007). Stem Cells on Patrol. *Cell*. https://doi.org/10.1016/j.cell.2007.11.010

Xie, Y., Davies, S. M., Xiang, Y., Robison, L. L., & Ross, J. A. (2003). Trends in leukemia incidence and survival in the United States (1973-1998). *Cancer*, *97*(9), 2229–2235. https://doi.org/10.1002/cncr.11316

Xu, H., Yang, W., Perez-Andreu, V., Devidas, M., Fan, Y., Cheng, C., … Yang, J. J. (2013). Novel susceptibility variants at 10p12.31-12.2 for childhood acute lymphoblastic leukemia in ethnically diverse populations. *Journal of the National Cancer Institute*, *105*(10), 733–742. https://doi.org/10.1093/jnci/djt042

Yang, L., Kowalski, J. R., Yacono, P., Bajmoczi, M., Shaw, S. K., Froio, R. M., … Luscinskas, F. W. (2006). Endothelial cell cortactin coordinates intercellular adhesion molecule-1 clustering and actin cytoskeleton remodeling during polymorphonuclear leukocyte adhesion and transmigration. *The Journal of Immunology*, *177*(9), 6440–9. https://doi.org/10.4049/jimmunol.177.9.6440

Yu, J., Chen, L., Chen, Y., Hasan, K., Ghia, E. M., Zhang, L., … Kipps, T. J. (2017). Wnt5a induces ROR1 to associate with 14-3-3ζ for enhanced chemotaxis and proliferation of chronic lymphocytic leukemia cells. *Leukemia*, (March), 1–8. https://doi.org/10.1038/leu.2017.132

Zuna, J., Madzo, J., Krejci, O., Zemanova, Z., Kalinova, M., Muzikova, K., … Trka, J. (2011). ETV6/RUNX1 (TEL/AML1) is a frequent prenatal first hit in childhood leukemia. *Blood*, *117*(1), 368–369. https://doi.org/10.1182/blood-2010-09-309070