Characterization of Oncogenic Role of ROR1 and PTK7 in B-ALL

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Proteiinikiinaasi superperheeseen kuuluvilla pseudokinaaseilla on mutaatioita tyrosiinikinaasidomeenin konservoituneissa alueissa, jotka estävät pseudokinaasien katalyyttisen aktiivisuuden. Pseudokinaasit ollaan yhdistetty useisiin sairauksiin, minkä vuoksi niiden säätelyyn ja signalointiin liittyvien mekanismien ymmärtäminen on tärkeää. ROR ja PTK7 ovat pseudokinaaseja, jotka toimivat reseptoreina epäkanonisella Wnt-signalointireitillä. ROR1 ja PTK7 reseptorien ollaan havaittu ilmentyvän useissa syövissä, mutta niiden toiminnallista roolia ei vielä täysin tunneta. Tässä tutkielmassa karakterisoimme ROR1 ja PTK7 reseptorien onkogeenistä roolia B-solu akuutissa lymfaattisessa leukemiassa (B-ALL).

Tutkimusmenetelmät: Tässä tutkimuksessa käytimme B-ALL t(1;19) solulinjoja. Tutkimme ROR1 ja PTK7 reseptorien ilmenemistä sekä solujen sisäistä Wnt signalointireittiä, käyttäen Western Blottia ja FACS analyysia. Teimme lisäksi immunopresipitaatioita selvittääksemme Wnt5a ligandin vuorovaikutusta ROR1 ja PTK7 reseptorien kanssa. Tutkimme ROR1 inhibition vaikutusta solujen sisäiseen signalointireittiin inhiboimalla ROR1 reseptoria monoklonaalisella vasta-aineella sekä RNA interferenssi systeemillä. Selvitimme myös ROR1 ja PTK7 reseptorien vaikutusta B-ALL solujen elinvoimaisuuteen inhiboimalla niiden ekspressiota RNA interferenssi systeemillä.

Tutkimustulokset: ROR1 ja PTK7 ilmenivät B-ALL soluissa, mutta vain ROR1 vaikutti solujen elinvoimaisuuteen. Vastaavasti reseptoreista vain ROR1 kykeni sitomaan Wnt5a ligandia. Havaitsimme ROR1 reseptorin inhibition alentavan RhoA, STAT3 ja NF-κB proteiinien ekspressiota.

Johtopäätelmät: ROR1 reseptorilla on tärkeä rooli B-ALL t(1;19) solujen elinvoimaisuuden ylläpitämisessä epäkanonisella Wnt-signalointireitillä. ROR1 inhibitio alentaa solujen jakautumista, minkä vuoksi ROR1 reseptoriin kohdennetut täsmälääkkeet voivat soveltua B-solu akuutin lymfaattisen leukemian hoidoksi.

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Abstract

Background and aims: Pseudokinases are members of the protein kinase superfamily that have mutations in their canonical motifs required for proper catalytic activity. Many pseudokinases are involved in human diseases, therefore it is important to understand their signaling mechanisms and regulation. Among pseudokinases family, receptor tyrosine kinase-like orphan receptors (RORs) and protein tyrosine kinase 7 (PTK7) are Wnt-binding receptors from non-canonical Wnt signaling pathway. These proteins are expressed in several cancers, but their functional role needs more investigation. In this thesis, we investigated the oncogenic role of ROR1 and PTK7 in B-cell acute lymphoblastic leukemia (B-ALL).

Methods: In this study, we used B-ALL cells with t(1;19) translocation. We investigated the expression of ROR1 and PTK7 as well as intracellular Wnt signaling pathway using Western Blotting and FACS analysis. We also examined the interaction of Wnt5a ligand with ROR1 and PTK7 using immunoprecipitations. To examine molecular pathway downstream from ROR1 and the relevance of ROR1 and PTK7 in cell survival, we targeted ROR1 with monoclonal antibody and both ROR1 and PTK7 with RNA interference system.

Results: Our results demonstrate that both ROR1 an PTK7 are expressed on B-ALL cells with t(1;19) translocation but only ROR1 is important for B-ALL cell survival. We showed that ROR1 interacts with Wnt5a indicating that ROR1 is a mediator of non-canonical Wnt signaling. Also, we demonstrated downregulation of RhoA, STAT3 and NF-κB after ROR1 targeting.

Conclusion: ROR1 has an important role maintaining B-ALL t(1;19) cell viability in non-canonical Wnt signaling pathway. ROR1 targeting reduces cell survival indicating it as a possible candidate for targeted therapy.

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ABBREVIATIONS

AML	Acute myeloid leukemia
B-ALL	B-cell acute lymphoblastic leukemia
CCK4	Colon carcinoma kinase 4
CLL	Chronic lymphocytic leukemia
CTG	CellTiter-Glo™ proliferation assay
DsRNA	Double stranded RNA
EMT	Endothelial to mesenchymal transition
FACS	Fluorescence activated cell sorting
Fz	Frizzled
LRP	Low-density lipoprotein receptor-related proteins
mAb	Monoclonal antibody
PCP	Planar cell polarity
PI	Propidium iodide
PTK7	Protein tyrosine kinase 7
ROR	Receptor tyrosine kinase-like orphan receptor
RTK	Receptor tyrosine kinase
shRNA	Short hairpin RNA
TKD	Tyrosine kinase domain
WB	Western Blot

1 INTRODUCTION

Wnt signaling has major roles in multiple cancers which can be either oncogenic or cancer suppressing. This research focuses on two receptor tyrosine kinases (RTK), receptor tyrosine kinase-like orphan receptor 1 (ROR1) and protein tyrosine kinase 7 (PTK7), which mediate mainly non-canonical Wnt signaling and their oncogenic role in B-cell acute lymphoblastic leukemia (B-ALL).

In the literature review we will explain Wnt signaling pathway and its role in cancer. We will introduce ROR1 and PTK7 receptor tyrosine kinases and their role in Wnt signaling focusing on B-cell acute lymphoblastic leukemia.

1.1 Wnt signaling

Wnt (*Dorsophilia wingless (wg)* and mouse *Int* human homolog *Wnt)* signaling is part of core developmental pathways in vertebrates and invertebrates (van Amerongen & Nusse, 2009). It is mostly active during embryonic patterning, where it has multiple functions related to differentiation, proliferation, motility and adhesion (Marwa S Asem, Steven Buechler, Rebecca Burkhalter Wates, Daniel L Miller, & M Sharon Stack, 2016; Zhan, Rindtorff, & Boutros, 2017). Wnt pathways are expressed in all metazoan, indicating their conserved role in core developmental processes (Willert & Nusse, 2012).

Humans have 19 different Wnt glycoprotein ligands, which can interact with over 15 receptors and co-receptors to induce high range of cellular responses due to the amount of possible ligand-receptor interactions that lead to activation of different signaling cascades (van Amerongen & Nusse, 2009). Wnt interacting receptors include Frizzled (Fz), low-density lipoprotein receptor-related proteins (LRP), proteoglycan families and receptor tyrosine kinases (RTK) such as tyrosine-protein kinase (RYK), muscle-specific tyrosine-protein kinase receptor (MuSk), receptor tyrosine kinase-like orphan receptors 1 and 2 (ROR1/2) and protein tyrosine kinase 7 (PTK7) (Mendrola, Shi, Park, & Lemmon, 2013; Niehrs, 2012).

Wnt signaling can be divided into canonical and non-canonical pathways based on the intracellular responses following the Wnt ligand binding. In the canonical pathway Wnt signaling stabilizes β-catenin and Wnt-dependent stabilization of proteins (STOP) signaling cascades and is involved mainly in regulation of cell proliferation and cell fate (Masaru Katoh, 2017b; van Amerongen & Nusse, 2009). On the other hand, the non-canonical Wnt pathway activates planar cell polarity (PCP), intracellular Ca^{2+} influx or receptor tyrosine kinase related signaling which play major roles in cell polarity, adhesion and cell motility as well as proliferation (Katoh & Katoh, 2017).

1.1.1 Canonical and non-canonical Wnt pathway

The division of Wnt signaling into canonical and non-canonical is not well-defined since both signaling pathways demonstrate significant cross-talk in multiple cell-models. The level of cross-signaling makes it difficult to categorize specific factors into either pathway, but some ligands and receptors have been found to be associated more with the canonical or the non-canonical Wnt signaling pathway (van Amerongen & Nusse, 2009). Below we will depict canonical and non-canonical Wnt signaling pathways separately and their cross-signaling cascades (Figure 1).

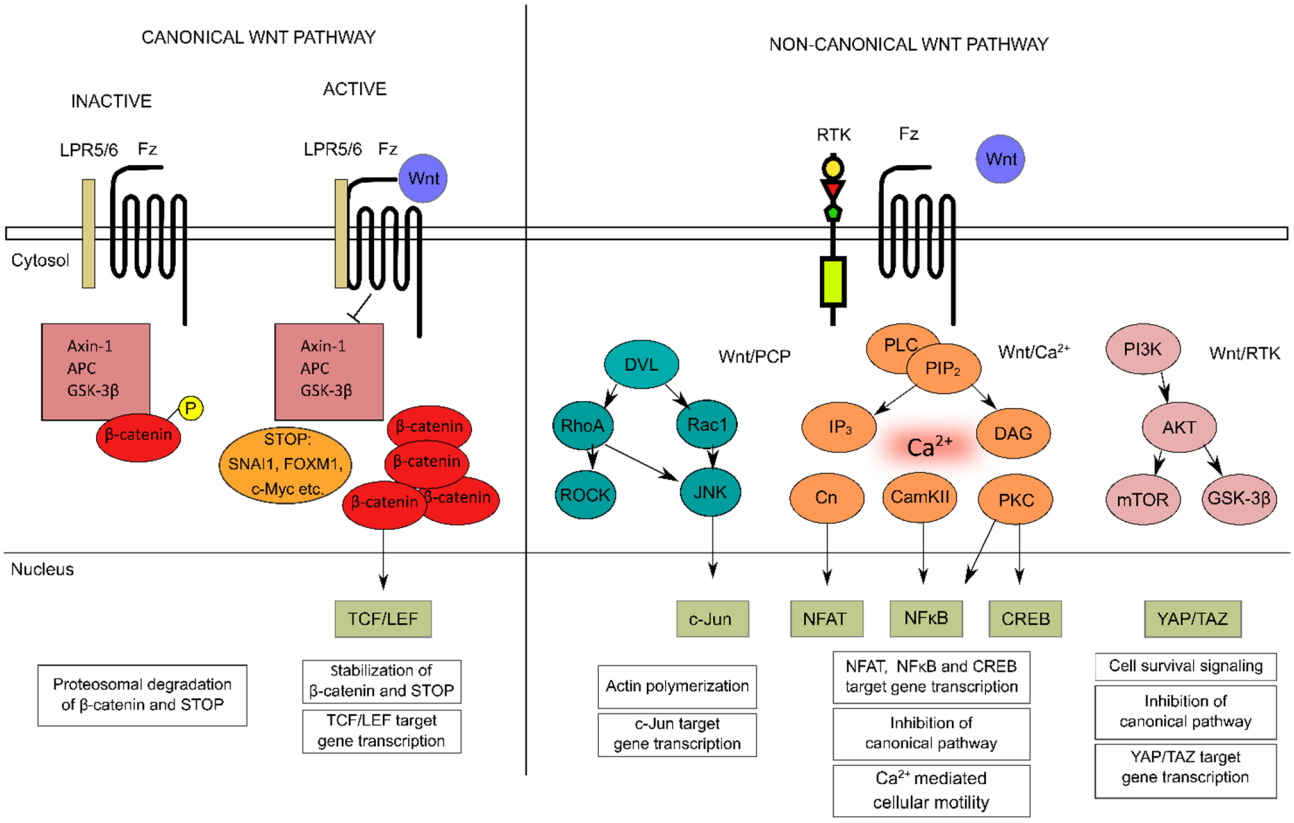

Figure 1. *Schematic representation of canonical and non-canonical Wnt pathway. When canonical Wnt pathway is not active, β-catenin and STOP proteins are phosphorylated and subsequently degraded by the proteasomes in the cytoplasm. Wnt signaling activation by ligand binding leads to the β-catenin destruction inhibition and subsequently to β-catenin and STOP stabilization and β-catenin mediated TCF/LEF transcription complex activation. On the other hand, non-canonical Wnt/PCP pathway induces planar cell polarity by regulating actin polymerization and c-Jun target gene transcription. Wnt/Ca^{2+} pathway induces NFAT, NF-κB and CREB mediated transcription and Ca^{2+} mediated cellular motility as well as inhibition of TCF/LEF transcription and canonical Wnt signaling. Wnt/RTK pathway also induces PI3K/AKT cell survival signaling pathway and YAP/TAZ target gene transcription. STOP: Wnt-dependent stabilization of proteins, PCP: planar cell polarity, RTK: receptor tyrosine kinase.*

Canonical Wnt signaling involves β-catenin stabilizing pathway that leads to β-catenin target gene transcription. The activation of canonical pathway takes place when Wnt ligand binds to and heterodimerizes extracellular receptors Fz and LPR5/6. In the absence of ligand binding, β-catenin signaling is inhibited by the destruction complex containing Axin-1, casein kinase I (CKI), tumor suppressor protein encoded by *adenomatous polyposis coli* (APC) and glycogen synthase kinase-3β (GSK-3β). When β-catenin is bound to the destruction complex, Axin-1 and APC enable GSK-3β mediated phosphorylation of β-catenin, leading to ubiquitination and proteasomal degradation (Asem, Buechler, Wates, Miller, & Stack, 2016; Mikels & Nusse, 2006; Peifer & Polakis, 2000). The activation of canonical signaling cascade via LPR 5/6 and Fz leads to activation of Dishevelled (DVL) that inhibits GSK-3β activation preventing β-catenin phosphorylation (H. Berger, Wodarz, & Borchers, 2017; Mikels & Nusse, 2006). This enables β-catenin to shuttle into the nucleus where it can form a transcription complex with transcription factor-7 (TCF)/lymphoid enhancer-binding factor (LEF). Without β-catenin, TCF is bound to Wnt repression complex with Groucho, and together with histone deacetylases (HDAC) it prevents β-catenin-targeted gene transcription (Masaru Katoh, 2017b). In addition to β-catenin stabilization, inhibition of GSK3β prevents phosphorylation of STOP (Wnt-dependent stabilization of proteins) target proteins and their degradation. STOP proteins include for example zinc finger protein SNAI1, forkhead box protein M1 (FOXM1), transcriptional coactivator YAP1 (YAP), tafazzin (TAZ) and Myc proto-oncogene protein (c-Myc) (Masaru Katoh, 2017b; van Amerongen & Nusse, 2009).

Wnt pathways that do not activate β-catenin are denominated as non-canonical pathways. These include PCP, Ca^{2+} mediated cell motility and RTK pathways that have pleiotropic effects in cell polarity, adhesion, proliferation, differentiation and survival (Masuko Katoh & Katoh, 2017).

Planar cell polarity (PCP) induces cells to polarize along epithelial plane perpendicular to apical-basal axis, which enables multicellular structure organization, tissue remodeling and cell movement and migration (Munoz-Soriano, Belacortu, & Paricio, 2012). PCP regulation takes place via conserved core PCP proteins including Van Gogh, Prickle, Flamingo, Diego and DVL (Green, Kuntz, & Sternberg, 2008; Vladar, Antic, & Axelrod, 2009). In the non-canonical Wnt/PCP pathway intracellular DVL is activated when Wnt binds to Frizzled and other co-receptor that could be RTK (ROR, MuSk, RYK, PTK7), glycoprotein glycan or G-protein related receptor Syndecan (Niehrs, 2012). DVL thereafter activates Ras homolog gene family member A (RhoA) and Ras-related protein Rac1 (Rac1) GTPases leading to activation of c-Jun N-terminal kinase (JNK) enabling transcription factor AP-1 (c-Jun) mediated transcription. RhoA activates also Rho-associated protein kinase 1 (ROCK) that enables actin polymerization. Together these pathways modulate cell polarization and

migration (Masuko Katoh & Katoh, 2017; Niehrs, 2012). During embryonic development PCP pathway is responsible for example for convergent extension, stereocilia orientation in inner ear, neural crest cell migration, neural tube closure and heart morphogenesis (Lu et al., 2004; Munoz-Soriano et al., 2012). In adults, PCP pathway is active in wound healing and cancer cell migration and metastasis (Munoz-Soriano et al., 2012).

Intracellular Ca^{2+} pathway increases cell movement and can influence transcriptional activity as well as suppress canonical signaling (Masaru Katoh, 2017b). Similar to Wnt/PCP pathway, intracellular Ca^{2+} signaling pathway is activated via Wnt-ligand engagement of Fz with ROR1/2 as co-receptor (De, 2011). The intracellular response involves activation of protein lipase C (PLC) which activates phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to inositol triphosphate (IP_3)/diglyceride (DAG) pathway activation. IP_3 induces Ca^{2+} release from ER (endoplasmic reticulum) which activates calcineurin and calcium calmodulin-dependent protein kinase II (CaMKII), that in turn activates nuclear factor of activated T-cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The increased Ca^{2+} level and hydrolyzation of PIP2 activates DAG that induces protein kinase C (PKC) activation and increases NF-κB and CREB transcription activation. NF-κB and CREB are both nuclear transcription factors and NFAT is T-cell associated nuclear factor that induces pro-inflammatory response (Komiya & Habas, 2008).

Wnt/RTK pathway is activated through receptor tyrosine kinases that are typically pseudokinases such as RYK, ROR1/2 and PTK7 (Mendrola et al., 2013). Wnt-ligand binding to these receptors induces intracellular signaling cascades that involves Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Protein kinase B (AKT) and YAP/TAZ downstream signaling pathways (Asem et al., 2016; Masuko Katoh & Katoh, 2017). Activation of PI3K/AKT activates serine/threonine-protein kinase (mTOR) that is one of the key regulators of cell growth, protein synthesis and metabolism (Asem et al., 2016). YAP and TAZ are transcription factors that regulate cell proliferation during organogenesis and tissue renewal. YAP/TAZ signaling, can also regulate β-catenin levels in cytosol (Anastas et al., 2014). In cytosol, Wnt signaling pathway regulates TAZ levels via destruction complex, while Wnt/RTK and Wnt/PCP pathways are able to increase nuclear localization of YAP and TAZ via PI3K/AKT and RhoA signaling initiating YAP/TAZ mediated transcription (Elbediwy, Vincent-Mistiaen, & Thompson, 2016; Piccolo, Dupont, & Cordenonsi, 2014).

Wnt ligands can induce multiple pathways activation at the same time causing crosstalk and integration of downstream signaling cascades. Moreover, same receptors can work in multiple

pathways either as main or co-receptors (van Amerongen & Nusse, 2009). For example, PCP, Ca^{2+} and RTK pathways can be activated via ROR. Non-canonical signaling can also interact with canonical pathway or inhibit canonical signaling cascades either by modulating the levels of canonical ligands or by targeting downstream signaling cascades (H. Berger et al., 2017; Green et al., 2008).

The cellular context is very important when predicting signaling responses. Most of the ligands and receptors working on Wnt pathway have been found to activate both canonical and non-canonical signaling depending on cellular contexts. For example Wnt5a that is described mostly as non-canonical Wnt ligand to activate PCP, Ca^{2+} and RTK pathways has also been found to activate β-catenin pathway in presence of Fz4 and LPR5 or Fz5 (De, 2011; Mikels & Nusse, 2006). Also, receptors such as ROR2 that typically interacts with Wnt5a can interact with the canonical ligand Wnt1 to potentiate canonical Wnt signaling and TCF/LEF expression in osteosarcoma cells (Billiard et al., 2005; Green et al., 2008).

1.1.2 Wnt signaling in cancer

Due to their important roles in developmental processes, cell survival and motility, it is no surprise that aberrant activation of Wnt pathways is associated with multiple malignancies. Both canonical and non-canonical signaling pathways have been involved in different cancers, where canonical pathway is related to cancer stem cells (CSC) self-renewal, increased cell proliferation and differentiation of cancer progenitor cells, while non-canonical pathway is related to CSC maintenance as well as cancer cells migration, endothelial to mesenchymal transition (EMT) and canonical signaling inhibition (Masaru Katoh, 2017b).

Disruption of β-catenin destruction complex is observed in multiple cancers leading to increased β-catenin expression (Bainbridge et al., 2014). The effects of this can affect cancer cell survival and proliferation directly or indirectly by affecting immune response or cell microenvironment. The cell proliferation is directly increased by β-catenin mediated upregulation of fibroblast growth factor (FGF), Snai1, Cyclin D1 (CCND1), CD44 and c-Myc transcription as well as suspended degradation of STOP target proteins including FOXM1, YAP and TAZ. The upregulation of growth factors enables better growth environment for the cancer cells by effecting tissue microenvironment and immune defense (Masaru Katoh, 2017b). Canonical Wnt signaling can also increase EMT, where destabilization of E-cadherin/β-catenin complex disrupts cell-cell contacts that are important in cell adhesion. β-catenin upregulation is also associated with chemoresistance and multidrug resistance (MDR) where it can inhibit apoptosis and increase cell proliferation by

increasing expression of cell cycle regulators c-Myc and CCND1 (Asem et al., 2016; Hung et al., 2014).

Non-canonical Wnt signaling can have very diverse roles in cancer that can be either oncogenic or cancer suppressor. The role of non-canonical Wnt signaling is dependent on cancer type and profile (Marwa S Asem et al., 2016). For example, non-canonical Wnt5a expression is down regulated in neuroblastoma and in some pancreatic and breast cancers, but it is upregulated in gastric cancer, embryonic tumors and in B-ALL (Masuko Katoh & Katoh, 2009). Like canonical Wnt signaling, non-canonical Wnt signaling can affect cancer indirectly by affecting inflammation or cancer cell environment as well as directly by affecting for example cell senescence, cancer stem cells (CSC), cell proliferation or metabolism. It also effects metastasis formation by promoting EMT, migration and invasion. As canonical signaling, non-canonical signaling has also been found to affect therapy outcome by increasing chemoresistance (Marwa S Asem et al., 2016).

A deeper understanding of Wnt signaling pathways and their oncogenic roles could endorse new targets for cancer therapy.

1.2 Receptor tyrosine kinases

Receptor tyrosine kinases (RTK) are a part of protein tyrosine kinase family with 20 subfamilies (Table 1) containing total of 58 proteins. Tyrosine kinases phosphorylate proteins which often leads to activation of pathways that are related to cell-to-cell interactions such as cell differentiation, adhesion, motility, migration, proliferation and apoptosis (Lemmon & Schlessinger, 2010). This is indicated also by their ligands that contain growth factors, insulin and Wnt (Hubbard & Till, 2000). Due to their important role in cell interaction and growth they often play a key role in multiple diseases including cancers (Lemmon & Schlessinger, 2010).

Table 1. *Receptor tyrosine kinase families and their extracellular characteristics (Ségaliny, Tellez-Gabriel, Heymann, & Heymann, 2015)*

Class	Family name	Characterization of extracellular domain
I	EGFR	2 cysteine-rich domains
II	Insulin R	2 chains α and β, with one cysteine-rich and 2 FNIII domains
III	PDGFR	5 Ig-like domains
IV	VEGFR	7 Ig-like domains
V	FGFR	3 Ig-like domains, 1 acidic box
VI	PTK7/CCK4	7 Ig-like domains
VII	NGFR	2 Ig-like domains, rich leucin domains
VIII	HGFR	1 transmembrane α chain linked with one extracellular β chain
IX	EPHR	1 Ig-like, 1 cysteine-rich and 2 FNIII-like domains
X	AXL	2 Ig-line, 2 FNIII-like domains
XI	TIE	2 Ig-like, 1 EGF, and 3 FNIII-like domains
XII	RYK	1 transmembrane β chain linked with one extracellular α chain
XIII	DDR	1 discoidin-like domain
XIV	RET	1 cadherin-like domain
XV	ROS	6 FNIII-like domains
XVI	LTK	1 cysteine-rich domain
XVII	ROR	1 Ig-domain, 1 cysteine-rich Frizzled-like domain and one Kringle-like domain
XVIII	MuSk	4 Ig-like and 1 cysteine-rich domains
XIX	LMR	A short extracellular domain
XX	Undetermined	A short receptor chain with a short extracellular domain

Receptor tyrosine kinases are anchored into the cell membrane via a transmembrane 20 amino acid α-helix. Their extracellular N-terminus is involved in ligand binding and homo- and/or heterodimerization of the receptor (Ségaliny et al., 2015). Due to large number of ligands, there is a big variety between compositions of the extracellular domain in different RTK families. For instance, extramembrane domain may contain cysteine-rich domains (e.g. ROR and MuSK, immunoglobulin-like domains (e.g. PTK7 and vascular endothelial growth factor receptor (VEGFR)) or fibronectin repeats (e.g. insulin receptor) (Ségaliny et al., 2015; Shabani et al., 2007). The intracellular C-terminus is composed of three elements: a juxtamembrane domain with 40-80 aa that is localized very close to plasma membrane, tyrosine kinase domain (TKD) and a possible C-terminal domain (Ségaliny et al., 2015).

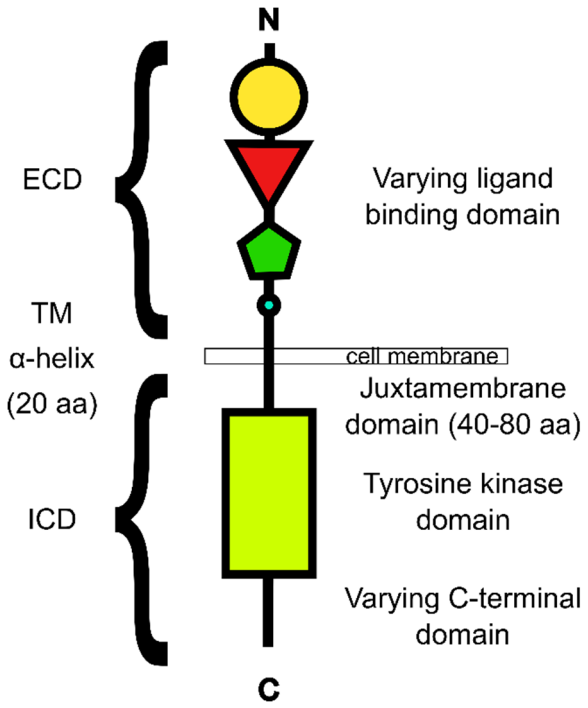

Figure 2. *General structure of receptor tyrosine kinases is composed of extracellular, transmembrane and intracellular domains. The extracellular N-terminal domain contains varying subdomains depending on RTK family that can bind a specific ligand or ligands. RTK is anchored into the cell membrane via transmembrane α-helix containing 20 amino acids. The intracellular domain contains a juxtamembrane domain, a tyrosine kinase domain and other C-terminal domains. ECM: extracellular domain, TM: transmembrane, ICD: intracellular domain.*

The activation of receptors takes place when ligand binding induces RTK homo/heterodimerization that stabilizes receptor chains (Lemmon & Schlessinger, 2010). Dimerization leads to ATP-mediated autophosphorylation of the tyrosine residues in the kinase domain and binding of intracellular signaling molecules that will initiate the intracellular signaling cascade (Hubbard & Till, 2000; Ségaliny et al., 2015).

From protein tyrosine kinases a subpopulation can be separated, that lacks autophosphorylation and catalytic kinase activity and these are called pseudokinases. The TKD of pseudokinases contains amino acid alterations in the conserved canonical motifs of the kinase domain leading to decreased or totally absent catalytic activity (Boudeau, Miranda-Saavedra, Barton, & Alessi, 2006). Even without a kinase activity, pseudokinases can play an essential role in transmitting intracellular signaling (Prebet et al., 2010). Pseudokinases can be activated allosterically by interacting with other kinases leading to transphosphorylation of the pseudokinase domain (Boudeau et al., 2006). Alternatively, if the allosteric activator is not an active kinase, activation can induce

intracellular effector protein binding leading to activation of downstream signaling cascade (Mendrola et al., 2013).

This study focuses on ROR1 and PTK7 that are both pseudokinases from the non-canonical Wnt-signaling pathway.

1.2.1 ROR1

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) was first discovered in 1992 (Masiakowski & Carroll, 1992). ROR1 belongs in ROR RTK family that comprises of 2 receptors, ROR1 and ROR2. RORs possess about 50 % sequence similarity, both genes encoding 104 kDa proteins with similar structure indicating similar functional mechanisms (Bainbridge et al., 2014; Green et al., 2008; Rebagay, Yan, Liu, & Cheung, 2012). They mediate multiple cellular functions such as cell polarity, migration and proliferation via non-canonical Wnt signaling, which indicates their important roles during development (Green et al., 2008). ROR orthologs are evolutionary conserved, for they have been found for example in fruit flies (*Dorsophilia melanogaster*), zebrafish (*Danio rerio*) and in mice (*mus musculus*) (Lyashenko et al., 2010).

ROR1 is organized similarly to other receptor tyrosine kinases. It is a class XVII receptor tyrosine kinase, indicating that the extracellular region contains an immunoglobulin-like domain (Ig), Frizzled-like cysteine rich domain (FZD) in the middle and Kringle-like domain (KRD) proximal to the cell membrane. The intracellular region contains the tyrosine kinase domain and two serine-threonine-rich (S/T) domains separated by a proline-rich domain (Figure 3) (Bainbridge et al., 2014; Green et al., 2008; Matsuda et al., 2001). Due to mutations in canonical motifs of TKD, ROR1 lacks autophosphorylation and catalytic activity *in vitro* and is described as pseudokinase (Bainbridge et al., 2014; Murphy et al., 2014). ROR1 also has three splice variants: variant 1 encodes 934 amino acid (aa) transmembrane protein, variant 2 encodes 549 aa intracellular protein and is also known as truncated ROR1 (t-ROR1) lacking extracellular domain and variant 3 encodes 393 aa intracellular or secreted protein that lacks both transmembrane and intracellular domain (Balakrishnan et al., 2017; Rebagay et al., 2012).

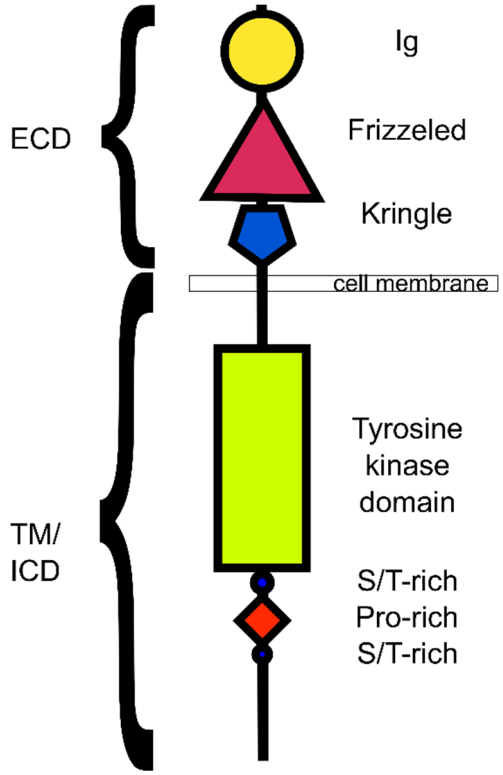

Figure 3. *ROR1 contains Ig-, Frizzled- and Kringle-like domain in the extracellular region. In intracellular region ROR1 contains the tyrosine kinase domain as well as two serine/threonine-rich domains divided by proline-rich domain. ECD: extracellular domain, TM: transmembrane, ICD: intracellular domain.*

Extracellular cysteine-rich FZD-like domain resembles FZD domains in receptors such as Frizzled, Smoothened and LRP that all bind Wnt ligands, suggesting that ROR is an Wnt binding receptor (Green et al., 2008). ROR ligand was initially unknown, leading to 'orphan' denomination, but later on non-canonical Wnt5a ligand has been demonstrated to interact with ROR1 and ROR2 via FZD-like domain (Ho et al., 2012; Jian Yu et al., 2016). From the other extracellular domains Ig-domain is also found in Wnt interacting receptors such as MuSk and PTK7 while KRD-like domain is found for example in hepatocyte growth factors and coagulation proteins where it mediates protein-protein interaction (Borcherding, Kusner, Liu, & Zhang, 2014; Ségaliny et al., 2015).

The catalytic activity of ROR1 has been controversial, but latest studies have determined ROR1 as pseudokinase lacking autophosphorylation and catalytic activity due to amino acid alterations in conserved regions of tyrosine kinase domain (Murphy et al., 2014). Despite lacking catalytic activity, the tyrosine kinase domain of ROR1, can be transphosphorylated by other kinases such as activated MuSk, hepatocyte growth factor receptor (MET) and proto-oncogene tyrosine-protein kinase (SRC) (Gentile, Lazzari, Benvenuti, Trusolino, & Comoglio, 2011; Karvonen,

Summala, Niininen, Barker, & Ungureanu, 2018). ROR1 can also interact with other pseudokinases such as ROR2. ROR1-ROR2 heterodimerization takes place in the presence of Wnt5a binding leading to GEF recruitment and activation of RhoA/RAC1 GTPases. RhoA mediates chemokine induced cell migration and RAC1 induces cell proliferation in leukemic cell lines (Jian Yu et al., 2016).

ROR1 expression is high during embryonic development. In mouse development, ROR1 is first detected in migrating neural crest after which it is observed in mesenchymal cells derived from neural crest cells and mesoderm. In facial development it is detected in lateral nasal and pharyngeal arches formation and in brains in diencephalon and in dorsal parts of mid-hind brain boundary. ROR1 is expressed in mesodermal somites, dermomyotomes and sclerotomes. In limb development ROR1 is expressed in proximal ends of limb buds and in limb mesenchyme. Later it is observed in necrotic zones and interdigital regions. ROR1 is expressed in developing eye lens, hearts myocardium, atrium, septum and aortic valve and lungs (Matsuda et al., 2001). Gene knockout studies have demonstrated that mice with *Ror1*$^{-/-}$ mutation are mostly born viable and resemble closely wild type mice (Ho et al., 2012). However, *Ror1*$^{-/-}$ mice life expectancy has been shown to decrease significantly due to postnatal growth retardancy (Lyashenko et al., 2010). Mice with both *Ror1*$^{-/-}$ and *Ror2*$^{-/-}$ mutations are not carried full term and exhibit facial malformations and limb truncations closely resembling Wnt5a deficient mice phenotype (Ho et al., 2012).

ROR1 expression is almost absent in healthy mature tissues, but it has been found in low levels in adipose tissue, heart, lung and kidney and in smaller concentrations in placenta, pancreas and in skeletal muscle (Balakrishnan et al., 2017; Borcherding et al., 2014; Fagerberg et al., 2014). It is also expressed in intermediate state of B-cell development and in their early precursor state (Rebagay et al., 2012).

1.2.2 PTK7

Protein tyrosine kinase-7 (PTK7) was first characterized in colon cancer as upregulated gene and was named colon carcinoma kinase 4 (CCK4) (Mossie et al., 1995). It belongs in RTK family VI and is composed of 7 extracellular Ig-like domains, a transmembrane domain and an intracellular tyrosine kinase domain (Figure 4) (Masaru Katoh, 2017a). Similarly, to ROR1, TKD of PTK7 contains amino acid alterations in conserved motifs involved in the kinase activity, leading to its denomination as pseudokinase (Murphy et al., 2014). The lack of catalytic activity is demonstrated in humans as well as in mice and chicken homologs (Peradziryi, Tolwinski, & Borchers, 2012).

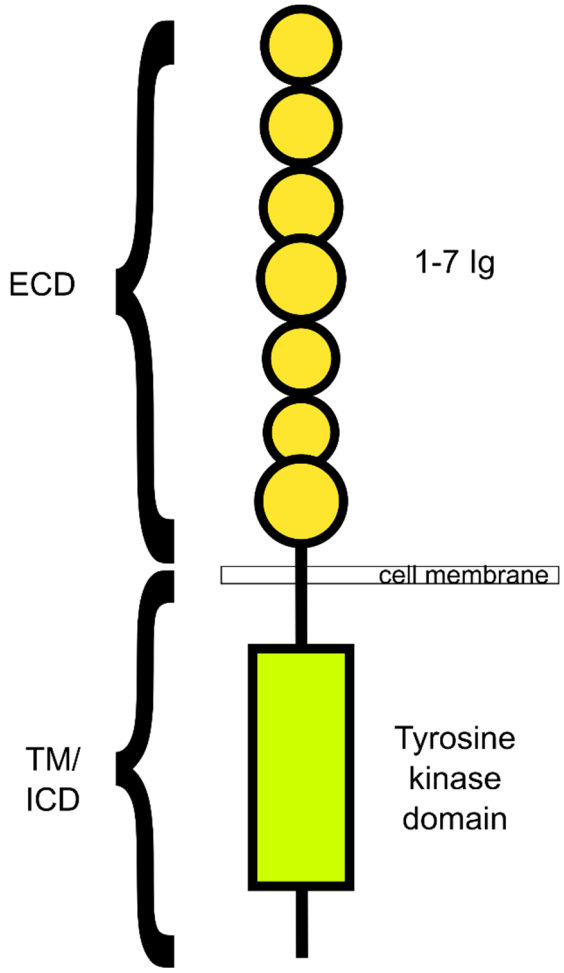

Figure 4. *PTK7 contains 7 Ig-like domains in the extracellular region and in the intracellular region it contains an inactive tyrosine kinase domain. ECD: extracellular domain, TM: transmembrane, ICD: intracellular domain.*

PTK7 belongs to the non-canonical Wnt pathway where it can activate PCP pathway (H. Berger et al., 2017; H. Berger et al., 2017; Masaru Katoh, 2017a). PTK7 activates PCP pathway by translocating DVL at the cell membrane via receptor of activated protein kinase C (RACK). Its downstream signaling pathway remains elusive, but over-expression of PTK7 has been observed to induce phosphorylated JNK translocation into nucleus, indicating also PCP pathway activation (Peradziryi et al., 2012).

PTK7 can interact with ROR2 and Fz7 receptors from non-canonical Wnt pathway. Interaction with ROR2 is initialized by Wnt5a that induces ROR2 PTK7 heterodimerization leading to activation of downstream signaling cascades involving PI3K/AKT, DAG/PKC, RhoA/ROCK, Rac1/JNK and IP_3/Ca^{2+}. ROR2/PTK7 dimerization is observed to increase cell migration in embryogenesis as well as in cancer (Masaru Katoh, 2017a; Podleschny, Grund, Berger, Rollwitz, & Borchers, 2015). PTK7 and Fz7 interaction on the other hand, leads to inhibition of canonical Wnt

signaling by binding Wnt3a and preventing its binding to canonical Wnt-receptors (H. Berger et al., 2017; Masaru Katoh, 2017a).

PTK7 has also a role in canonical Wnt pathway, for it has been observed to interact with β-catenin and LRP6. PTK7 β-catenin interaction is observed for example in cell-cell complexes composed of β-catenin and E-cadherin, where PTK7 interacts with β-catenin but not with E-cadherin. The interaction is Wnt regulated for Wnt3a has been observed to reduce the amount of co-immunoprecipitated PTK7 and β-catenin (Puppo et al., 2011). PTK7 LRP6 interaction is hypothesized to work in PCP and canonical-Wnt signaling regulation. PTK7 stabilizes LRP6 levels in embryogenesis and together they can inhibit PCP (Bin-Nun et al., 2014).

PTK7 can also interact with VEGFR2 that has very similar structure as PTK7, biggest difference being the structure of the tyrosine kinase domain. Together they activate vascular endothelial growth factor (VEGF) pathway, leading to similar downstream signaling effects as non-canonical Wnt pathway, and it can also activate MEK/ERK and FAK/Paxillin signaling cascades (Masaru Katoh, 2017a).

PTK7 has also a soluble form, sPTK7, that is observed after PTK7 interaction with membrane type-1 matrix metalloproteinase (MT1-MMP). MT1-MMP is membrane anchored metalloproteinase that acts in normal embryogenesis and cancer cell invasion by cleaving extracellular matrix (ECM) proteins, activating soluble MMP and by effecting signaling receptors and cell adhesion complexes. MT1-MMP cleaves PTK7 between 6^{th} and 7^{th} Ig domain, forming sPTK7 that activates RhoA-ROCK complex increasing cell migration. This cleavage reduces PTK7 mediated PCP activation, thus reducing cell invasion inhibition. In embryogenesis, both MT1-MMP and PTK7 inhibition lead to similar birth defects, caused by incomplete convergent extension (Golubkov et al., 2010). In addition to MT1-MMP disintegrin and metalloprotease ADAM-17 and γ-secretase are PTK7 shredding proteins that interfere with PCP signaling (Lhoumeau et al., 2016; Na, Shin, Ludwig, & Lee, 2012)

Similar to ROR1, PTK7 is active in embryonic development where it is expressed in intestinal stem cells (LGR5 and ASCL2) and in hematopoietic stem cells (HSC) (Jung et al., 2015; Lhoumeau et al., 2016). In intestinal stem cells PTK7 expression is associated with better self-renewal capability (Jung et al., 2015; Masaru Katoh, 2017a). PTK7 expression in adult tissue is mostly found in liver, pancreas, melanocytes and kidney tissue as well as in placenta. Low expression is observed in ovary, prostate, thyroid gland, brain, heart and skeletal muscle (Fagerberg et al., 2014). Mouse knockout studies have demonstrated that *Ptk7*-deficient mice die perinatally with severe defects in

neural tube closure, gastroschisis and forelimb truncations. These mice also exhibit lower levels of hematopoietic stem and progenitor cells compared to wild type mice (Lhoumeau et al., 2016).

1.2.3 ROR1 and PTK7 expression in hematological malignancies

ROR1 and PTK7 abnormal expression has been observed in multiple diseases and malignancies (Borcherding et al., 2014). Increased ROR1 expression was first detected in chronic lymphocytic leukemia (CLL), and later has been identified for example in acute lymphoblastic leukemia (ALL), hairy cell leukemia (HCL) and in subsets of Non-Hodgkin's lymphoma (NHL) including mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) (Borcherding et al., 2014; Amir Hossein Daneshmanesh et al., 2013; Shabani et al., 2007). The highest expression level of ROR1 has been detected in CLL, HCL and MCL, while other malignancies express either lower levels of ROR1 or the expression pattern alters between cancer types (Amir Hossein Daneshmanesh et al., 2013).

ROR1 upregulation has been associated with increased cell survival and proliferation as well as chemoresistance via activation of MEK/ERK, PI3K/AKT/mTOR and NF-κB (Mohammad Hojjat-Farsangi et al., 2014; Karvonen et al., 2017). In MCL and B-ALL ROR1 has also been detected to cross-talks with pre-B-cell receptor (pre-BCR). This cross-talk maintains the immature state of cancer cells and induces cell proliferation and survival (Bicocca et al., 2012). In CLL ROR1/ROR2 heterodimerization induced by Wnt5a has been associated with increased cancer cell proliferation and migration via activation of RhoA and Rac1 GTPases (Jian Yu et al., 2016).

PTK7 is expressed AML, T-ALL and B-ALL (Jiang et al., 2012; Lhoumeau et al., 2016). PTK7 expression in acute myeloid leukemia (AML) is found in two thirds of patients (Jiang et al., 2012; Prebet et al., 2010; Shabani et al., 2011). AML is characterized by poorly differentiated malignant myeloid cells, which leads to accumulation of lymphocytes into bone marrow impairing normal blood cell development (De Kouchkovsky & Abdul-Hay, 2016). High expression of PTK7 in AML has been found to be associated with poor prognosis due to increased chemoresistance against anthracycline therapy *in vitro* and *in vivo* because of increased cell survival and migration (Prebet et al., 2010). Similar effects have also been observed in PTK7 transfected Ba/F3 cell-lines (Prebet et al., 2010).

1.2.4 B-ALL

B-cell acute lymphoblastic leukemia (B-ALL) is the most common pediatric cancer that accounts for 25 % of all childhood cancers and is typically observed in patient under 6 years old (Bicocca et al.,

2012; Dave et al., 2012). Patients typically express cytopenia, which is bone marrow failure that consists reduced number of mature blood cells, (Loghavi, Kutok, & Jorgensen, 2015).

B-ALL cells resemble immature B-cells and these cells are hypothesized to be derived from pro- or precursor B-cells (Dave et al., 2012; Müschen, 2015). Alternative explanation could be attributed to mature B-cells loss of identity that could be caused by loss of PAX5 expression, which maintains B-cell maturity (Carotta & Nutt, 2008). This common characteristic enables diagnosis to be made by detecting markers of immature B-cell from peripheral blood or bone marrow samples with flowcytometry followed by immunophenotyping and cytogenetic testing (Loghavi et al., 2015; Terwilliger & Abdul-hay, 2017).

About 75 % of all B-ALL cases include chromosomal alterations that constitute aneuploidy and chromosomal rearrangements (Table 2) (Mullighan, 2012). Aneuploidy means reduced or increased number of specific chromosome. High hyperdiploidy means that number of chromosomes is increased with at least five chromosomes, (typically > 50 chromosomes total) and it is observed in 20-30 % of all B-ALL cases. The prognosis in hyperdiploidic cases is good but the biological basis of the cancer with increased number of chromosomes is not well understood (Mullighan, 2012; Woo, Alberti, & Tirado, 2014). However, about 80 % of patient with hyperdiploidy express also other genetic alterations, which could be caused by genetic instability (Woo et al., 2014). In contrary, hypodiploidy means presence of under 46 chromosomes and is associated with poor prognosis. In most hypodiploid B-ALL cases patient has 45 chromosomes but there are also cases of high hypodiploidy (40-44 chromosomes), low-hypodiploidy (33-39 chromosomes) and near-haploidy (23-29 chromosomes) with reduced number of chromosomes. Common characteristics in haploidy are increased PI3K signaling via Ras and TP53 mutations (Woo et al., 2014).

Table 2. *Typical chromosomal alterations in B-ALL* (Bicocca et al., 2012; Loghavi et al., 2015; Mullighan, 2012; Woo et al., 2014).

Genetic abnormality	**Transgene**	**Prognosis**	**Frequency (%)**	**Typical phenotype or mutations**	**Typical patient age**
Aneuploidy					
High-hyperdiploidy (>50 chromosomes)		good	20 - 30	*FLT3* mutations	
Hypodiploidy (< 46 chromosomes)		poor	5-8	Alterations in *TP53, RB1* and *IKZF2 (Helios)* or alterations in Ras and RTK signaling, IKZF3 (Aiolos) mutations.	
Translocations					
t(12;21) p(13;q22)	ETV6-RUNX1 (TEL-AML1)	Good	22		
t(1;19)(q23;p13)	TCF3-PBX1 (E2A-PBX1)	Intermediate	4-7	Increased *ROR1* expression	
t(17;19)(q22;p13)	TCF3-HLF (E2A-HLF)	Very poor	rare	Increased *ROR1* expression	Associated with older age
t(9;22)(q34;q11)	BCR-ABL1 (Ph)	Intermediate	3-5	Alterations in JAK-STAT signaling, *IKZF1* (*Ikaros*) deletions	Associated with older age
MLL (11q23) rearrangements: t(4;11)(q21;23), t(9;11)(p22q23), t(11;19)(q23;p13.3), t(10;11) (p13-14;q14-21)	MLL-AFF1 (AF4) MLL-MLLT3 (AF9) MLL-ENL MLL-MLLT10 (AF10)	Poor	6	*FLT3* mutations	Typical in infants (80 % of infant ALL)
t(5;14) (q31;q32)			rare	Eosinophilia caused by increased IL3 levels	
PAX5 rearrangement t(7;9), t(9;12), t(9;20)			2		
CRLF2 rearrangement					
JAK mutations					

Translocations are chromosomal rearrangement that lead to gene fusion. The most common translocations in B-ALL is t(12;21), which is detected in 15-25 % of B-ALL cases and it is associated with good prognosis. In t(12;21) two genes associated with normal hematopoiesis are fused (*ETV6* and *RUNX1*) which converts RUNX1 into transcriptional repressor (Mullighan, 2012; Woo et al., 2014). The fusion protein induces tyrosine-protein kinase JAK and signal transducer and activator of

transcription (STAT) signaling and progenitor B-cell self-renewal but it does not promote leukemia without secondary genetic events (Woo et al., 2014).

Another typical translocation is Philadelphia chromosome (Ph) caused by t(9;22)(q34;q11.2) translocation, which leads to *BCR-ABL1* fusion gene. This is more common in adult B-ALL and it is detected in 3-5 % of all B-ALL cases (Loghavi et al., 2015; Woo et al., 2014). The fusion protein BCR-ABL1 is an active tyrosine kinase that upregulates cell proliferation. In Ph or in *BCR-ABL1*-like B-ALL, *IKZF1* deletion is often observed, which results into more aggressive cancer. *IKZF1* gene encodes Ikaros protein that is cancer suppressive. This deletion increases JAK-STAT activation and Bruton tyrosine kinase (BTK) expression is common (Loghavi et al., 2015). JAK mutations are also typical in BCR-ABL1-like B-ALL with *CRLF2* mutations, which are especially seen in down-syndrome patients with B-ALL (Woo et al., 2014).

With infant patients *MLL* rearrangements are most typical and are observed in almost 80 % of infant B-ALL cases. In contrary older patient rarely have *MLL* mutations (Loghavi et al., 2015). The most common translocation concerning *MLL* is t(4;11)(q21;q23) leading to *MLL-AFF1* gene fusion which is observed in about 50 % of *MLL* rearrangements. Other typical rearrangements are t(9;11)(p22;q23), t(11;19)(q23;p13.3) and t(10;11)(p13-14;q14-21). Protein that *MLL* gene codes takes part in histone methyltransferase activity that is required for activation of *HOX* genes that regulate hematopoiesis (Woo et al., 2014).

4-7 % of B-ALL cases express translocations t(1;19) with *TCF3-PBX1* (E2A-PBX1) fusion protein (Bicocca et al., 2012; Dave et al., 2012; Eldfors et al., 2017). *TCF3* encodes transcription factors E12 and E47 that are necessary for early lymphoid development. The fusion with homeobox gene *PBX1* reduces E12 and E47 expression. At the same time the fusion impairs *PBX1* homeodomain protein transcription that are associated with lymphoid precursor development (Mullighan, 2012; Woo et al., 2014). Other translocation containing *TCF3* gene is t(17;19)(q22;p13) that leads to *TCF3-HLF* fusion gene. This translocation is rear and is correlated with older age and very poor prognosis. The fusion protein TCF3-HLF can control apoptosis regulating genes in lymphoid progenitors (Mullighan, 2012).

Recently, ROR1 expression in B-ALL was correlated with t(1;19) and t(17;19) translocation and/or lack of CD34 expression (Broome, Rassenti, Wang, Meyer, & Kipps, 2011). ROR1 expression is correlated with increased cancer cell survival and chemotherapy resistance (Dave et al., 2012).

ROR1 is normally expressed in intermediate state of B-cell development in $CD34^{-}CD38^{+}CD19^{+}CD10$ cells (Hudecek et al., 2010). The fact that in B-ALL ROR1 is expressed

both in developing and mature cancer cells, suggests that ROR1 helps in maintaining cell viability. In developing B-cells, pre-B-cell receptor (pre-BCR) is present and induces cell survival and proliferation via PI3K/AKT and MEK/ERK pathways. However, there is evidence that ROR1 and pre-BCR crosstalk in B-ALL. Evidence shows that inhibition of pre-BCR leads to ROR1 mediated MEK/ERK pathway activation and AKT activation. This has led to conclusion that ROR1 upregulation after pre-BCR inhibition maintains cell viability. In mature B-cells pre-BCR light chain is internalized and ROR1 is no longer expressed which allows cells to differentiate into mature B-cells but this leads to loss of AKT mediated cell survival. For B-ALL cells, it is hypothesized that fusion protein arrests cells into pre-B-cell state so that cell viability can be supported in cancer cells (Bicocca et al., 2012).

According to one study, PTK7 is also expressed in 27 % of B-ALL in patient sample analysis (Prebet et al., 2010). It is also found in early B-cell precursors from bone marrow (Jiang et al., 2012). However, the role of PTK7 in B-ALL is not yet well understood.

The survival rate is for B-ALL is about 80 % after initial chemotherapy (Bicocca et al., 2012; Dave et al., 2012; Terwilliger & Abdul-hay, 2017). The cancer is typically treated with chemotherapy in three steps: induction, consolidation and long-term maintenance (Terwilliger & Abdul-hay, 2017). After induction therapy 85 - 90% of the patient go into remission but some subsets are refractory and relapses after remission are very common (Terwilliger & Abdul-hay, 2017). In addition, treatment with chemotherapy can cause longtime toxicity especially for developing brain with psychological and neurocognitive defects (Dave et al., 2012).

1.2.5 RTK targeting in cancer

Chemotherapy can cause serious defects especially in developing children, which makes it important to develop therapies that target only cancer cells with minimal cytotoxic effects to healthy tissues (Masaru Katoh, 2017a). Receptor tyrosine kinases are often major regulators of cancer cell survival, which has led to development of RTK targeting treatments that have been observed to reduce viability of cancer cells (Takeuchi & Ito, 2011). RTK targeting drugs have shown promising effects against multiple cancers and they could be used in combination therapy, leading to decreased therapy resistance and toxicity. Some of the possibilities are: monoclonal antibodies, antigen-drug conjugates (ADC), bispecific antibodies (bsAb) or modified T-cells with chimeric antigen receptor (T-CAR) (Masaru Katoh, 2017a).

Monoclonal antibodies (mAb) are genetically identical antibodies that target one specific epitope. The mAbs used in therapy are humanized or human antibodies. Both are typically produced

in mice, but in human antibody production mice are transgenic with human immunoglobulin sequence, while humanized mAbs are produced in mice with wild type sequence. Hypervariable domain of humanized mAbs therefore must be modified afterwards to resemble human antibody framework (Mallbris et al., 2016).

Targeting ROR1

Internal ROR1 gene silencing with siRNA has been observed to significantly decrease t(1;19) B-ALL cell viability and similar effects have been observed with extracellular targeting of ROR1 with monoclonal antibodies in CLL (Bicocca et al., 2012; A. H. Daneshmanesh et al., 2012). Because of this, ROR1 monoclonal antibodies (mAb) have arisen as therapeutic agents against ROR1 expressing malignancies. When mAb binds to ROR1, the receptor is partially internalized leading to antibody-dependent cellular cytotoxicity (Baskar, Wiestner, Wilson, Pastan, & Rader, 2012; Yang et al., 2011). This partial internalization of ROR1 after mAb exposure could also be used to deliver cytotoxic agents into cancer cells (Rebagay et al., 2012). ROR1 targeting with mAbs has been observed to decreased breast cancer cell metastasis, which could broaden ROR1 mAb medical applications to solid malignancies as well (Borcherding et al., 2014).

ROR1 targeting mAb Cirmtuzumab (or UC961) is now in phase II clinical trials against CLL (Somovilla-Crespo, Cuesta-Mateos, Alcaraz-Serna, & Muñoz-Calleja, 2018). Cirmtuzumab is observed to compete with ROR1 ligand, Wnt5a, in CLL cells to impair ROR1/ROR2 binding as well as downstream signaling effects including RhoA and RAC1 activation (Choi et al., 2018; J. Yu et al., 2017). Cirmtuzumab epitope binding is located to the junction between Ig-like and CRD-like domain and exhibits highly cytotoxic and specific effects against ROR1. Antibodies that target other extracellular parts of ROR1 have also been tested and CRD- and Kringle-like domains targeting antibodies induced apoptosis in cancer cells better than antibodies that target Ig-like domain (A. H. Daneshmanesh et al., 2012).

However, owning to low expression level of ROR1 on the surface of B-cells, the cytotoxic effects of ‘naked’ ROR1 antibodies can be weak, which could make armed ROR1 antibodies or T-CARs a more attractive alternative to mAbs (Rebagay et al., 2012). T-CARs against ROR1 have already been developed and tested in rhesus monkeys and no significant off-target cytotoxic effects were observed (C. Berger et al., 2015). T-CARs could be even safer to mAbs because T-cell delivery requires active inflammation, which is observed in cancerous tissue, but not in healthy tissues (Hudecek et al., 2010). Small molecule drug KAN0439834 has also been developed to target intracellular parts of ROR1. KAN0439834 targets tyrosine kinase domain of ROR1 and has been

shown to induce apoptosis in CLL patient samples but not in normal T and B cells (M. Hojjat-Farsangi et al., 2018).

Targeting PTK7

PTK7 silencing has not been researched in correlation with B-ALL but in AML PTK7 silencing has been observed to decrease cell migration and survival (Prebet et al., 2010).

PTK7 targeting drug PF-06647020 is in phase I trials against advanced solid tumors (Sachdev et al., 2016). PF-06647020 is an antibody-drug conjugate (ADC) where PTK7 antibody is conjugated to microtubule inhibitor Aur0101. The ADC has given good antitumoral response in patient xenografts. It targets especially tumor initiating cells which have been observed to reduce cancer recurrence. Experiments done in monkeys demonstrates that because the drug conjugate, Aur0101, requires high antigen level to function, the ADC does not cause target dependent toxicity in non-cancerous cells despite the PTK7 expression in them. This drug could also affect tumor microenvironment by targeting plasmacytoid dendritic cells and adverting their immune suppressive functions (Damelin et al., 2017).

Further knowledge of oncogenic role of ROR1 and PTK7 in cancer signaling, enables targeting their downstream signaling as well. Potential pathways are for example PI3K/AKT/mTor, RhoA/RAC1 and MEK/ERK pathways, that are both important in maintaining cell survival in cancer cells (Blenis, MacKeigan, & Murphy, 2005). Because ROR1 and PTK7 can both be either oncogenic or cancer suppressing depending on cancer cell origin and subtype, it is important to specify the oncogenic role of these receptors before targeted therapy can be used.

2 AIMS OF THE RESEARCH

Aim of this research is the analyze the of oncogenic role ROR1 and PTK7 in B-ALL and use this information to improve B-ALL cancer therapy by targeting these pseudokinases.

3 MATERIALS AND METHODS

1.3 Tissue culture

Cell lines used in this study are introduced in Table 3 with corresponding growth conditions. Our main focus was on B-ALL cell lines Kasumi-2, RCH-ACV and 697 with t(1;19) translocation (Bicocca et al., 2012; Eldfors et al., 2017). 293T cells were used in production of shRNA cell lines and JEKO-1, HeLa, MEC-1 and Ba/F3 cells were used as controls in Western Blot. All growth mediums were supplemented with 1 % of penicillin streptomycin (17-602E, Lonza), 1 % of L-glutamate (17-605E, Lonza) and 10 % of fetal bovine serum (FBS) (10500064, Life Technologies). In addition, Ba/F3 RPMI1640 growth medium was supplemented with 10 % IL-3 supernatant derived from WEHI cultured cells.

Table 3. *Cell lines used in this study (Bicocca et al., 2012; Eldfors et al., 2017)(https://www.dsmz.de/).*

Cell line	**Cell type**	**Fusion gene**	**Growth media**
Kasumi-2	B-ALL t(1;19)	TCF3-PBX1	RPMI1640 (31870025, Life Technologies)
RCH-ACV	B-ALL t(1;19)	TCF3-PBX1	RPMI1640 (31870025, Life Technologies)
697	B-ALL t(1;19)	TCF3-PBX1	RPMI1640 (31870025, Life Technologies)
293T	Embryonic kidney epithelial cells		DMEM (BE12-614F, Lonza)
JEKO-1	MCL		DMEM (BE12-614F, Lonza)
HeLa	Cervix carcinoma epithelial cells		RPMI1640 (31870025, Life Technologies)
MEC-1	B-CLL		IMDM
Ba/F3	mouse pro B cell line		RPMI1640 (31870025, Life Technologies) supplemented with 10 % WEHI supernatant.

1.4 Western Blot

Western Blot (WB) was done for cell lysates and immunoprecipitation (IP) samples. All steps in sample preparation were performed on ice. Cell lysates were prepared from harvested cells that were pelleted in 4 °C, 500 x *g* for 5 min. Supernatants were aspirated and resuspended into TritonX lysis buffer (Table 4). Cells were incubated on ice for 15 min after which they were centrifuged on full speed (21139 x *g*) for 30 min in 4 °C and supernatants were collected. Protein level was measured with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and with QuickStart™ Bradford Protein assay (Bio-Rad) (Bradford, 1976). In Bradford protein assay QuickStart™ Bradford 1x Dye-reagent (Bio-Rad, #5000205) was pipetted to clear bottom 96 well plate with 1:50 ratio of cell lysate. Luminescence was measured using Multiskan at 595 nm. Normalized lysates were mixed with 4x Laemlli Sample Buffer (Bio-Rad) (Table 4).

Table 4. *Reagents used for cell lysis*

Reagent	**Content**	**Proportion**
TritonX lysis buffer	TritonX-100 (T8787-50ML, Sigma)	1 %
	Glycerol	10 %
	NaCl	150 mM
	EDTA	1 mM
	NaF	50 mM
	Tris-HCl pH 7.5	50 mM
	Phosphatase Inhibitor Cocktail A (Bimake)	1 %
	Phosphatase Inhibitor Cocktail B (Bimake)	1 %
	Protease Inhibitor Cocktail (B15001, Bimake)	1 %
Laemlli Sample Buffer	Laemlli Sample Buffer 4x (1610747, Bio-Rad), Laemlli Sample Buffer 2x (1610737, Bio-Rad)	
	β-mercaptoethanol	

Immunoprecipitations were done with ROR1 6D4 and PTK7 antibodies (Table 6). Antibody was added on 1:100 ratio in cell lysate and they were incubated overnight in rotation at 4 °C. On the following day protein G beads (Protein G PLUS-Agarose, sc-2002, Santa Cruz) were washed twice with lysis buffer containing inhibitors and added on cell lysates. Lysates were incubated with protein G beads on rotation in 4 °C for 1,5 h. After incubation, beads were collected and washed twice with 500 µl of lysis buffer with inhibitors. Samples were resuspended into 20 µl 2x Laemlli Sample Buffer

(Bio-Rad). Both IP and cell lysates were boiled for 5 min in 98 °C before running the samples on gels.

The blotting was done for cell lysate and IP samples likewise. Reagents used in blotting are listed in Table 5. 7-12 % acrylamide SDS-PAGE gels were prepared and used depending on size of the examined proteins. Electrophoresis was done in 120 V, 400 mA for 1 h with Mini-PROTEAN Tetra System (Bio-Rad). After SDS-PAGE running, proteins were transferred to polyvinylidene fluoride (PDVF) membrane in semi-dry electro transfer system. The transfer was done in 12 V, 100 mA/ blot for 50 min on Trans-Blot SD (Bio-Rad).

Blocking of the membrane was done in 4 % bovine serum albumin (BSA), 0,05 % Tween TBS solution. Blots were incubated with primary antibodies (Table 6) O/N in 4 °C on shaker. After incubation blots were washed with decreasing TBS-Tween of buffers series and secondary antibodies (Table 7) were added for 1 h after which the washes were repeated. Washed blots were scanned with Odyssey (Li-Cor) imaging system and analyzed with Image Studio (Li-Cor).

Table 5. *Regents used in SDS-PAGE and Western Blot*

Step	**Reagents**	**Content**
Gel run	Gel	30 % Acrylamide/Bis Solution (161-0156, Bio-Rad)
		1,5 M Tris pH 8.8 with 0,4 % SDS
		0,5 M Tris pH 6.8 with 0,4 % SDS
		10 % Ammonium persulfate
		10 % TEMED
	Running buffer	25 mM Tris
		192 mM Glycine
		0,1 % SDS
	2x Laemmli Sample Buffer	2x Laemmli Sample Buffer
		β-mercaptoethanol
	Marker	PageRuler Plus Prestained protein ladder (Thermo Scientific)
Transfer	Transfer Buffer	30,8 mM Tris
		0,24 mM Glycine
		20 % methanol
Blocking	Blocking Buffer	0,05 % Tween TBS
		4 % BSA (Thermo Fisher)
Washing	TBS-Tween buffers	0,5 % Tween TBS
		0,1 % Tween TBS
		0,05 % Tween TBS
Antibody dilution	Antibody dilution buffer	0,05 % Tween TBS
		0,5 % BSA (Thermo Fisher)
		0,005 % NaN_3

Table 6. *Primary antibodies used in immunoblotting.*

Antibody	**Catalog number**	**Manufacturer**
ROR1 6D4		From dr. Riddell lab (Balakrishnan et al., 2017)
PTK7 (CCK-4) (D2Z1N)	25618S	Miltenyi Biotec
Wnt5a (C27E8)	2530S	BioNordika
β-tubulin (F-1)	SC-166729	Santa Cruz
AKT (B-1)	SC-5298	Santa Cruz
Phospho AKT (Ser473) (D9E)	4060S	Cell Signaling
MEK (H-8)	SC-6250	Santa Cruz
ERK 1 (MK1)	SC-135900	Santa Cruz
NF-κB (p56) (L8F6)	6956	Cell Signaling
STAT3 (124H6)	9139S	Cell Signaling
PI3K (p85)	13666S	Cell Signaling
DVL3	3218S	Cell Signaling
PARP (46D11)	9542S	Cell Signaling
β-catenin (6B3)	9582S	Cell Signaling
SAPK/JNK	9252S	Cell Signaling
RhoA (67B9)	2117S	Cell Signaling
RAC1 (CDC42)	PA1091X	Cell signaling

Table 7. *Secondary antibodies used in immunoblotting*

Antibody	**Product number**	**Manufacturer**
IRDye® 800CW Donkey Anti-Mouse IgG (H+L)	923-32212	Li-Cor
IRDye® 680CW Donkey Anti-Rabbit IgG (H+L)	926-69073	Li-Cor

On SDS-PAGE gels, cell lysates were ran with positive and negative controls. The positive control for Wnt5a expression level was HeLa cell extracts and for ROR1 expression levels was JEKO-1 cell extracts. Negative control for PTK7 expression was Ba/F3 cell lysates and for ROR1 expression was MEC-1 cell lysates.

1.5 FACS

Fluorescent activated cell sorting (FACS) was performed using BD Accuri™ C6 and corresponding software. Antibodies and buffer used in FACS are presented in Table 7. The antibodies are conjugated to R-Phycoerythrin (PE) fluorochrome that has excitation wavelength of 496 and 565 nm and emission wavelength of 575 nm, and allophycocyanin (APC) fluorochrome that has excitation wavelength of 645 nm and emission wavelength of 660 nm (http://www.biolegend.com/media_assets/support_resource/BioLegend_Fluorochrome-Instrument_chart.pdf).

For FACS sample preparation, over 50 000 cells were harvested and collected with centrifuge (500 x *g* for 10 min). Cells were resuspended into 50 µl of FACS buffer. Antibody was added 1:10 and cells were incubated for 10 min in 4 °C. After incubation cells were washed and resuspended into FACS buffer (amount depending on pellet size).

Table 7. *Reagents used in FACS*

Reagent	**Content**
FACS buffer	PBS
	0,5 % BSA (Thermo Fisher)
	2 mM EDTA
Antibody	Anti-human-ROR1-PE (130-098-317, Miltenyi Biotec)
	Anti-human-PTK7-PE (130-091-364, Miltenyi Biotec)
	Anti-human-PTK7-APC (130-112-678, Miltenyi Biotec)

Measurements were done with Accuri™ C6 right after sample preparation. For PE conjugate FL2 channel was used and samples ran until total number of events was 10 000 with medium flow rate (60 µl/ min). Antibody stained samples were always ran with unstained samples that had gone through same protocol without addition of the primary antibody. In sample analysis, live cell population was gated separately for each cell line and doublets were gated out.

1.6 Cell counts

Cell counts were performed using automated cell counter Countess II (Thermo Fisher) with trypan blue to mark the dead cells and with FACS using propidium iodide (PI) staining. PI binds to double stranded DNA and by gating out positively stained events it was possible to determine the number of live cells in the sample. FACS was done with Accuri™ C6 and analysis with corresponding software.

1.7 CellTiter-Glo® proliferation assay

CellTiter-Glo® (CTG) (Promega) is a proliferation assay in which the amount of ATP is measured from cell lysate. CTG reagent contains Ultra-Glo™ Recombinant Luciferase which in presence of ATP, Mg^{2+} and O_2 mono-oxygenates into luminescing Oxy-Luciferin. The intensity of luminescence is directly proportional to the amount of ATP and to the number of live cells.

Before the start of the assay, CTG reagent was placed in dark room temperature (RT) to warmup. Cells were pipetted in 3 replicates to clear bottom 384 well plate. The CTG reagent was diluted 1:5 with RPMI1640 and added 1:1 on each well with cells. Plate was incubated for 30 min and 1 h in dark at RT after which the luminescence was measured using EnVision Multimode plate reader (Perkin Elmer).

1.8 ROR1 inhibition with monoclonal antibody

Monoclonal anti-hROR1(2A2) (MACS Miltenyi Biotec) antibody was used in three different concentrations 2,5 µg/ ml, 5 µg/ ml, and 10 µg/ ml and 10 µg/ ml of IgG (mouse isotype control, Thermo Scientific) was used as control. RCH-ACV, 697 and Kasumi-2 cells were diluted to 250 000 cells/ ml and plated on round bottom 96 well plate with antibodies and untreated negative controls. The cell proliferation was measured with CTG in three timepoints 0 h, 24 h and 48 h.

Intracellular downstream signaling and ROR1 levels after exposure to mAb were also investigated. In this experiment cells were treated with 10 µg/ ml of 2A2 mAb and incubated for 24 h. Cells were lysed and analyzed by Western Blot.

1.9 ROR1 gene silencing with shRNA

Short hairpin RNA (shRNA) was used to create stable gene knockdown system. Inside the cell shRNA degrades into small interfering RNA (siRNA) which attaches into RNA-induced silencing complex (RISC) and activates RNase complex. RNase complex attaches to sequence specific mRNA leading to mRNA degradation and gene silencing (Agrawal et al., 2003).

In this study we used single-vector inducible lentiviral transfection system (Figure 10) (Wee et al., 2008; Wiederschain et al., 2009) with pLKO Tet-On vector (Figure 5) to create stable shROR1 cell lines from RCH-ACV, 697 and Kasumi-2 (Wee et al., 2008; Wiederschain et al., 2009). With pLKO-Tet-On, shRNA production can be controlled with doxycycline (DOX) (Frank, Schulz, & Miranti, 2017; Paddison, Caudy, Bernstein, Hannon, & Conklin, 2002). Without DOX induction, shRNA production is repressed with TetR (Wiederschain et al., 2009).

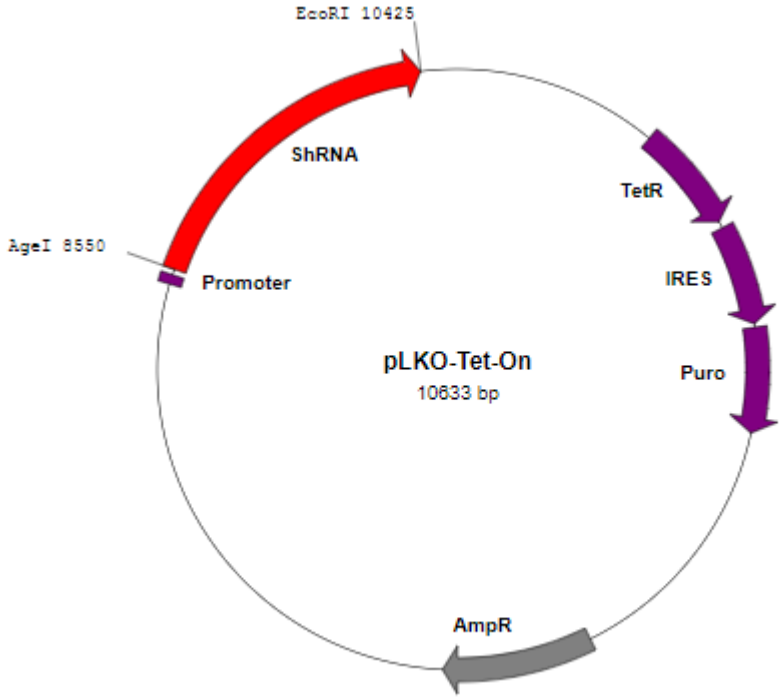

Figure 5. *pLKO-Tet-On vector map. Targeted shRNA can be cloned into pLKO-Tet-On vector with AgeI and EcoRI and cloned vector can be selected with ampicillin. Cells infected with lentiviruses containing pLKO-Tet-On constructs can be selected with puromycin. Without doxycycline TetR suppresses shRNA production. Figure drawn with http://rf-cloning.org/savvy.php.*

Three lentiviruses were produced using single-vector inducible lentiviral transfection system in 293T cells with pLKO-Tet-On transfer plasmid. Two ROR1 shRNA constructs, shROR1-1 and shROR1-2, and control shRNA construct, shCtrl, were previously cloned to pLKO-Tet-On vector to produce transfer plasmids (shRNA oligos shown on Table 8). 293T cells were plated on 6 well plate 24 h before transfections. In the morning of transfections, the culture medium was changed into fresh medium.

FuGENE HD transfection reagent (Promega) and Opti-MEM (Life Technologies) were mixed and incubated for 5 min. pLKO-Tet-On transfer plasmids were transfected together with two helper plasmids VSV-G envelope plasmid and dR8.91 packaging plasmid. Plasmids were mixed together and FuGENE was added on Opti-MEM solution and incubated for 15 min. Mixtures were pipetted on the 293T cells drop by drop and plates were carefully rocked side to side. Cells were incubated in 37 °C for 48 h.

Table 8. *ShRNA oligos for pLKO-Tet-On vector cloning. Sense sequence is shown in red and antisense in blue.*

ShROR1-1
Top: 3’ CCGGCTTTACTAGGAGACGCCAATACTCGAGTATTGGCGTCTCCTAGTAAAGTTTTT 5’
Bottom: 3’ AATTAAAAACTTTACTAGGAGACGCCAATACTCGAGTATTGGCGTCTCCTAGTAAAG 5’
ShROR1-2
Top: 3’ CCGGCGGAGAGCAACTTCATGTAAACTCGAGTTTACATGAAGTTGCTCTCCGTTTTT 5’
Bottom: 3’ AATTAAAAACGGAGAGCAACTTCATGTAAACTCGAGTTTACATGAAGTTGCTCTCCG 5’
ShCtrl
Top: 3’ CCGGTAGAGACTAGCTGCACGTATACTCGAGTATACGTGCAGCTAGTCTCTATTTTT 5’
Bottom: 3’ AATTAAAAATAGAGACTAGCTGCACGTATACTCGAGTATACGTGCAGCTAGTCTCTA 5’

After the incubation, cell media containing lentiviruses were collected from 293T cells. Media was filtered with 0,45 µm filter and 8 µg/ ml of polybrene was added on media to increase the infection efficiency (Davis, Morgan, & Yarmush, 2002). 3 x 10^6 cells were collected from RCH-ACV, 697 and Kasumi-2 cell lines for infections. Cells were pelleted (5 min, 300 x *g*) and each cell line was resuspended with 500 µl of filtrate for each infection: shROR1-1, shROR1-2 and shCtrl. Resuspended cells were centrifuged (1000 x *g*, 30 min, 30 °C) after which they were gently vortexed and incubated for 2 h in 37 °C after which centrifugation was repeated. Supernatants were aspirated, and cells were resuspended into normal RPMI1640 culture medium and incubated in 37 °C over three days. Infected cells were selected with puromycin treatment. Over duration of 3 weeks puromycin concentration was increased from 0,25 µg/ ml to 2 µg/ ml for RCH-ACV and 697 shRNA cell lines and to 1 µg/ ml for Kasumi-2 shRNA cell lines due to slower cell proliferation.

4 RESULTS

1.10 ROR1 and PTK7 expression in B-ALL cells and their interaction with Wnt5a

Previous studies have demonstrated uniform expression of ROR1 in B-ALL cells with t(1;19) translocation (Broome et al., 2011), but PTK7 expression in B-ALL cells with t(1;19) translocation has not been previously investigated. To confirm ROR1 expression and investigate PTK7 expression in B-ALL cells with t(1;19) translocations we used FACS and immunoblotting (Figure 6 and Figure 7 A).

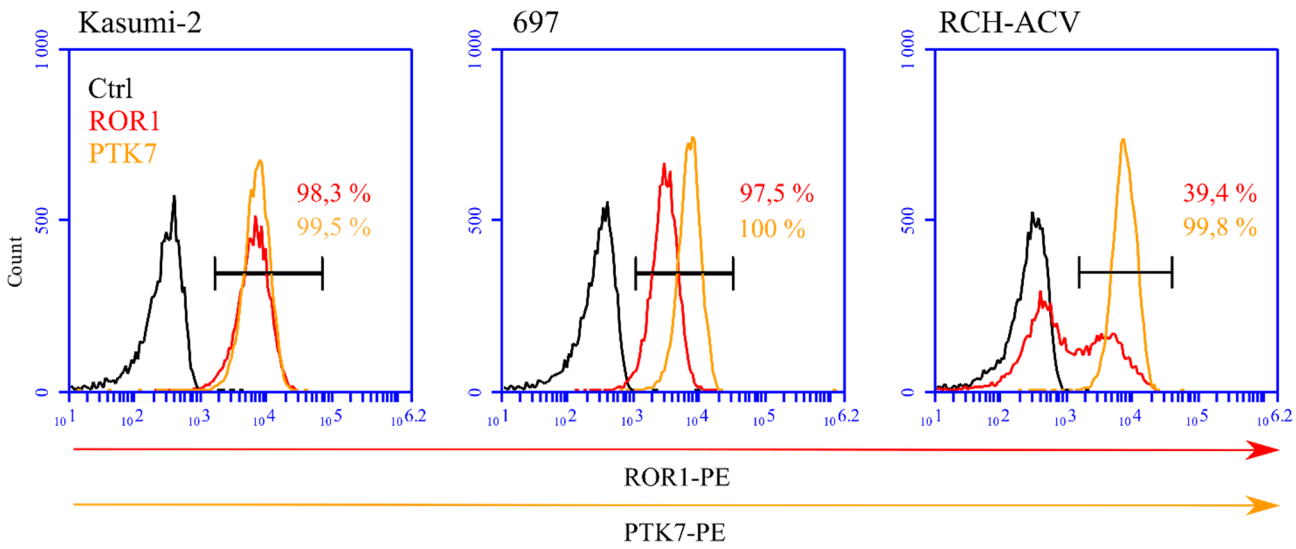

Figure 6. *FACS histogram with ROR1 and PTK7 staining demonstrates that ROR1 and PTK7 are expressed on cell surface of Kasumi-2, 697 and PTK7.*

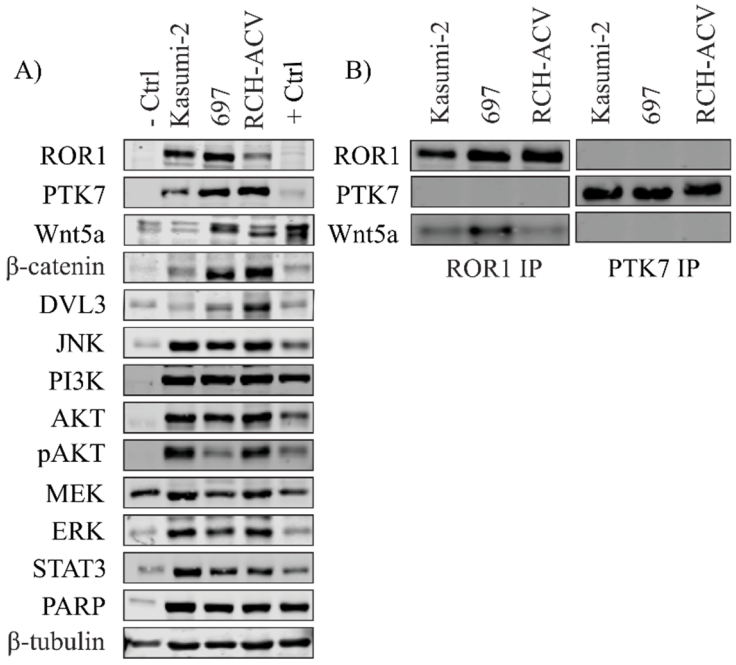

Figure 7. *A) Western Blot image analysis of Kasumi-2, 697 and RCH-ACV cell lysates shows that both ROR1 and PTK7 are expressed in these cell lines as well as Wnt5a ligand and multiple proteins from non-canonical and canonical Wnt pathway. B) Immunoprecipitation with ROR1 and PTK7 antibodies shows that Wnt5a binds to ROR1 but not to PTK7.*

FACS was used to measures protein expression on the cell surface by detecting fluorescence from ROR1-PE and PTK7-PE antibodies. Figure 6 A shows that PTK7 is expressed in Kasumi-2, 697 and RCH-ACV cells at similar levels. ROR1 expression is observed in all the cell lines. The expression is uniform in Kasumi-2 and 697 cell lines, but it is significantly more dispersed in RCH-ACV cell line. Similar expression has also been observed in previous studies (Bicocca et al., 2012). RCH-ACV cells, stained with ROR1-PE, showed two peaks in histogram figure from which one was in the same area as the unstained control indicating ROR1 negative population, not observed in Bicocca et al. paper. The other peak showed a ROR1 positive population indicating two cell populations in RCH-ACV cell line, from which one expresses ROR1 on cell surface and the other does not.

Since ROR1 and PTK7 expression was detected with FACS, we sought to confirm the expression using Western Blotting. Kasumi-2, 697 and RCH-ACV cell lysates were run on SDS-PAGE gels and the membranes were stained with primary antibodies (Figure 7 A). Similarly to FACS analysis, ROR1 and PTK7 expression was detected in B-ALL cell lines. ROR1 level was lower in RCH-ACV compared to 697 and Kasumi-2 cell lines. Ba/F3 cells were used as negative control for

PTK7 whereas MEC-1 was used as negative control for ROR1, and JEKO-1 as a positive control for ROR1.

Because both ROR1 and PTK7 are described to work in non-canonical Wnt signaling pathway (Green et al., 2008; Masaru Katoh, 2017a) we sought to investigate the non-canonical Wnt ligand, Wnt5a, expression and other effector proteins by Western Blotting (Figure 7 A). All the t(1;19) cell lines expressed endogenous Wnt5a at different levels, compared with Hela cell lysates that were used as Wnt5a positive control. Moreover, we detected downstream signaling effectors from non-canonical Wnt/PCP pathway, JNK as well as from Wnt/RTK pathway PI3K/AKT, to be expressed in all the cell lines. To investigate the pathway activation, pAKT antibody was used to detect AKT phosphorylation. Based on AKT phosphorylation the pathway was activated in RCH-ACV and Kasumi-2 cells. We also detected the expression of MEK and ERK as well as STAT3 proteins. We investigated also expression major mediator of canonical Wnt signaling pathway, β-catenin and DVL3, to be expressed in B-ALL cells. β-catenin expression was very low in Kasumi-2 cells compared to 697 and RCH-ACV cells.

Next, we sought to investigate whether ROR1 or PTK7 could interact with Wnt5a ligand in B-ALL cells. Previous results have indicated that ROR1 and Wnt5a could interact in CLL cells (Cui et al., 2016). To investigate the interaction in B-ALL cells, we used immunoprecipitation with ROR1 and PTK7 antibodies (Table 6). Immunoprecipitation demonstrated that only ROR1 binds Wnt5a indicating that ROR1-Wnt5a interaction in also functional in B-ALL cells. Co-immunoprecipitation shows highest Wnt5a level in 697 cells and lowest in RCH-ACV cells. Previous studies have demonstrated PTK7 to interact with Wnt5a in PTK7 and Wnt5a co-transfected 293T cells (Martinez et al., 2015). Based on our results of PTK7 immunoprecipitation, PTK7 does not bind Wnt5a in B-ALL cells indicating that Wnt5a and PTK7 do not interact directly.

1.11 Targeting ROR1 with monoclonal antibody reduces cell viability in B-ALL cells

Since ROR1 was expressed in B-ALL cell lines at different levels, we sought to investigate what are the effects to cell proliferation when non-canonical Wnt pathway via ROR1 is blocked using anti-human-ROR1 (2A2) monoclonal antibody. Previously it has been demonstrated that ROR1 (2A2) antibody recognizes Ig epitopes from the extracellular domain of ROR1 leading to partial internalization of the receptor (Baskar et al., 2012) and similar effects have been observed in ROR1 expressing CLL, MCL and B-ALL cells (Dave et al., 2012). The effects of ROR1 targeting with monoclonal antibody have previously been demonstrated to induce apoptosis in CLL cells (A. H.

Daneshmanesh et al., 2012) but the effects of ROR1 monoclonal antibody on B-ALL cells were not investigated.

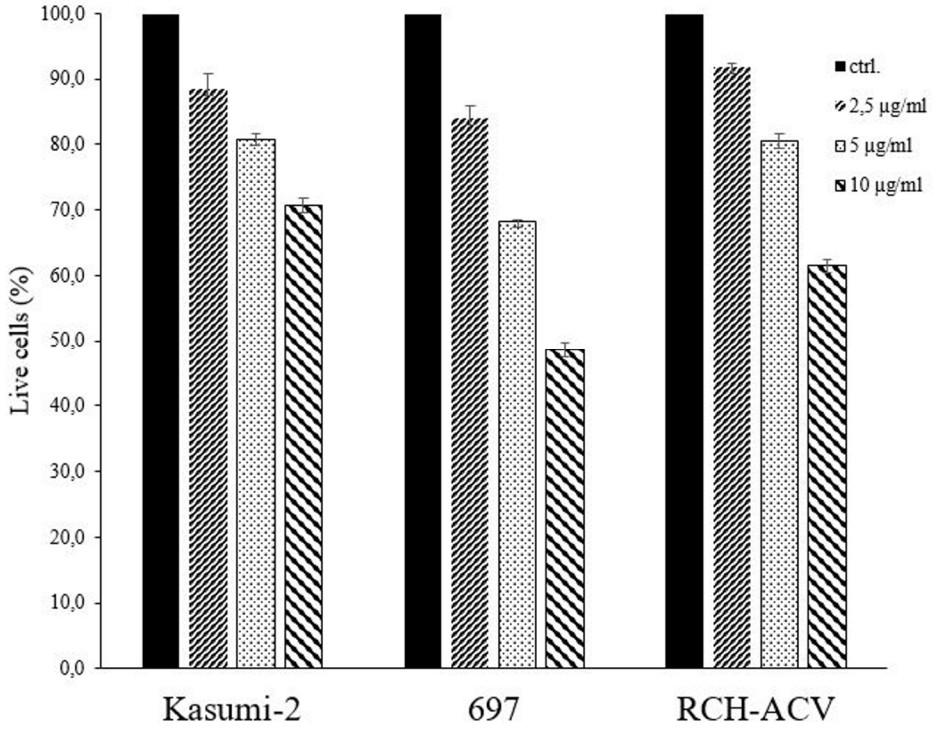

Figure 8. *CellTiter-Glo proliferation assay demonstrates dose dependent toxicity in Kasumi-2, 697 and RCH-ACV cell lines with four different ROR1 (2A2) monoclonal antibody concentrations in 24 hours after exposure to antibody.*

To investigate the effects of ROR1 targeting with mAb to cell proliferation we used CellTiter-Glo® assay. CTG analysis (Figure 8) demonstrated definite decrease of cell viability after 24 hours exposure to anti-hROR1 (2A2) antibody in a dose-dependent manner. Cell viability decreased in all the cell lines as mAb dose increased, indicating a dose dependent toxicity consistent with previous findings (Baskar et al., 2012). The decrease of cell viability was highest in 697 cell line where number of live cells decreased to under 50 % with 10 µg/ ml mAb dose compared to untreated control. The decrease of cell viability was less observed in Kasumi-2 cell line, where the cell viability stayed around 70 % with highest mAb concentration (10 µg/ ml).

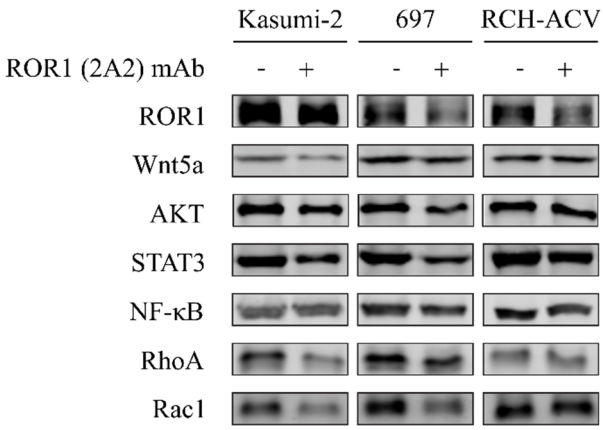

Figure 9. *Western Blot in the presence or absence of ROR1 mAb treatment was done to detect changes in protein levels after exposure to ROR1 antibody.*

Because the cell proliferation was decreased after ROR1 mAb exposure, we sought to investigate how ROR1, Wnt5a and downstream effector protein levels were affected by ROR1 targeting and accordingly, which pathways play a role in ROR1 mediated cell proliferation. To observe the changes in protein levels B-ALL cells were exposed for 24 h to 10 µg/ ml of ROR1 mAb after which cells were lysed and protein levels were investigated using Western Blotting. Figure (Figure 9) shows definite decrease of ROR1 protein level in mAb treated RCH-ACV and 697 samples but not as evident in Kasumi-2. ROR1 ligand Wnt5a protein level shows slight decrease after ROR1 mAb exposure in all the B-ALL cell lines.

Downstream signaling effectors of interest were RhoA and Rac1 as previously shown (Jian Yu et al., 2016), and also STAT3, NF-κB and AKT/PI3K pathway. According to WB Figure (Figure 9), AKT level decreases in all the B-ALL cell lines after mAb exposure. RhoA and Rac1 levels were observed to decrease in Kasumi-2 and 697 and less in RCH-ACV cell line. Moreover, STAT3 and NF-κB protein levels were also affected by ROR1 mAb exposure. STAT3 level decreased in all the cell lines and NF-κB decreased slightly in RCH-ACV and 697 cells.

1.12 **ROR1 knockdown using shRNA**

Next, we wanted to investigate the long-term effects of ROR1 silencing to cell viability and to intracellular signaling cascade in t(1;19) B-ALL by using shRNA gene knockout system. For this purpose, we created stable shRNA cell lines from Kasumi-2, 697 and RCH-ACV B-ALL using inducible lentiviral transfection system with pLKO-Tet-On vector containing shROR1-1, shROR1-2 and shCtrl constructs. The shRNA production was induced via doxycycline to silence ROR1 gene expression (Figure 10 A).

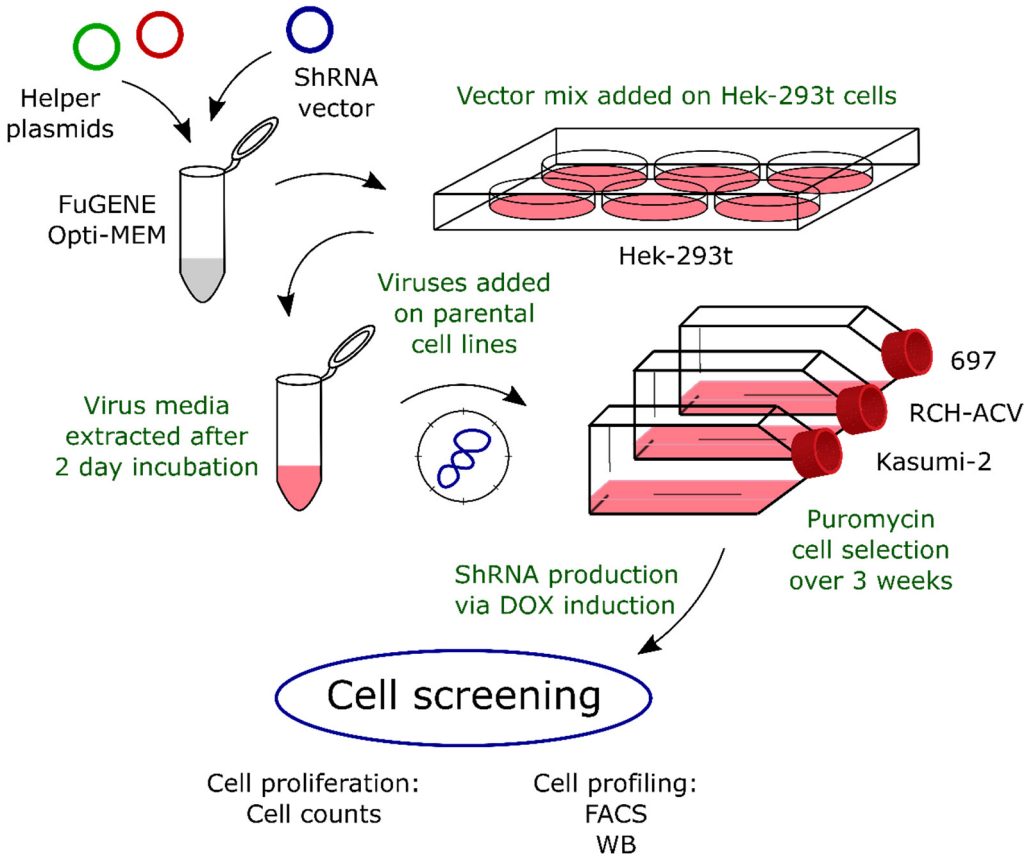

Figure 10. *ShRNA cell lines were created using single-vector inducible lentiviral transfection system. ShROR1-1, shROR1-2 and shCtrl cloned pLKO-Tet-On vectors were transfected to 293T cells with helper plasmids to create lentiviruses. RCH-ACV, 697 and Kasumi-2 cell lines were infected with lentiviruses and infected cells were selected using puromycin. ShRNA production from pLKO-Tet-On vectors was induced with doxycycline after which cells were screened by investigating cell proliferation via cell counts and ROR1 expression using FACS and WB.*

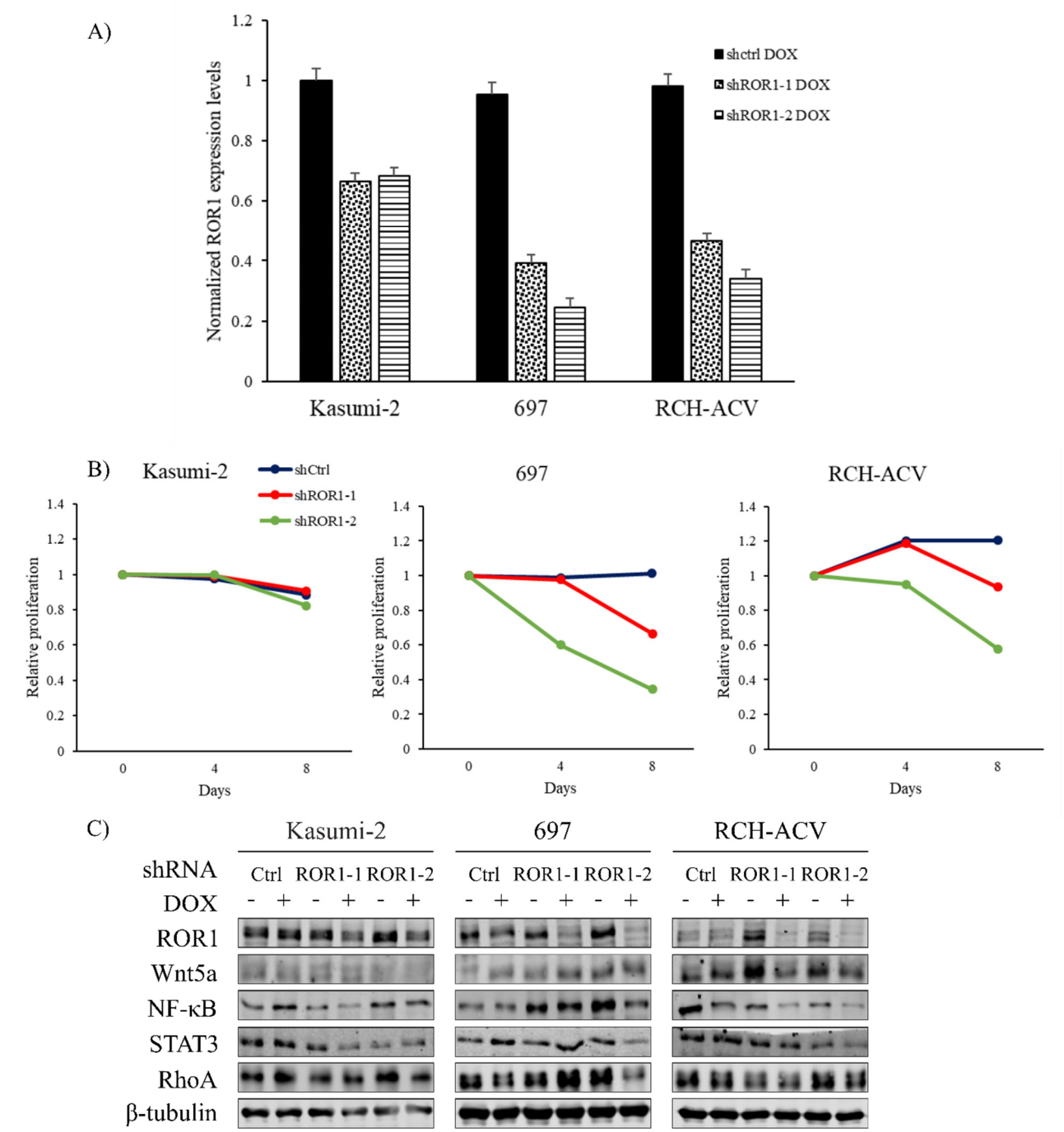

Figure 11. *A) The decrease of ROR1 level on cell surface was observed using FACS after shRNA production in B-ALL cell lines transfected with shRNA constructs, shROR1-1, shROR1-2 and negative control shCtrl. B) The cells were counted by FACS using propidium iodide staining. Cell counts were performed after 4 and 8 days and values were normalized using untreated samples. C) Western Blot was used to detect changes in protein levels induced by ROR1 knockdown.*

To demonstrate that shROR1 constructs were able to induce ROR1 knockdown when exposed to DOX, cell surface ROR1 level was measured using FACS with ROR1-PE antibody (Figure 11 A). The mean values from FACS were normalized with untreated samples. Compared to shCtrl, both

shROR1 constructs, shROR1-1 and shROR1-2, decreased ROR1 level in all the B-ALL cell lines. In 697 and RCH-ACV cell lines, shROR1-2 construct showed higher effects in ROR1 protein level downregulation compared to shROR1-1 construct. The decrease of ROR1 level was lowest in Kasumi-2 cell lines where both shROR1 constructs gave similar response. This may indicate that the infection efficiency or the selection of shRNA positive cells was not very successful.

To investigate the effects of ROR1 knockdown on cell viability, we used FACS with PI staining to count the live cells (Figure 11 B). Cells were counted after four and eight days in the absence or presence of DOX induction and results were normalized with untreated samples. Both shROR1 constructs in 697 and RCH-ACV cell lines demonstrated decreased in cell viability compared to shCtrl, shROR1-2 construct being most effective in both cell lines. However, in shROR1 Kasumi-2 cell lines, the cell viability remained similar to shCtrl after DOX induction. However, the efficiency of ROR1 knockdown in these cells was also low, around 20 %, therefore the results with Kasumi-2 cells are not very clear.

To determine molecular basis of loss of viability, ROR1 downstream signaling protein levels were investigated using Western Blotting at the time point of 8 days (Figure 11 C). ROR1 Western Blotting clearly demonstrates that both shROR1 constructs, shROR1-1 and shROR1-2, decreased ROR1 levels in B-ALL cells while shCtrl did not show effects to ROR1 protein levels. Similarly to FACS results (Figure 11 A), the decrease of ROR1 level was higher in 697 and RCH-ACV cell lines compared to Kasumi-2. According to Figure 11 C ROR1 gene silencing decreases Wnt5a level in RCH-ACV cell line. From downstream signaling cascades the decrease of RhoA was observed in all the cell lines especially with shROR1-2 construct. NF-κB decreased in all the cell lines in different levels and STAT3 level decreased in RCH-ACV and 697 cells with shROR1-2 construct.

1.13 **PTK7 knockdown using shRNA**

To investigate the role of PTK7 on cell proliferation we made shRNA cell lines with PTK7 targeting constructs. Due to maintenance brake in Arvo Virus Core and malfunctions with Accuri™ C6 flow cytometer I was not able to investigate shRNA PTK7 cell lines and measure effects of PTK7 interference to cell viability. However, these cells were investigated by Hanna Karvonen in the lab after I completed my work.

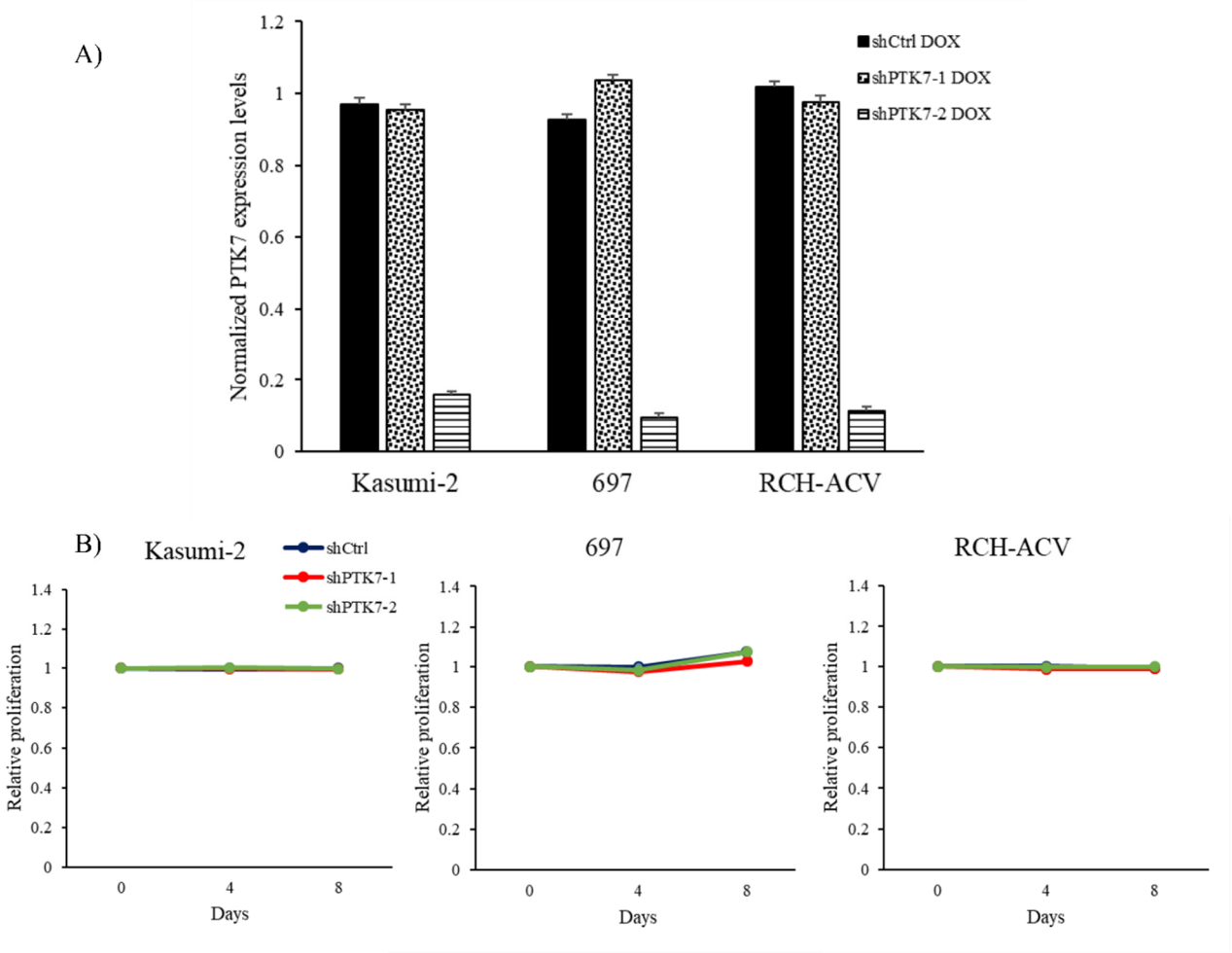

Figure 12. *A) The decrease of PTK7 level on cell surface was observed using FACS after shRNA production in B-ALL cell lines transfected with shPTK7 constructs. B) The cells were counted with FACS using propidium iodide staining. Cell counts were performed after 4 and 8 days and values were normalized using untreated samples.*

Mean values from FACS analysis with shPTK7 constructs using PTK7-APC antibody were normalized with untreated samples. Figure 12 A demonstrates that shPTK7-2 was the only construct that effected PTK7 expression level after DOX induction. Cell counts were also normalized with untreated samples and even though PTK7 expression decreased drastically with shPTK7-2 construct, we observed no effects on cell proliferation after 4 and 8 day DOX induction indicating that PTK7 is not important for B-ALL cell survival (Figure 12 B).

5 DISCUSSION

Multiple studies have demonstrated oncogenic role of ROR1 and PTK7 in non-canonical Wnt signaling pathway to increase cancer cell proliferation, viability and motility in different hematological malignancies (Green et al., 2008; Masaru Katoh, 2017a). The upregulation of ROR1 has been previously observed in B-ALL cells with t(1;19), and targeting ROR1 expression was associated with cell death (Bicocca et al., 2012). There are no previous studies focusing specifically to PTK7 functional analysis in B-ALL, but PTK7 expression has been observed in other blood malignancies with incompletely matured hematological cells, where PTK7 targeting has led to decreased cell viability indicating the importance of the protein in cell survival (Prebet et al., 2010). In this study, we investigated what are the downstream signaling pathways employed by these receptors in B-ALL.

RCH-ACV, Kasumi-2 and 697 B-ALL are cell lines containing t(1;19) translocation. Previous studies have shown high ROR1 expression in Kasumi-2 cells (Bicocca et al., 2012). In our experiments, FACS and WB analysis indicated high ROR1 expression in Kasumi-2 and 697 cell lines, whereas RCH-ACV contains two cell fractions, from which one does not express extracellular ROR1 indicating that RCH-ACV cells have higher dispersion in ROR1 expression level. Immunoprecipitation revealed ROR1 to interacted with Wnt5a indicating the oncogenic role of ROR1 in non-canonical Wnt signaling pathway. PTK7 was also uniformly expressed in cell lines but we detected no interaction with Wnt5a indicating that it does not activate non-canonical Wnt pathway via Wnt5a directly.

The expression of non-canonical Wnt signaling cascade proteins and AKT phosphorylation indicated cells to signal via non-canonical Wnt pathway. Cells expressed JNK that is activated in RhoA/Rac1 pathway and PI3K/AKT pathway proteins. B-ALL t(1;19) cells expressed also MEK and ERK that have been found to activate AKT (Bicocca et al., 2012) and STAT3 transcription factor. In this study we detected also canonical Wnt signaling mediator β-catenin to be expressed in RCH-ACV and 697 cell lines demonstrated in previous studies (Nygren et al., 2009), whereas β-catenin expression in Kasumi-2 cells was very low. However, β-catenin expression in leukemia cells has been located proximal to cell membrane with N-cadherin to promote cell-to-cell adhesion not acting as canonical Wnt signaling mediator and the canonical Wnt signaling in B-ALL with t(1;19) has been described to be very low or completely absent (Nygren et al., 2009).

To investigate the downstream signaling cascade from ROR1 we targeted B-ALL t(1;19) cells with ROR1 (2A2) monoclonal antibody and with shRNA mediated ROR1 interference system (Figure

9 and 11). Both ROR1 targeting methods impaired B-ALL cell proliferation. The highest decrease in cell viability was observed in 697 cells that expressed high level of ROR1 and Wnt5a. Accordingly, the decrease of ROR1 level was highest in 697 cells as shown by FACS analysis with ROR1 shRNA induction. The lowest decrease of cell survival was observed in Kasumi-2 cell line with both mAb and shRNA targeting. We targeted also PTK7 with shRNA and although we observed a decrease in protein level with PTK7 shRNA-2, we saw no effects on cell survival after shRNA induction (Figure 12 A and B) indicating that PTK7 does affect B-ALL cell survival.

In this study we observed downregulation of RhoA, STAT3 and NF-κB protein expressions after ROR1 targeting (Figure 9 and 11 C). RhoA has been identified as ROR1 downstream regulator (Jian Yu et al., 2016). RhoA downregulation was observed in all cell lines with shRNA targeting and with mAb in Kasumi-2 and 697 cell lines. STAT3 was downregulated in 697 and RCH-ACV cells with shROR1-2 construct and in all the cell lines treated with ROR1 mAb. A link between ROR1 and STAT3 expression has been previously demonstrated in CLL where STAT3 was found to bind two ROR1 promoter sites to induce ROR1 activation and STAT3 shRNA has been observed to downregulate ROR1 production (Li et al., 2010). NF-κB downregulation was observed in RCH-ACV cells with both shRNA constructs and in 697 cells with shROR1-2 and in Kasumi-2 with shROR1-1 construct. NF-κB was also downregulated with mAb induction in RCH-ACV and 697 as demonstrated before in MCL (Karvonen et al., 2017). ROR1 and Wnt5a co-expression in 293T cells has been previously observed to induce NF-κB expression indicating a direct link between ROR1 and NF-κB (Fukuda et al., 2008).

In conclusion, these results show that although both ROR1 and PTK7 are expressed in B-ALL cells, only ROR1 is important for cell survival and not PTK7, indicating that ROR1 is a good candidate for targeted therapy. Also, targeting ROR1 downregulates STAT3. Moreover STAT3 is hypothesized to activate non-canonical Wnt signaling pathway by inducing ROR1 and Wnt5a expression (Li et al., 2010). We also see a reduction in NF-κB levels after ROR1 downregulation as demonstrated before in AML (Karvonen et al., 2017).

6 CONCLUSION

This study investigated the oncogenic role of two receptors, ROR1 and PTK7, from non-canonical Wnt signaling pathway in B-ALL cancer cells. Results show that ROR1 is important for cancer cell survival, but PTK7 is not. Therefore, targeting ROR1 will have potential to kill cancer cells and can be used in anti-cancer therapies.

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