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THE FUNCTIONAL CONSEQUENCES OF ZNF414 OVEREXPRESSION IN PANCREATIC CANCER CELLS

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ABSTRACT

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Background and aims: All cancers are known to arise from genetic mutations in specific driver genes, including oncogenes, tumor suppressor genes and DNA repair genes. DNA alterations in these genes lead to malfunction of transcriptional regulation that normally controls various biological processes including cell differentiation and development, maintenance of intracellular balance, and cell cycle progression. Transcription is tightly regulated by a diverse array of DNA-binding proteins including transcription factors. Zinc-finger proteins form the largest group of eukaryotic transcription factors and their altered expression levels are known to be associated with development and progression of cancer. Zinc-finger protein 414 (ZNF414) is one of the newly discovered regulators of pancreatic cancer cell growth. Knockdown of ZNF414 expression has been shown to result in a distinct G1 arrest and thereby decreases the growth of cancer cells. Still, there are many unresolved issues related to cellular function of ZNF414 in pancreatic cancer. Therefore, the ultimate aim of this study was to examine the functional consequences of ZNF414 overexpression in pancreatic cancer cells.

Methods: In this study, lentivirus-based Lenti-X™ Tet-One® Puro Inducible Expression system was used to generate stable SU.86.86 and Hs700T pancreatic cancer cell lines overexpressing ZNF414. The Lenti-X™ Tet-One® Puro includes the pLVX-TetOne-Puro vector which was used to transduce the gene of interest, ZNF414, into the target cells. The vector also contains necessary components for tightly controlled expression of ZNF414 when the cells are cultured in the presence of doxycycline. Prior to functional studies, the functionality of the pLVX-TetOne-Puro-ZNF414 was examined in transduced SU.86.86 and Hs700T cells by comparing the ZNF414 expression between induced and uninduced cells. To characterize the functional consequences of ZNF414 overexpression in pancreatic cancer cells, the cell cycle and proliferation of ZNF414-overexpressing cells were examined by flow cytometry and crystal violet staining, respectively.

Results: The ZNF414 gene was successfully cloned from the pMK-RQ plasmid into the pLVX-TetOne-Puro vector. After transduction, the SU.86.86 cells expressed high levels of ZNF414 when the expression was induced with doxycycline. Similar effect in transduced Hs700T cells was not detected and transduction was thus considered unsuccessful in the Hs700T cells. Functional studies were performed by using the SU.86.86 cells as a model for studying the cellular phenotype of ZNF414 overexpression. The results showed no difference in the proliferation and cell cycle between ZNF414-overexpressing SU.86.86 cells and control cells.

Conclusions: The proliferation and cell cycle of pancreatic cancer cells are not affected by the ZNF414 overexpression. The generalizability of the results is limited by narrow sampling since the functional studies were only performed with SU.86.86 cells. In the future, the study should be repeated with additional cancer cell lines in order to confirm our findings and to gain a thorough insight into the role of ZNF414 overexpression in cancer.

Keywords: cancer, transcriptional regulation, transcription factors, ZNF414

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TIIVISTELMÄ

Janika Perttala: ZNF414:n yliekspression funktionaaliset vaikutukset haimasyöpäsoluissa
Pro Gradu
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Työn tausta ja tavoitteet: Syöpä saa alkunsa geneettisistä mutaatioista solun kriittisiä toimintoja säätelevissä geeneissä kuten proto-onkogeeneissä, tuumorisuppressorigeeneissä sekä DNA:n korjaus -geeneissä. DNA-muutokset kyseisissä geeneissä johtavat transkriptionaalisen säätelyn toimintahäiriöön, joka normaalisti kontrolloi monia biologisia tapahtumia, kuten solujen erilaistumista ja kehittymistä, solun sisäisen tasapainon ylläpitoa sekä solusyklin etenemistä. Transkription säätelyssä on mukana monipuolinen joukko DNA:han sitoutuvia proteiineja, johon lukeutuvat esimerkiksi transkriptiotekijät. Sinkkisormiproteiinit muodostavat suurimman transkriptiotekijäryhmän, joiden muuttunut ekspressiotaso on osoitettu johtavan syövän kehittymiseen ja etenemiseen. Sinkkisormiproteiini 414 (ZNF414) on vastikään löydetty haimasyöpäsolujen kasvua säätelevä proteiini. Alennetun ZNF414:n ekspression tuloksena haimasyöpäsolut pysähtyvät G1-faasiin, joka johtaa solujen kasvun vähentymiseen. On kuitenkin olemassa vielä monia ratkaisemattomia kysymyksiä koskien ZNF414:n toimintaa haimasyövässä. Tästä johtuen, tämän tutkimuksen päätavoitteena oli tutkia ZNF414:n yliekspression funktionaalisia vaikutuksia haimasyöpäsoluissa.

Menetelmät: Työssä käytettiin lentivirus-pohjaista Lenti-X™ Tet-One® Puro Inducible Expression System -menetelmää ZNF414:ä yliekspressoivien SU.86.86- ja Hs700T-haimasyöpäsolulinjojen kehittämiseksi. Lenti-X™ Tet-One® Puro sisältää pLVX-TetOne-Puro vektorin, jonka avulla ZNF414-geeni transdusoidaan haimasyöpäsoluihin. Vektorin sekvenssistä löytyvät myös tarvittavat komponentit tiukasti säädeltävään ZNF414:n ekspressioon, kun soluja viljellään doksisykliinin kanssa. Ennen funktionaalisia tutkimuksia, pLVX-TetOne-Puro-ZNF414 vektorin toiminta varmistettiin transdusoiduissa SU.86.86- ja Hs700T-soluissa vertaamalla indusoitujen ja indusoimattomien solujen ZNF414 ekspressiota. ZNF414:n yliekspression funktionaalisia vaikutuksia haimasyöpäsolujen solusykliin ja kasvuun tutkittiin käyttämällä virtaussytometria- ja kristalliviolettivärjäys-menetelmiä.

Tulokset: ZNF414-geeni kloonattiin onnistuneesti pMK-RQ-plasmidista pLVX-TetOne-Puro-vektoriin. Transdusoinnin jälkeen ZNF414-ekspressiotasot nousivat SU.86.86-soluissa doksisykliinilla indusoitaessa, kun taas samanlaista eroa ekspressiotasoissa ei näkynyt indusoitujen ja indusoimattomien Hs700T-solujen välillä. Funktionaaliset tutkimukset suoritettiin käyttämällä SU.86.86 soluja mallina, jotta voitiin tutkia ZNF414:n yliekspression sellulaarista fenotyyppiä. Tulokset osoittivat, ettei ZNF414:ä yliekspressoivien SU.86.86-solujen ja kontrollisolujen proliferaatiossa ja solusyklissä ollut tilastollisesti merkittäviä eroja.

Johtopäätökset: ZNF414:n yliekspressiolla ei todettu olevan vaikutusta haimasyöpäsolujen kasvuun eikä solusykliin. Tuloksien yleistämistä rajoittaa funktionaalisten tutkimusten rajoittunut näytemäärä, sillä tutkimus suoritettiin vain yhdellä haimasyöpä-solulinjalla. Tulevaisuudessa tutkimus pitäisi toistaa useammalla eri haimasyöpäsolulinjalla, jotta voitaisiin varmistua tulosten oikeellisuudesta ja saada perusteellinen käsitys ZNF414:n yliekspression roolista haimasyövässä.

Avainsanat: syöpä, transkriptionaalinen säätely, transkriptiofaktorit, ZNF414

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Janika Perttala

TABLE OF CONTENTS

ABBREVIATIONS

3D	Three-dimensional
AKT	Protein kinase B
bHLH	Basic helix-loop-helix
C2H2	Cys2His2
cPCR	Colony PCR
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle Medium
Dox	Doxycycline
E. coli	Escherichia coli
EMT	Epithelial-mesenchymal transition
GAPD	Glyceraldehyde-3-phosphate dehydrogenase gene
GOI	Gene of interest
GTF	General transcription factor
HIV-1	Human immunodeficiency virus 1
JAK2	Janus kinase 2
KLF	Krüppel-like factor
KPNA7	Karyopherin alpha 7
KRAB	Krüppel-associated box
LMCV	Lymphocytic choriomeningitis virus
LMCV-WE	Lymphocytic choriomeningitis virus (strain WE)
MAZ	MYC-associated zinc-finger protein
MCS	Multiple cloning site
MNT	MAX-binding protein
MXD1	MAX dimerization protein 1
NC	Negative control
ORF	Open reading frame
PBS	Phosphate-buffered saline
PI	propidium iodide
Pol II	RNA polymerase II
PS	Positive control
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RE-digestion	Restriction enzyme digestion
RPMI-1640	Roswell Park Memorial Institute 1640 medium
TF	Transcription factor
TSS	Transcriptional start site
VSV-G	Vesicular stomatitis virus G-protein
ZAD	Zinc finger-associated domain
ZF	Zinc-finger
ZNF414	Zinc-finger protein 414
WT1	Wilms' tumor 1

1 INTRODUCTION

Cancer is one of the leading causes of human deaths worldwide with an average of 18.1 million new cancer cases and 9.6 million cancer deaths being reported in 2018.[1] Studies on cancer genomics have focused on finding the underlying mechanisms of cancer origin, that initiates the development and progression of cancer. All cancers are known to emerge from genetic mutations in driver genes, including proto-oncogenes, tumor suppressor genes and DNA repair genes.[2–5] The vast majority of the DNA alterations in these genes are attributed to somatic mutations and environmental factors, yet around 10% of the cancer cases are suggested to arise from hereditary, germline mutations.[5] Generally, inactivation of tumor suppressor genes often contributes as a loss-of-function of their gene product[5], whereas mutational activation of oncogenes leads to overexpression of oncogenic proteins that can promote uncontrolled proliferation and viability of cancer cells.[6]

Driver genes encode proteins which are crucial for various developmental and signaling pathways[7,8], such as cell survival and proliferation.[9,10] More specifically, mutations can promote the altered expression of these genes followed by abnormal functionality or loss of the gene product.[11] This leads to malfunction of the regulatory systems that normally prevent uncontrolled cell growth and proliferation, eventually resulting in cancer.[2,8,12] In addition to the driver genes, epigenetic changes can drive the progression of cancer through the dysregulation of DNA methylation, noncoding RNAs, and histone modifications, all of which can directly affect gene expression.[7,8,13]

Among other regulatory proteins, altered expression levels of transcription factors have been reported to be associated with the onset and progression of several human diseases, including cancer.[7,14–16] Transcription factors cover a large proportion of the identified oncogenes[14], but they also include several tumor suppressor genes.[7] Studies have demonstrated that a great variety of zinc-finger proteins are involved in cancer progression through their transcription factor function.[17] MYC-associated zinc-finger protein (MAZ), for instance, is distinctly overexpressed in pancreatic cancer cells thereby promoting their proliferative, invasive, and metastatic ability.[18] However, the cellular function of a majority of zinc-finger proteins is yet to be discovered and further studies are required in order to gain comprehensive insight into their role in cancer.

2 LITERATURE REVIEW

2.1 Regulation of gene expression

2.1.1 Overview of gene expression

Every cell of an individual organism contains the same genetic information stored in DNA as genes. Instead of expressing the entire repertoire of genes continuously, the cell regulates the expression of different genes independently giving the cell its characteristic phenotype. Regulation of gene expression also enables the cell to adjust the rate of expression according to its needs. Cells are constantly under internal and external stimuli due to changes occurring in their intracellular and extracellular environments. Exposure to such diverse signals requires the cells to recognize them and respond through changes in gene expression. This leads to proper physiological response which is essential for cell survival and maintenance of the cell homeostasis.[19] Gene expression, however, is a complex and precise process that needs cooperative and combinatorial actions of various cellular factors.

The aim of gene expression is to convert the genetic information provided by the target gene into a protein or other functional gene product. In brief, the process consists of different steps starting from the DNA transcription where genetic information of expressed gene is copied into a single-stranded RNA. Beginning of the transcription requires the opening and unwinding of a DNA double helix at the region of particular gene.[20] This exposes the bases in both DNA strands for readout, but only one of the two strands acts as a template for the RNA synthesis. In eukaryotic cells, transcription is performed by an RNA polymerase enzyme that moves stepwise along the template in the 5′-to-3′direction and catalyzes the formation of phosphodiester bonds in the RNA strand.[21] The synthetized RNA molecules can be messenger RNAs (mRNAs) that are recoded into proteins.[22] However, the final gene product can also be the RNA molecule itself. Such RNA molecules are known as noncoding RNAs, a group comprising of ribosomal and transfer RNAs, microRNAs (miRNA), long noncoding RNAs, as well as other functional RNAs.[2,22]

In the case of protein production, pre-mRNA is further modified before protein synthesis. First step is to add a small cap to 5′and 3′ends of the RNA sequence, which helps to distinguish the mRNA from the noncoding RNAs.[23] Secondly, noncoding intervening sequences, known as

introns, are removed from the sequence by RNA splicing. As a result, expressed sequences, exons, are rearranged in a way that correspond with the desired protein. mRNA is then ready to be transported into cytoplasm where it is translated into the primary amino acid sequence in ribosomes. Ribosome is a catalytic complex comprised of a small and a large ribosome subunit which together ensure and maintain the accuracy and the correct reading frame during translation.[24,25] However, mRNAs are not capable of forming the amino acid sequence on their own. Since mRNAs contain the genetic information for the protein synthesis, adaptor molecules called transfer RNAs (tRNAs) are required for the transportation of amino acids into the ribosomes.[25] Moreover, tRNAs can recognize and pair with the codons, complementary sets of three nucleotides in mRNA sequence by their base-pair matching anticodons.[25,26] In this way, the correct amino acid is added to the C-terminal end of the newly formed amino acid sequence at the right time. The end of translation is signaled by stop codons, since tRNAs cannot recognize them, and they do not specify any amino acids. After translation, primary amino acid sequence starts to fold into tertiary structure and goes through additional modifications within the cell before protein is fully functional.

2.1.2 Transcriptional regulation of gene expression

Every step of the gene expression is tightly regulated, especially the initiation of the transcription. Transcriptional regulatory system controls various biological processes such as cell cycle progression, maintenance of metabolic and physiological balance as well as cell differentiation and development. Thus, transcription is considered to play a key regulatory role in gene expression.[27] Defective regulation of transcription, therefore, leads to altered gene expression, and thereby to many disorders such as inflammation, metabolic abnormalities, infections, and cancer.[19] Transcription initiation is regulated at multiple levels, including the level of chromatin state, preinitiation complex assembly, as well as transcription factor (TF) and enhancer function.[27,28]

Chromatin plays important role in gene expression by regulating the accessibility of DNA, and thereby approachability of regulatory binding sites to TFs and enhancers.[29] Nucleosomes are the structural units of the chromatin, and one nucleosome consist of a long DNA segment wrapped around proteins known as histones.[20] Chromatin affects gene expression through its structural changes, which can be mediated in multiple ways. For example, N-terminal end of the histones can be post-translationally modified in a way of acetylation, phosphorylation,

methylation, ubiquitylation or sumoylation.[8,20] These modifications activate or repress the transcription by affecting the configuration of the chromatin as well as the chromatin-DNA interactions.[8] Still, post-translational modifications of histones alone are rarely sufficient for transcription initiation. Combinatorial actions with DNA methylation and noncoding RNAs, however, should be enough to determine whether the target gene is expressed or not.[8] Secondly, structure of the chromatin can also be changed with chromatin modifying enzymes, ATP-dependent remodeling complexes and transcriptional regulators such as TFs, which are regulated via signal-dependent manner.[8,20,29] Signal transduction pathways contain protein kinases which activate these cellular factors by phosphorylation.[8] Since the protein kinases are mostly cytoplasmic, they are considered to affect chromatin structure only indirectly.[8] However, several studies have demonstrated that various kinases, such as protein kinase B (AKT) and Janus kinase 2 (JAK2), are also able to modify the structure of the histones independently.[30,31]

Preinitiation complex is a transcription machinery required for recruitment of RNA polymerase II (Pol II) to the transcriptional start site (TSS).[2,21,27] The core promoter act as a platform for the assembly which is located near the transcriptional start site, upstream on DNA.[21] The complex comprises of a diverse group of proteins that form the first transcriptional regulatory element of the assembly.[27] These specific proteins include general transcription factors (GTFs) and cofactors, which mediate the transcription according to intracellular and extracellular signals.[2] Preinitiation complex is estimated to be over a megadalton in size, which covers over one hundred base pairs of DNA around the core promoter region.[2] Most of the regulatory information of the assembly, however, is carried by other regulatory sequences located either upstream or downstream from core promoter.[27,32] By interacting with different cellular factors, they activate or repress the preinitiation complex assembly on target core promoter, thereby determining the level of gene transcription.[27]

Transcription initiation is regulated through the integrated function of different cis-regulatory elements that are located in various regulatory sequences on DNA, including core promoters, enhancers, insulators, silencers, and other regions of DNA.[32–35] While promoters are close to the TSS, other regulatory regions can be localized at greater distances from the genes that they regulate.[32,36] Among the extensive set of different regulatory sequences, enhancers and their associated TFs are in a key role in the transcription initiation. Enhancers initiate the transcription by interacting with TFs through their specific DNA motifs which act as a platform

for TF binding.[36] The DNA-binding of TFs (including GTFs) is typically regulated directly or indirectly via intracellular signal transduction pathways that involve protein phosphorylation and dephosphorylation.[29] The binding of transcriptional repressor Wilms' tumor 1 (WT1), for instance, is inhibited when its two serine residues in the zinc-finger motif are phosphorylated.[29] In addition to DNA-binding, protein phosphorylation and dephosphorylation regulate the transcription through TF function in at least four other ways: by controlling cellular localization of TFs and the length of time spent in the nucleus, by regulating the protein-protein interactions between TFs and their coregulators, by modifying the structure of chromatin, and by targeting TFs or their coregulators for proteolytic degradation.[29] Other regulatory features that are known to contribute to the TF-DNA interactions, and thereby to transcriptional regulation, are DNA methylation[37], three-dimensional (3D) structure and flexibility of TFs and their DNA-binding regions[38–40], accessibility of chromatin and nucleosome occupancy[41–45], the presence of cofactors[46,47] and the nucleotide sequence of DNA[48–52]. Furthermore, all of these factors are known to interact with each other, which is suggested to correspond the tissue- and cell-type-specific regulation.[15,53]

2.2 Transcription factors

2.2.1 Functions of transcription factors

As described above, TFs regulate the transcription mainly by binding to core promoters and distal regulatory regions on DNA.[54] They recognize these sequence-specific regions by their structurally organized DNA-binding domains (DBDs).[32–35] However, in certain situations, binding can be also nonspecific in which case TFs interact with the DNA backbone instead of the bases.[50] Individual TF can interact with DNA by only one single DBD or it can be comprised of multiple DBDs such as C2H2-zinc finger domains.[15] In addition to DBDs, other types of effector domains can also be found in the sequences of TFs.[54,55] These functional units are suggested to control subcellular localization, and participate in DNA-binding and gene regulation by interacting with other transcription factors and cellular factors.[28,56,57]

TFs have generated a wide network of functions where they aid each other in myriad ways, for example by facilitating DNA interactions in a process called cooperative binding.[15,53] In general, cooperative binding is comprised as an interaction between two (or more) TFs that provide additional stability for binding.[15,38,58,59] Most of the known TFs bind cooperatively on

DNA as homodimers, heterodimers, or homomultimers[14,15], but the binding of individual TF can also affect the shape of DNA in a way that facilitates the binding of other TFs.[15] TFs also work together by promoting the DNA accessibility, since DNA-binding sites have to be in an accessible mode before TFs can regulate the gene expression.[20] For this, TFs can indirectly cooperate by binding[60,61] or competing[62] with nucleosomes which remodels the structure of chromatin, and thereby promote the accessibility of DNA.[14,22,24,27]

Activation or repression of target gene expression through TF functions is known to be a key metabolic and developmental regulator in eukaryotic cells. TFs are generally represented as regulators of cell growth and proliferation[15,28], but they also have important roles in many other cellular processes including cell differentiation, stemness maintenance and autophagy.[15,56] TFs can also control stimulatory response pathways, such as immune responses and sensory perceptions[15], or processes like signal transduction.[14] Furthermore, TFs are demonstrated to play a crucial part in embryogenesis, especially in epithelial-mesenchymal transition (EMT).[63] Together with microenvironmental signals, EMT-inducing TFs activate trans-differentiation by interacting with epigenetic regulators.[63] These interactions lead to the expression of multiple proteins that are involved, for example, in cell–cell contact, extracellular matrix degradation, cytoskeleton structure, and cell polarity.[63] Still, TF functions are highly gene and interaction partner specific.[15,36] For example, MAX acts as an transcriptional inhibitor when bound by MAX-binding protein (MNT) or MAX dimerization protein 1 (MXD1), but functions as an activator when interacting with MYC.[15]

2.2.2 Transcription factor classification

Classification of TFs is commonly based on the structure of their DNA-binding motifs. The three largest TF classes are known as classical zinc-fingers (C2H2-zinc fingers), homeodomains, and basic helix-loop-helix (bHLH) factors[34,54,56] which cover over 80% of the entire TF repertoire.[14,64] Dividing TFs by their motifs has provided insights into their evolutionary origin and taxonomical history, as well as deepened the knowledge on how specific groups of TFs recognize and bind to their targets. Even though the structure of the DNA-binding domain does not predict the functions of the TF, some generalizations have been made. bHLH proteins, for instance, have been linked to neurogenesis, myogenesis and sex determination[65], whereas homeodomain-containing proteins are involved in developmental processes including axial patterning, cell identity and proliferation.[14,66,67] Since the amount of

information has increased in time, each TF class has been further subclassified based on more detailed differences in the structure and specificity within the classes (Table 1).[50]

Table 1. **Classification of eukaryotic transcription factors based on their DNA-binding domains**. The three largest superclasses are further categorized into classes, families and subfamilies. Information gathered from TFClass.[33] *One of the three largest TF classes

Superclass	Classes	Families	Subfamilies
Basic domains	Basic leucine zipper factors (bZIP)	8	14
	Basic helix-loop-helix factors (bHLH)*	7	22
	Basic helix-span-helix factors (bHSH)	1	0
Zinc-coordinating DNA-binding domains	Nuclear receptors with C4 zinc fingers	7	14
	Other C4 zinc finger-type factors	1	2
	C2H2 zinc finger factors*	5	114
	DM-type intertwined zinc-finger factors	1	0
	CXXC zinc finger factors	1	0
	C2HC	5	0
	C3H zinc finger factors	4	0
	C2CH THAP-type zinc finger factors	1	0
Helix-turn-helix domains	Homeo domain factors*	11	98
	Paired box factors	2	4
	Fork head / winged helix factors	3	21
	Heat shock factors	1	0
	Tryptophan cluster factors	3	14
	TEA domain factors	1	0
	ARID	1	7

bHLH proteins are characterized by highly conserved bipartite domains required for interaction with DNA and other proteins.[65] The other motif is formed of basic residues that mainly interact with the consensus of hexanucleotide E-box. The second motif is comprised of hydrophobic residues that allow the protein to form homo- and heterodimers. In addition, the second motif

is usually referred to as the HLH domain since its structure contains two α-helixes and the loop between them.[65]

Homeodomain-containing proteins are characterized by the helix-turn-helix DNA-binding motif, also known as the homeodomain, that consists of the 180 bp homeobox region.[67] The region is formed of three helices, folded into a globular structure in which helices I and II are positioned parallel to each other, across from the third helix.[67] The third helix is known to form specific bonds with the DNA bases while the other helices interact with the backbone of DNA.[67]

Classical zinc finger proteins forms the largest group of eukaryotic transcription factors.[19,56,68] Zinc-finger (ZF) proteins were first identified as sequence-specific DNA-binding proteins, but studies have found them to interact also with RNA, lipids and proteins.[28,56,57] Similarly to other TFs, biological functions of ZF proteins vary according to their DNA-binding domains.[17,56] By combining different zinc-finger motifs or by adding multiple clones of the same motif type, ZF proteins can expand their binding specificity to different DNA sequences and other ligands.[19,56,57] As a result, they can control several cellular processes through gene expression, such as cell development, differentiation and tumor suppression. In addition to cell homeostasis, studies have demonstrated the crucial role of ZF proteins in cancer development and progression mainly due to their transcription factor function.[17,56,64]

2.3 C2H2-zinc finger proteins

2.3.1 Structure of C2H2-zinc fingers proteins

Classical C2H2-zinc finger motif is the most common and widely studied group of all zinc-finger motif classes.[19,56,69] The C2H2-ZF is 28-30 amino acid long protein motif with conserved pairs of two cysteine and two histidine residues that fold into a finger-like structure after interacting with zinc ions (Figure 1A).[56,57,68,70] Motif consists of two ß-strands in N-terminal half, and one α-helix in C-terminal half of the motif (Figure 1B).[56,57,69] Characteristic feature of C2H2-ZF proteins is their ability to bind to relatively long, approximately 20-40 base pair DNA sequences.[69] C2H2-ZF motif interacts with DNA by its specifically positioned residue located in the N-terminal part of the α-helix.[28,57,68,69] This four amino acid long residue enables the binding by interacting with hydrogen donors and acceptors found on the major groove of DNA (Figure 1C).[28,57,68,71] In addition, the binding specificity is also defined by the loop

between the ß-strands, number of zinc-fingers within the protein, the amino acid sequence of the zinc-fingers as well as the linker between them.[17,57,71] Although there are multiple and various types of C2H2-ZF proteins, most of them can be classified into one of the following three groups: triple-C2H2, multible-adjacent-C2H2, and separated-paired-C2H2.[28,57,69] Classification is not always accurate, since C2H2-ZF proteins can be constructed of different motif combinations from the three groups above.[28] However, two to three C2H2-ZF motifs are demonstrated to be the most appropriate number for DNA binding.[56]

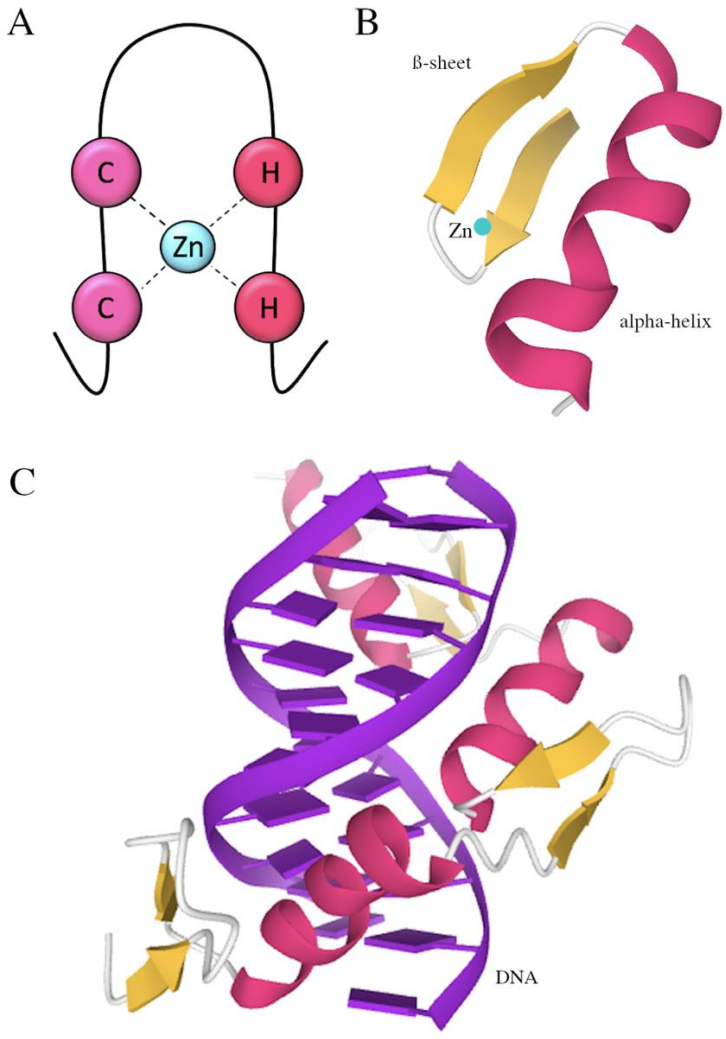

Figure 1. A) Illustrative image of the C2H2-ZF motif with cysteine (C) and histidine (H) residues that interact with a zinc ion with coordination bonds. B) Three-dimensional structure of C2H2-ZF motif composed of α-helix and antiparallel ß-structure (PDB ID: 1ZNF). α-helix forms the hydrophobic core in C-terminal half of the motif whereas the ß-structure is located in N-terminus. C) 3D-model of the interactions between DNA and C2H2-ZF domain composed of three zinc-fingers motifs (PDB ID: 1A1J). Insertion of the α-helix into the major groove enables the binding of C2H2 motif on DNA. (Pictures modified from https://www.rcsb.org/)

In the triple-C2H2 proteins, C2H2-ZF motifs are located close to each other as a cluster of three, either in the middle or at the C-terminal part of the protein.[69] Several known TF families belong to this group, for example Krüppel-like factors (KLFs) and SP1-like factors.[28,57] C2H2-ZF proteins containing the triple-C2H2 cluster in the C-terminus usually have an additional effector domain, such as Krüppel-associated box (KRAB) or zinc finger-associated domain (ZAD), in the N-terminus.[28,69,70] Most of the C2H2-ZF proteins with the triple-ZF cluster in the middle, however, do not have any other functional domains in their structure.[19,69] Nonetheless, proteins with three C2H2 motifs do not necessarily need conserved domains to interact directly with the DNA.[68]

Multiple-adjacent-C2H2 proteins contain four or more zinc fingers that are located close to each other at regular intervals.[57] Number of fingers vary greatly within this group as some proteins are known to contain over 30 zinc-fingers. In most of the proteins, only 24-75 % of the total number of fingers participate in DNA-binding.[57] The remaining fingers are suggested to have different role in protein function, including binding to RNA and other proteins.[57]

Like the multiple-adjacent-C2H2 proteins, separated-paired-C2H2 proteins can contain several C2H2 motifs in their protein structure starting from two fingers.[28,57] However, characteristic for this group is that their motifs are always paired and usually widely separated. This group is clearly the smallest of C2H2 classes, yet it includes several annotated TFs such as tramtrack and basonuclin.[28]

2.3.2 Zinc-finger protein 414

Zinc-finger protein 414 (ZNF414) is one of the C2H2-ZF proteins belonging to the group of Krüppel C2H2-type zinc-finger proteins.[72] Like the KLFs, it can be categorized into triple-C2H2 proteins since it has a cluster of three C2H2 motifs in the middle of its amino acid sequence (Figure 2). There are two confirmed variants of human ZNF414, a shorter isoform consisting of 312 amino acids and a longer isoform of 390 amino acids. The shorter variant, isoform 1, has been chosen as the canonical sequence of the ZNF414 protein by UniProt (https://www.uniprot.org/uniprot/Q96IQ9, 17.4.2020), and thereby all information provided in this chapter refers to it.

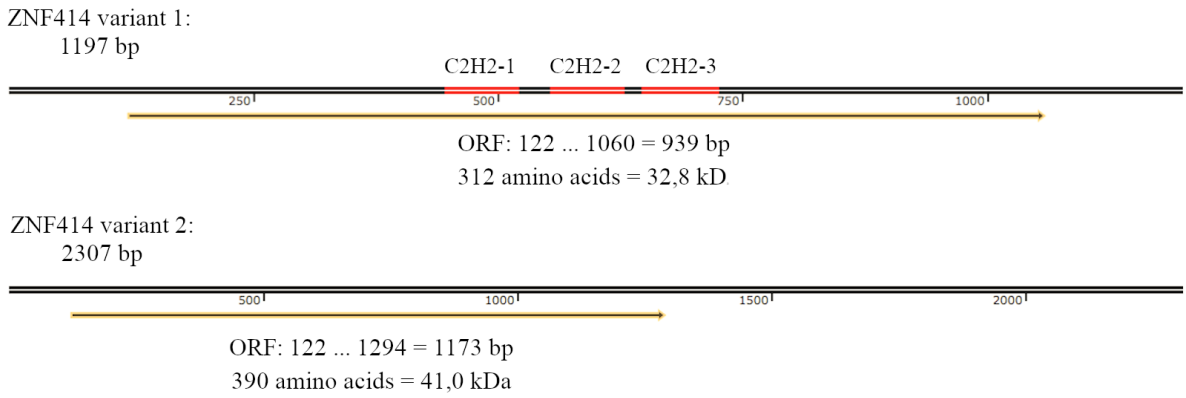

Figure 2. **Shorter and longer variant of the ZNF414**. Cluster of three C2H2-ZF motifs (highlighted in red in variant 1) are localized in the middle of the open reading frame (ORF) sequence.

Studies have suggested that the ZNF414 is involved in transcriptional regulation which is supported by the fact that ZNF414 is primarily localized in the nucleus (https://www.proteinatlas.org/ENSG00000133250-ZNF414, 22.4.2020). The ZNF414 has low tissue specificity as its mRNA expression is detected in every tissue type in human body. In previous studies of our group, the ZNF414 was confirmed to be the cargo protein of the nuclear import protein, karyopherin alpha 7 (KPNA7).[73] Moreover, the downregulation of ZNF414 by siRNA based gene silencing resulted in a distinct G1 arrest in the cell cycle of pancreatic cancer cells, leading to a major decrease in cell proliferation. Due to the lack of research, there are no other reports in terms of ZNF414 function either in normal or cancerous pancreatic tissues indicating that the effects of ZNF414 overexpression on cancer cell growth are still unknown and require further investigation.

3 AIMS OF THE STUDY

In previous studies of our group, the silencing of ZNF414 led to severe inhibition of cancer cell growth making it interesting target for further studies. Since there are no other reports on the ZNF414 function in cancer cells, the aim of this study was to examine the cellular phenotype of ZNF414 overexpression. First step was to clone a synthetized ZNF414 gene into pLVX-TetOne-Puro vector and further generate pancreatic cancer cell lines overexpressing ZNF414. These cell lines are used in functional assays, such as proliferation and cell cycle analyses, to characterize the functional consequences of ZNF414 overexpression in pancreatic cancer cells. The detailed aims were:

1. To clone a synthetized ZNF414 gene into pLVX-TetOne-Puro vector
2. To generate ZNF414-overexpressing Hs700T and SU.86.86 pancreatic cancer cell lines
3. To study the functional consequences of ZNF414 overexpression in pancreatic cancer cells

4 MATERIALS AND METHODS

4.1 Cell lines

Human pancreatic cell lines Hs700T and SU.86.86 (ATCC, American Type Culture Collection) were used to investigate the effects of ZNF414 overexpression on cancer cell growth. Hs700T cells are human epithelial adenocarcinoma cells from a 61-year-old Caucasian male, whereas human epithelial ductal carcinoma cells, SU.86.86, are derived from a 57-year-old Caucasian female. Both cell lines were grown in recommended culture mediums, Hs700T in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, Missouri, USA), and SU.86.86 in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Sigma-Aldrich, St. Louis, Missouri, USA). Medium was changed in every two to three days.

4.2 Cloning of the ZNF414 into lentiviral vector

In this study, ZNF414-overexpressing Hs700T and SU.86.86 cell lines were generated by lentivirus-based Lenti-X™ Tet-One® Puro Inducible Expression System (Clontech, Mountain View, California, USA). pLVX-TetOne-Puro, vector of the Lenti-X™ Tet-One® Puro kit, is designed to transduce foreign DNA into mammalian cells. After pLVX-TetOne-Puro has been integrated into the cell genome, components of pLVX-TetOne-Puro enables controlled induction of target gene expression in the presence of doxycycline. Thereby, first aim of the study was to clone our gene of interest (GOI), ZNF414, into the pLVX-TetOne-Puro vector (Figure 3). Based on our previous studies, the shorter transcript of the ZNF414 gene was selected.

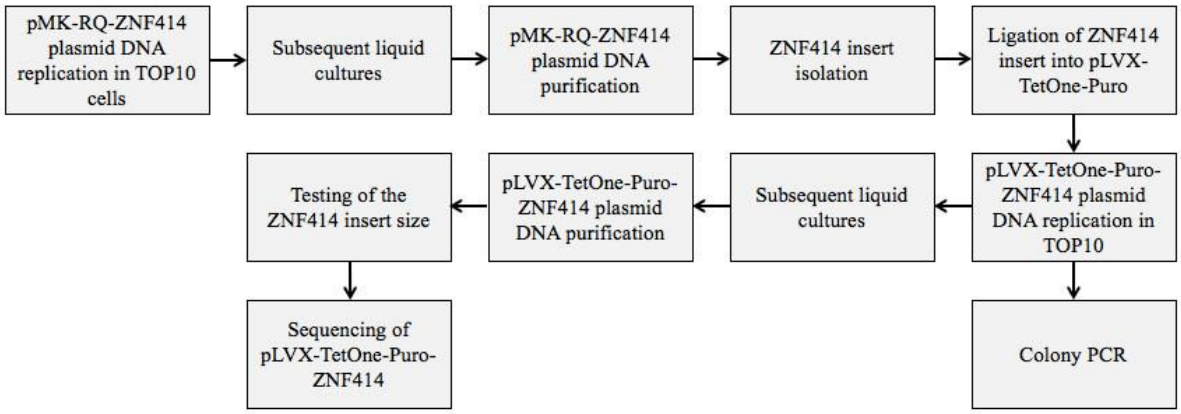

Figure 3. **Overview of the cloning workflow.**

4.2.1 Transformation of chemically competent cells

A commercial GeneArt™ service (Thermo Fisher Scientific, Darmstadt, Germany) was used to synthetize and clone the ZNF414 gene, combined with a V5 tag, into the pMK-RQ (KanR) vector backbone, herein called pMK-RQ-ZNF414. Based on the V5 tag, the presence and amount as well as the localization of ZNF414 can be verified after transduction of Hs700T and SU.86.86 cells. Chemically competent bacterial cells were then transformed with pMK-RQ-ZNF414 vector in order to replicate the plasmid DNA. Transformation was carried out using the One Shot™ TOP10 Chemically Competent Escherichia coli (E. coli) (Invitrogen, Darmstadt, Germany) protocol as instructed. Transformed bacteria were transferred into kanamycin containing LB agar plates and incubated at +37°C overnight. Kanamycin prevents the growth of colonies that lack the pMK-RQ-ZNF414 plasmid since they do not contain the kanamycin resistance gene. The following day, individual colonies were collected for subsequent liquid cultures. In addition, plasmid DNA from subsequent liquid cultures were purified for insert extraction using Nucleobond Xtra Midi Plus EF kit (Macherey-Nagel, Düren, Germany) by following the manufacturer's instructions.

4.2.2 Insert isolation and ligation

Restriction enzyme digestion (RE-digestion) was performed for purified plasmid DNA to isolate the ZNF414 insert from the pMK-RQ plasmid. Restriction enzymes, EcoRI and BamHI, were specifically chosen to cut pMK-RQ-ZNF414's multiple cloning site (MCS) on both sides of the insert. Reagents for the RE-digestion are presented in Table 2. Solution was quickly vortexed and centrifuged before incubation at +37°C for 5 minutes. 10 µl of RE-digestion mix was ran in 1 % agarose gel and the digested ZNF414 insert was cut out from the gel with a scalpel. Insert was extracted from the agarose gel by QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Table 2. **Used restriction-digestion mixture for insert extraction.**

Restriction-digestion mix	1x
Nuclease-free H_2O	15 µl
10x FastDigest Green Buffer	2 µl
plasmid DNA (up to 1µg)	1 µl
FastDigest EcoRI	1 µl
FastDigest BamHI	1 µl

In order to clone the ZNF414 insert into the pLVX-TetOne-Puro plasmid, DNA ligation was conducted. First step was to set up restriction digests for the pLVX-TetOne-Puro plasmid with same restriction enzymes, EcoRI and BamHI, as used in insert isolation. In this manner, the ZNF414 insert is cloned into the receiving plasmid with correct orientation and location. In addition, dephosphorylation was performed for the pLVX-TetOne-Puro plasmid after RE-digestion in order to prevent premature closure until ligation is completed. Dephosphorylation and ligation of the pLVX-TetOne-Puro plasmid was performed using the Rapid DNA Dephos and Ligation kit (Roche, Mannheim, Germany) following the standard protocol of manufacturer. In addition, 2 µl of ligation solution was transformed into the One Shot™ TOP10 Chemically Competent E. coli (Invitrogen, Darmstadt, Germany) and added on ampicillin containing LB agar plates according to the manufacturer's instructions. Lastly, individual colonies were collected for colony PCR (cPCR) and subsequent liquid cultures which were grown overnight for plasmid DNA purification.

4.2.3 Colony PCR

cPCR was performed to verify the presence of the pLVX-TetOne-Puro-ZNF414 plasmid in selected colonies. First step was to collect selected colonies from LB agar plates (containing ampicillin). Each individual colony was divided into two halves, one half was collected for subsequent liquid culture and the other half was submerged into dH_2O for cPCR. In order to get the template DNA for the PCR reaction mix (Table 3), dH_2O was quickly vortexed and incubated at +95°C water bath for 5 minutes. After cooling the dH_2O on ice, solution was centrifuged at 13 000 g for 10 minutes. Supernatant was then collected and used as a DNA

template in the cPCR reaction. The sequences of ZNF414 primers used in the PCR reaction were: 5′-GCC TGA CCA GCA TAG TCTCC-3' (forward) and 5'-GAC GCT GGG AAA ACTG AGG-3' (reverse). Purified pMK-RQ-ZNF414 plasmid DNA and the cPCR mix without the template DNA were used as positive and negative controls.

Table 3. **PCR reaction mixture used in the cPCR.**

Reagent	1x
dH_2O	15,2 µl
10x Dynazyme buffer	2 µl
10 mM dNTPs	0,4 µl
10 µM F primer	0,5 µl
10 µM R primer	0,5 µl
Template DNA	1 µl
Dynazyme II	0,4 µl

After completing the PCR program (Table 4), PCR products were ran on 1 % agarose gel at 80V for 40 minutes. Colonies were considered as pLVX-TetOne-Puro-ZNF414 positive if clear band was detected in the gel image. Based on the cPCR results, plasmid DNA from selected subsequent liquid cultures was purified using Monarch Plasmid Miniprep kit (New England BioLabs, Ipswich, UK) by following manufacturer's instructions.

Table 4. **Detailed information of the PCR program.** PCR was performed using MJ Research PTC-200 Thermal Cycler.

Step of the PCR program	Temperature (°C)	Time	Number of cycles
initialization	+95	5 minutes	1
denaturation	+98	10 seconds	30
annealing	+57	20 seconds	
elongation	+72	40 seconds	
final elongation	+72	5 minutes	1

4.2.4 Insert size and orientation verification

After DNA purification, RE-digestion was conducted to examine if the ZNF414 insert was cloned into the pLVX-TetOne-Puro-ZNF414 plasmid in correct size. RE-digestion mix (Table 5) was vortexed and centrifuged before 5 minutes of incubation at +37°C. The digest was loaded on a 1 % agarose gel and the results were analyzed based on the size of the insert. The final content of the pLVX-TetOne-Puro-ZNF414 plasmid and insert orientation were verified by sequencing.

Table 5. **Used restriction-digestion mixture used for verifying the correct size of ZNF414 insert in pLVX-TetOne-Puro-ZNF414.** Restriction enzymes, EcoRI and BamHI, were used to cut the pLVX-TetOne-Puro-ZNF414 plasmid on both sides of the ZNF414 insert.

Restriction-digestion mix	1x
Nuclease-free H_2O	12 µl
10x FastDigest Green Buffer	2 µl
plasmid DNA (up to 1µg)	4 µl
FastDigest EcoRI	1 µl
FastDigest BamHI	1 µl

4.3 Production of cancer cell lines overexpressing ZNF414

After the cloning was completed, the pLVX-TetOne-Puro-ZNF414 contained all the necessary components for regulated ZNF414 induction, including the puromycin selection marker, Tet-On 3G transactivator, and the ZNF414 gene under the tight control of a TRE3GS promoter (P_{TRE3GS}) (Lenti-X™ Tet-One® Inducible Systems User Manual). A pLVX-TetOne-Puro-ZNF414 vector map is presented in Figure 4. The next step was to transduce Hs700T and SU.86.86 cells with pLVX-TetOne-Puro-ZNF414 lentiviral vector in order to generate ZNF414-overexpressing cancer cell lines.

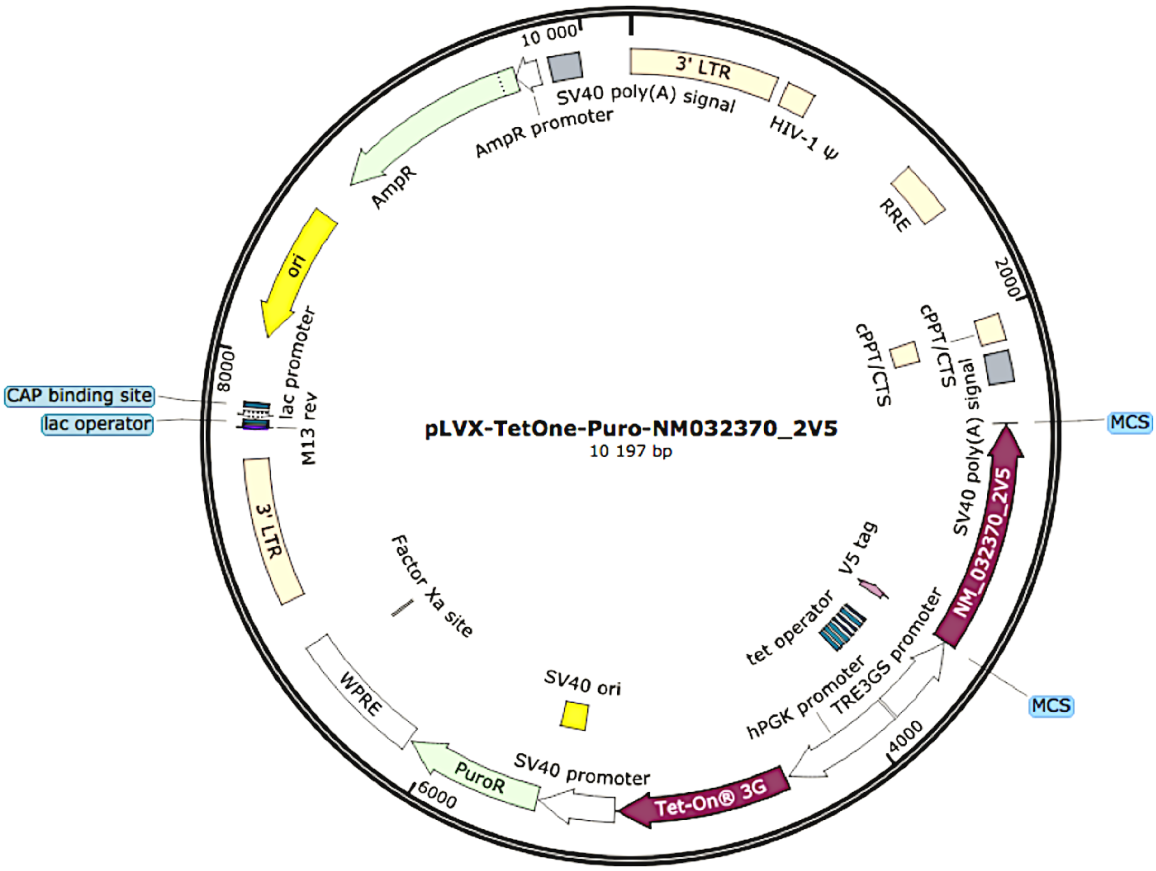

Figure 4. **Vector map of the pLVX-TetOne-Puro-ZNF414.** Synthetic ZNF414 gene, ‘‘NM_032370_2V5’’, was cloned into pLVX-TetOne-Puro vector with N-terminal V5 tag.

4.3.1 Puromycin kill curve experiment

Before the transduction of Hs700T and SU.86.86 cells, antibiotic kill curve experiments were performed to determine the lowest concentration of an antibiotic that can kill the non-transduced cells that do not contain the pLVX-TetOne-Puro-ZNF414 vector and thereby the puromycin resistance gene. Designed time point for analyzing was selected to be on day 3. At first, Hs700T and SU.86.86 cells were seeded on a 24-well plate and incubated at +37°C for 24 hours. Plating density for both cell lines was approximately 40 000 cells per well. After 24 hours, puromycin was added with varying concentrations (0, 0.5, 1, 1.5, 2 and 2.5 µg/ml) into the cell medium and replenished once after two days. Experiment was performed with three replicates and repeated twice.

4.3.2 Transient plasmid transfection

Prior to lentiviral supernatant production, the pLVX-TetOne-Puro-ZNF414 vector was tested for induction efficiency by transient plasmid transfection. The ZNF414 gene is expressed only when target cells are cultured in the presence of a synthetic tetracycline derivative called doxycycline. When bound by doxycycline, Tet-On 3G protein is capable of binding to *tet* operator sequences in P_{TRE3GS} promoter, initiating the expression of ZNF414 gene. Thus, efficiency of doxycycline induction was verified by measuring ZNF414 expression levels after transient plasmid transfection.

At first, SU.86.86 cells were plated on 12-well plates with a density of 120 000 cells per well and incubated for 24 hours at +37°C. The following day, cells were transiently transfected using the Lipofectamine® 3000 Transfection kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. 24 hours after transfection, 2.0 µg/ml puromycin was added into cell medium and sustained until the end of the experiment. After 72 hours of transfection, 500 ng/ml doxycycline was added into the cell medium to induce the expression of the ZNF414 gene in the transfected cells. Designed time point was set at 24 hours post-induction as instructed, and the transfected SU.86.86 cells were then lysed for mRNA purification. mRNA was isolated from lysed cells using the NucleoSpin® RNA Plus kit (Macherey-Nagel, Düren, Germany) and cDNA synthesis was conducted with the SuperScript III First Strand Synthesis kit (Invitrogen, Darmstadt, Germany). mRNA expression was measured with real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) using the LightCycler instrument (Roche, Mannheim, Germany) to verify the upregulation of the ZNF414 gene. In addition, ZNF414 expression levels were normalized against a house-keeping gene GAPD (Roche's Reference Gene Assay) expression. Detailed sequences of the primers and the quantitative RT-PCR program used in the experiment are presented in Table 6 and 7. The study was performed with four replicates and pLVX-TetOne-Puro-ZNF414 vector cells cultured in the absence of doxycycline were used as a negative control.

Table 6. **Sequences of the forward (for) and reverse (rev) primers used in the qRT-PCR experiment.**

Gene	Primers	Sequence (5' → 3')
ZNF414	ZNF414_qPCR_for	GGA GCC GCA GAA ACA AGA
	ZNF414_qPCR_rev	CCT TGT CGG TCT CAC TGGA

Table 7. **Detailed information of the quantitative RT-PCR program used to measure mRNA expression levels of ZNF414.**

Step of the PCR program	Temperature	Time
denaturation	+95°C	10 minutes
amplification (45 cycles)	+95°C	10 seconds
	+60°C	30 seconds
	+72°C	1 second
cooling	+40°C	30 seconds

4.3.3 Production of lentiviral supernatants

293T/17 cells were seeded on 100mm culture plates approximately 24 hours before transfection. The cells were plated with seeding density of 3 million per plate and cultured in DMEM until suitable cell confluence was achieved. At the time of transfection, pLVX-TetOne-Puro-ZNF414 vector was combined with Lenti-X Packaging Single Shots (VSV-G), an optimized packaging pre-mix lyophilized with Xfect Transfection Reagent, and added into 293T/17 cell plates according to manufacturer's instructions. After 48 hours of transfection, high-titer lentiviral supernatant was harvested by collecting the medium from the 293T/17 cell plates. The collected medium was filtered with Whatman® 0.45µm filter (Sigma-Aldrich, St. Louis, Missouri, USA) prior further use.

4.3.4 Transduction of target cells

Hs700T and SU.86.86 cells were transduced with the lentiviral supernatant with the aim of transferring the pLVX-TetOne-Puro-ZNF414 plasmid inside the cancer cells and thereby enabling integration into a host cell genome. For both Hs700T and SU.86.86, approximately 75 000 cells per well were seeded on 6-well plates. The following day, transduction was performed by replacing either 1 ml or 2 ml (three wells each) of the old cell medium with lentiviral 293T/17 medium. Antibiotic selection with 2.0 µg/ml puromycin was initiated after 72 hours of transduction and was regularly replenished at every medium change. All in all, Hs700T and SU.86.86 cells were cultured for 3 weeks at the virus facilities to ensure that the virus particles were entirely removed from the cell medium as a result of medium changes. Verification of the matter was done by performing a p24 test for mixed populations of Hs700T and SU.86.86. The p24 test is ELISA-based detection of the viral capsid protein p24 conducted by the virus core facility personnel. Prior to further studies, transduced Hs700T and SU.86.86 cells were tested for induction of the ZNF414 expression by quantitative RT-PCR.

4.4 Functional assays

4.4.1 Cell proliferation assay

Crystal violet staining was performed to determine the proliferation of the ZNF414-overexpressing cells in the presence and absence of doxycycline (Dox). For the cell proliferation assay, SU.86.86 cells were cultured in advance in two separate T-25 cell culture flasks with (500 ng/ml) and without doxycycline. Three days post-induction, cells were plated on 24-well plates with exactly the same cell suspension volume per each well in order to transmit acquired difference in cell proliferation to well plates. After three days of culture, cells were fixed by adding 50 µl of 11 % glutaraldehyde into the cell medium. Samples were incubated for 15 minutes on a plate shaker, washed three times with dH_2O, and air dried in a fume hood overnight. On the next day, 500 µl of 0,1 % crystal violet dye was added to each sample followed by 20 minutes of incubation on a plate shaker. In addition, cells were washed three times with dH_2O again and air dried for 7 hours. Crystal violet was dissolved to 1 ml of 10 % acetic acid and cells were incubated in the solution for 15 minutes on a plate shaker. 100 µl of acetic acid was then pipetted to a 96-well plate and absorbance was measured using the

Wallac Victor^3 1420 Multilabel Plate Counter (PerkinElmer, Shelton, Connecticut, USA) with a wavelength of 590nm. All experiments were performed with six replicates and repeated twice.

4.4.2 Cell cycle analysis

For the cell cycle analysis, SU.86.86 cells were cultured in advance similarly as described in the section 4.5.1. Three days post-induction, the cells were plated on 12-well plates with appropriate cell density. At the designed time point, 48 hours later, the cells were collected and suspended in 400 µl of hypotonic propidium iodide staining buffer (0.1 mg/ml sodium citrate tribasic dehydrate, 0.03% Triton X-100, 50 µg/µl propidium iodide (PI), 2 µg/ml RNase A). Quantitation of DNA content was measured using the BD Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, Michigan, USA) and analyzed with the ModFit LT software (Verity Software House Inc, Topsham, Maine, USA). All experiments were conducted with six replicates and repeated three times.

4.4.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, California, USA) to analyze statistical significance of the functional study results. Unpaired t-test was used to compare means of induced and uninduced (control) groups. Results were considered significant if p-value < 0.05.

5 RESULTS

5.1 Generation of inducible pLVX-TetOne-Puro-ZNF414 vector

The lentiviral pLVX-TetOne-Puro vector was selected for this study as it enables sustained and tightly regulated expression of the transgene after the vector DNA has integrated into target cell genome. Since the synthetized ZNF414 gene was received in a commercial pMK-RQ plasmid, the first step of the study was to extract the ZNF414 from the pMK-RQ plasmid and stably clone it into the pLVX-TetOne-Puro with the correct size and orientation.

5.1.1 ZNF414 insert extraction from the pMK-RQ plasmid

The ZNF414 insert was first cleaved from the pMK-RQ plasmid by using restriction enzymes EcoRI and BamHI. These enzymes were specifically chosen for the RE-digestion since the restriction site of EcoRI is located in the N-terminal end and BamHI's in the C-terminal end of the ZNF414 insert. As a result, the pMK-RQ plasmid was divided into two DNA fragments, the ZNF414 insert and the pMK-RQ backbone, which were separated from each other by gel electrophoresis (Figure 5). Since the ZNF414 is shorter in size than the plasmid backbone (1002 bp vs. 2318 bp with V5 tag, 957 bp vs. 2318 bp without V5 tag), it ran further in the gel within the given time. This enabled the verification of the ZNF414 size followed by DNA extraction of the ZNF414 from the agarose gel. To decrease the amount of workload, further studies were performed only with the ZNF414 insert containing V5 tag.

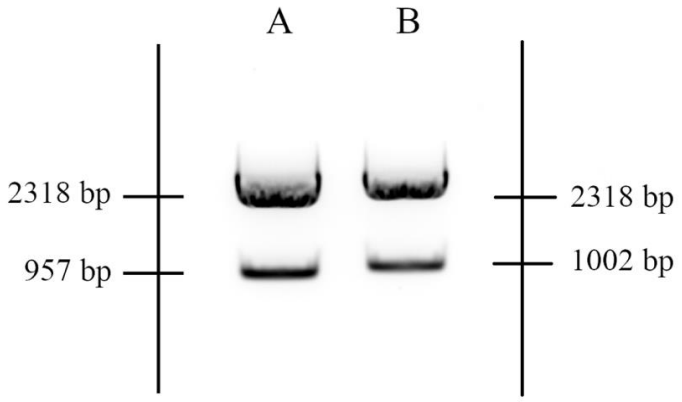

Figure 5. **The ZNF414 insert was extracted from the pMK-RQ plasmid by RE-digestion and gel electrophoresis.** Before the insert was cut out from the gel for DNA purification, its size was verified. A) represent the digested pMK-RQ plasmid + ZNF414 insert without the V5 tag and B) the pMK-RQ plasmid + ZNF414 insert with the V5 tag.

5.1.2 Determining the presence of the ZNF414 insert in the pLVX-TetOne-Puro plasmid

After the ligation of the pLVX-TetOne-Puro and the ZNF414 insert (with V5 tag), plasmid DNA was replicated in TOP10 cells. 10 individual colonies (L1 to L10) were then selected from the LB agar plate for colony PCR (cPCR) to determine the presence or absence of the ZNF414 insert in pLVX-TetOne-Puro plasmid. Since the cPCR assay did not require additional culturing of individual colonies or preparation of the plasmid DNA, cPCR enabled rapid screening of the presence of pLVX-TetOne-Puro-ZNF414 plasmid in used E. coli. To analyze the PCR results, PCR duplicates of each sample were pipetted on an agarose gel directly after the PCR and were run by electrophoresis. Results were analyzed based on the size and the level of brightness of the DNA fragment bands. Brighter DNA bands in gel represent colonies where the desired plasmid, pLVX-TetOne-Puro-ZNF414, is present.

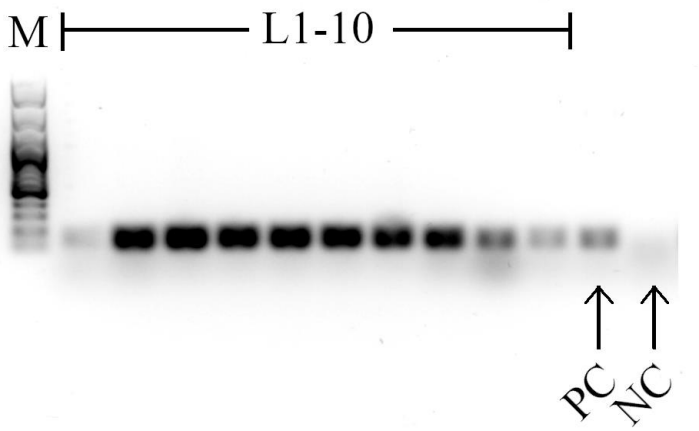

Figure 6. **Agarose gel image from the cPCR performed with the aim of determining the presence of the pLVX-TetOne-Puro-ZNF414 plasmid in the selected colonies**. 10 individual colonies were marked from L1 to L10, ladder as M, and PC and NC represent positive and negative controls, respectively.

Results from the cPCR experiment are shown in Figure 6. The purified pMK-RQ-ZNF414 plasmid was used as a template in the positive control (PC) while the PCR mix without the template DNA was used as a negative control (NC). By comparing test samples L1-10 with the negative control, each 10 samples indicated to be positive regarding the presence of the pLVX-TetOne-Puro-ZNF414 plasmid especially the colonies from L2 to L8. However, bands L1, L9, and L10 were less distinguishable than the others, thereby left out of the consideration for further use. Since there were no great differences in the bands from L2 to L8, subsequent liquid cultures corresponding to the samples L2 and L5 were selected for plasmid DNA purification.

5.1.3 Verifying the correct size and orientation of the ZNF414 insert

RE-digestion was performed for samples L2 and L5 in order to examine if the ZNF414 insert was successfully cloned into the pLVX-TetOne-Puro plasmid in correct size. Results of the RE-digestion were analyzed by running the digested L2 and L5 samples on an agarose gel and further visualized by imaging (Figure 7). Normally, at this point of the process, orientation of the ZNF414 insert would be verified. However, shorter ZNF414 variant did not include suitable restriction sites for that matter. The correct size of the ZNF414 insert (1002 bp with V5 tag) was therefore verified by using the same restriction enzymes, EcoRI and BamHI, as in the ligation described in a section 4.2.2. The final content of the pLVX-TetOne-Puro-ZNF414 plasmid, the insert orientation and possible mutations were confirmed by sequencing.

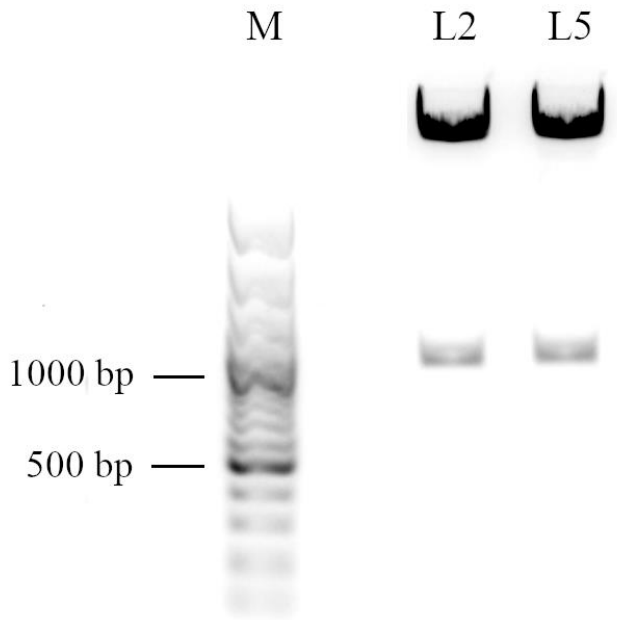

Figure 7. **Size of the ZNF414 insert was verified by RE-digestion and gel electrophoresis**. The ZNF414 insert was cleaved from the pLVX-TetOne-Puro plasmid with EcoRI and BamHI. After running the digested test samples L2 and L5 in the agarose gel, results were analyzed by comparing the size of the ZNF414 insert (1002 bp) with the marker (M).

5.2 Generation of stable ZNF414-overexpressing cancer cell lines

The second aim of the study was to generate ZNF414-overexpressing Hs700T and SU.86.86 pancreatic cancer cell lines in order to use them as models for studying cellular phenotypes of ZNF414 overexpression. The SU.86.86 and Hs700T cell lines were selected for this study based on their different endogenous ZNF414 expression levels. In our previous studies, relative gene expression of ZNF414 was found to be endogenously low in SU.86.86 cells as compared to the Hs700T cell line in which endogenous ZNF414 expression levels are significantly higher (unpublished data).

5.2.1 Optimal puromycin concentration for the selection of transduced cell lines

Antibiotic kill curve experiments were performed to determine the lowest concentration of an antibiotic that can kill the non-transduced cells in three days by subjecting cancer cells to increasing amounts of puromycin. Puromycin kills the non-transduced cells since they do not contain the pLVX-TetOne-Puro-ZNF414 vector, and thereby the puromycin resistance gene. In addition, the kill curve experiment is a critical step before transducing the Hs700T and SU.86.86 cells with the pLVX-TetOne-Puro-ZNF414 plasmid since the selection of stable cell lines requires accurate culturing conditions. By ensuring the optimal puromycin concentration, non-transduced cells can be eliminated without reaching cytotoxic levels of puromycin. Since antibiotic sensitivity differs between each mammalian cell line, antibiotic kill curve experiments were executed for both cell lines separately. Selected puromycin concentrations were 0, 0.5, 1.0, 1.5, 2.0 and 2.5 µg/ml.

Results from puromycin kill curve experiment for SU.86.86 cells are represented in Figure 8. When cultured without the presence of puromycin, SU.86.86 cells proliferated normally through the entire experiment until day 3. Addition of puromycin, however, resulted in a massive cell loss already on day 2 time point irrespective of the puromycin concentration. In order to facilitate the analysis on day 3 time point, dead and unattached cells were removed by replacing the puromycin containing medium with phosphate-buffered saline (PBS). As compared to the day 2, number of viable cells was significantly decreased in puromycin concentrations of 0.5-1.5 µg/ml, yet some cells were able to survive. In concentrations of 2.0 and 2.5 µg/ml, only unattached cells were floating in PBS, otherwise wells were completely

blank. As the aim was to determine the lowest concentration that can kill the cells in three days, 2.0 µg/ml of puromycin was selected for generation of stable transduced SU.86.86 cell lines.

In the case of Hs700T cells, the puromycin kill curve experiment was carried out with the same concentrations as for SU.86.86 cells (Figure 9). In the absence of puromycin, Hs700T cells proliferated normally until day 3 since they formed wider cell rafts with each passing day which is typical for their character. Regardless of the concentration, majority of the Hs700T cells cultured with puromycin had already lost their normal morphology and detached from the well bottom on day 2 time point. Since so many dead cells were floating in the medium, puromycin containing medium was replaced with PBS on day 3 time point as in the kill curve experiment for SU.86.86 cells. Up to the concentration of 1.5 µg/ml, a small proportion of Hs700T cells managed to survive until day 3. However, no large difference was noticed between 2.0 and 2.5 µg/ml since Hs700T cells were all dead. Due to this observation, puromycin concentration of 2.0 µg/ml was selected for the generation of stable transduced Hs700T cell lines.

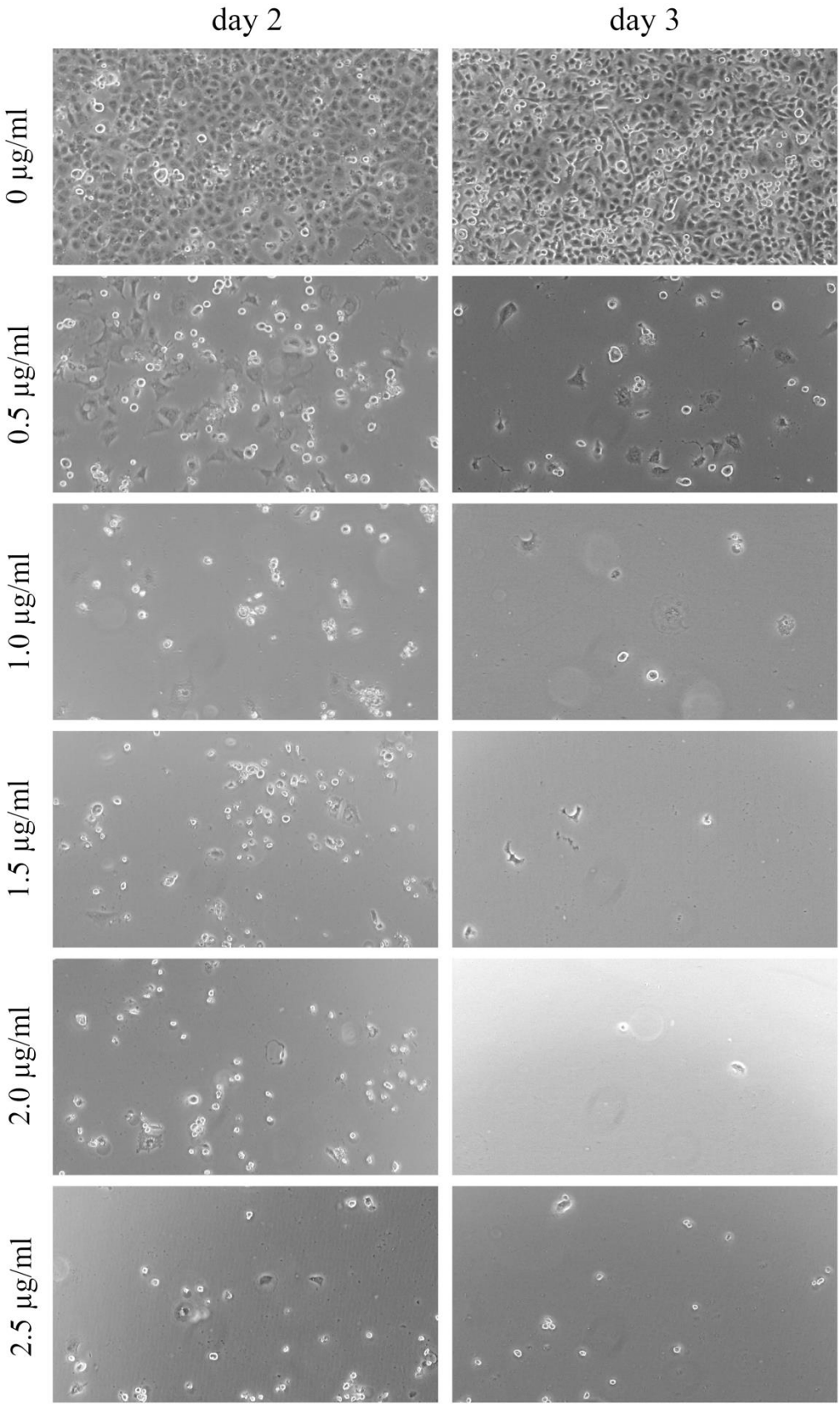

Figure 8. **Images of SU.86.86 cells from puromycin kill curve experiment on day 2 and day 3 time points.** SU.86.86 cells were cultured in six different puromycin concentrations. Lighter, round shaped cells represent dead, unattached cells. Images were taken with a light microscope using 4x objective.

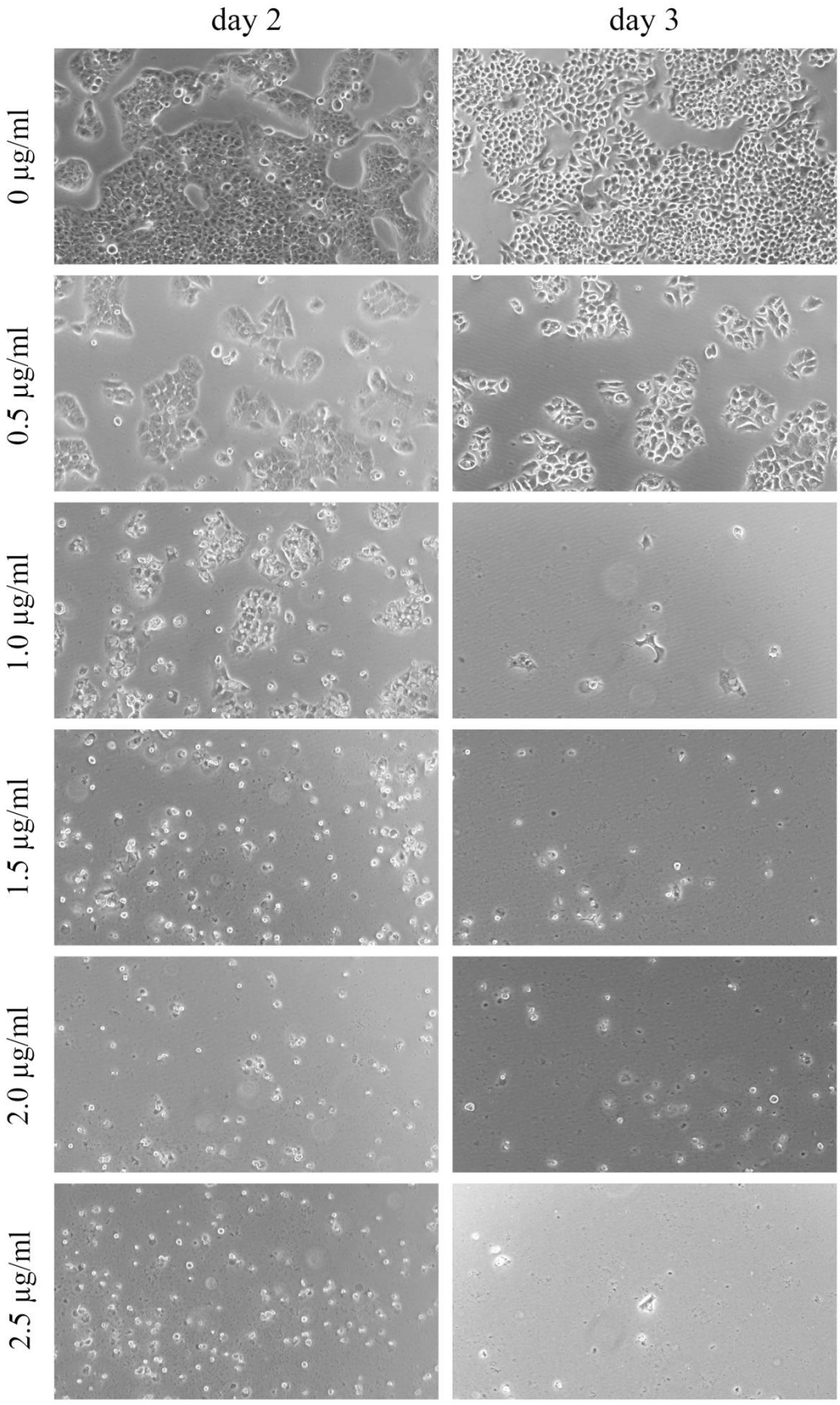

Figure 9. **Images of Hs700T cells from puromycin kill curve experiment on day 2 and day 3 time points**. Hs700T cells were cultured in six different puromycin concentrations. Lighter, round shaped cells represent dead, unattached cells. Images were taken with alight microscope using 4x objective.

5.2.2 Induction efficiency of the pLVX-TetOne-Puro-ZNF414 plasmid

The pLVX-TetOne-Puro-ZNF414 plasmid was tested for functionality by transient plasmid transfection. The lentiviral construct should always be tested prior to transduction to verify the efficiency of transgene induction. The experiment was conducted on SU.86.86 cells that were transfected using the Lipofectamine® 3000 Transfection kit (Invitrogen, Carlsbad, California, USA). After culturing for 24 hours in the presence or absence of doxycycline, cells were collected, and their mRNA was extracted for qRT-PCR. Relative gene expression of ZNF414 was then compared between the induced and uninduced SU.86.86 cells (Figure 10). The relative gene expression of ZNF414 was confirmed to be 6 times higher in the induced SU.86.86 cells than in the uninduced control cells. Therefore, transgene induction was considered to be fully functional.

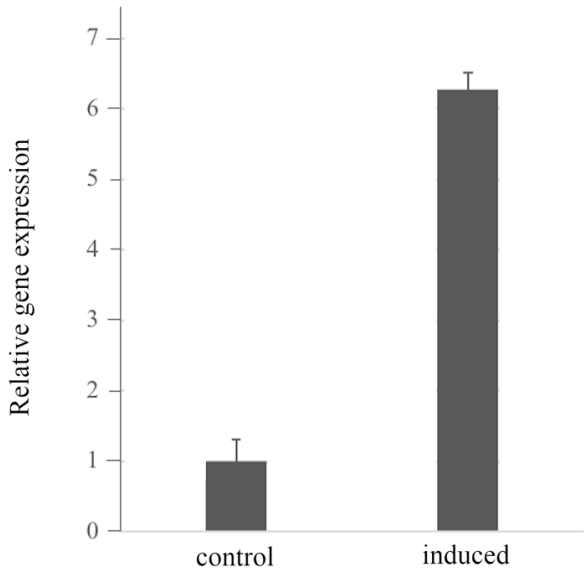

Figure 10. **Relative gene expression of ZNF414 in induced and uninduced SU.86.86 cells.** Transient plasmid transfection was performed using SU.86.86 cells which were transfected with pLVX-TetOne-Puro plasmid containing ZNF414. Four replicates were treated with doxycycline and other four were left as a control (uninduced SU.86.86 cells). ZNF414 expression was normalized against the housekeeping gene GAPD before comparing the results.

5.2.3 Quantitation of ZNF414 expression in transduced cells

After stable transduced cell lines were generated, their ZNF414 induction was tested for the verification of successful target cell transduction. When transduced cells are induced with doxycycline, expression levels of the ZNF414 should increase, thereby suggesting that the

pLVX-TetOne-Puro-ZNF414 vector is successfully integrated into target cell genome. Thus, the transduced SU.86.86 and Hs700T cells were cultured for 24 hours with doxycycline followed by the measurement of ZNF414 expression levels with quantitative RT-PCR.

Doxycycline was added into SU.86.86 culture medium with varying concentrations of 0, 10, 50, 100 and 500 ng/ml. As shown in Figure 11A, addition of doxycycline resulted in a dramatic increase in the expression of ZNF414. Furthermore, the expression levels increased as the concentration of doxycycline kept rising. For instance, with concentration of 500 ng/ml, the expression level of ZNF414 was up to 41 times higher when compared to uninduced SU.86.86 cells. After 5 days of the latest doxycycline change, ZNF414 expression was found to be still at the same level as on day 1 time point. Consequently, this suggested that the transduction was successful in SU.86.86 cells. Since SU.86.86 cells expressed high levels of ZNF414 when cultured with 500 ng/ml of doxycycline, functional studies were decided to be performed with the particular concentration.

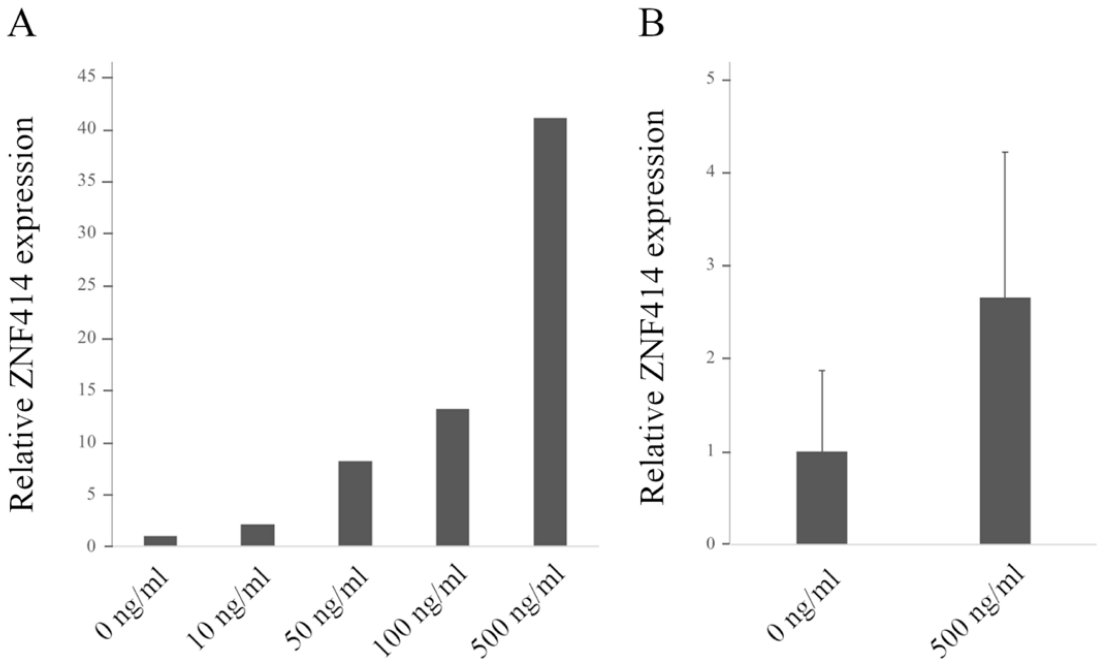

Figure 11. **Relative gene expression of ZNF414 in transduced SU.86.86 and Hs700T cells.** A) Dose-response of SU.86.86 cells when cultured with varying amounts of doxycycline. B) Hs700T cells were induced only with 500 ng/ml of doxycycline since particular concentration increased ZNF414 expression significantly in SU.86.86 cells. However, no significant difference in ZNF414 expression was detected when comparing induced and uninduced Hs700T cells.

The induction of transduced Hs700T cells was conducted only with 500 ng/ml of doxycycline since the particular concentration showed distinctively higher expression of ZNF414 in the transduced SU.86.86 cells compared to the uninduced SU.86.86 control. However, the results did not show any difference in the ZNF414 expression between the induced and uninduced Hs700T cells (Figure 11B). Although the doxycycline concentration was increased to 1000 ng/ml, or even 2000 ng/ml, expression levels of ZNF414 did not rise. Results were verified by performing Western blot for the induced SU.86.86 and Hs700T cells. Hence, transduction of the Hs700T cells was considered unsuccessful, and thereby left out of the functional studies.

5.3 Functional characterization of SU.86.86 cells overexpressing ZNF414

The ultimate aim of this study was to examine the functional consequences of ZNF414 overexpression for pancreatic cancer cell growth. Since the transduction of the Hs700T cells was considered unsuccessful, functional studies were performed using the transduced SU.86.86 cells as a model. Possible differences in the cell cycle and proliferation of ZNF414-overexpressing cells were analyzed by comparing the results between induced and uninduced SU.86.86 cells.

5.3.1 Effects of ZNF414 overexpression on SU.86.86 cell proliferation

For the cell proliferation assay, transduced SU.86.86 cells were cultured in the presence or absence of doxycycline until the designed time point of day 6. The effects of ZNF414 overexpression on cell proliferation were examined by crystal violet staining. Crystal violet dye binds to proteins and DNA of attached cells, thereby correlating with a number of living cells. After the results were analyzed, no significant difference was observed in the cell proliferation between the induced cells and the uninduced controls. The assay was repeated twice, both of which referred to a similar result. A representative example of the results is presented in Figure 12.

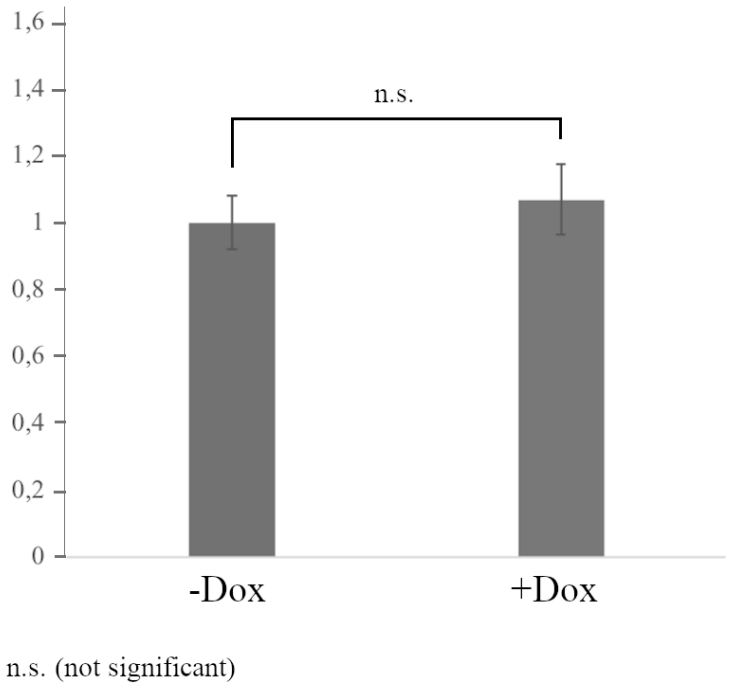

Figure 12. **Representative results of the cell proliferation assay performed for transduced SU.86.86 cells**. Absorbance was measured from induced (+Dox) and uninduced (-Dox) SU.86.86 cells on the day 6 time point. The results were compared in order to examine the impact of ZNF414 overexpression on cell proliferation. No statistical significance was detected between +Dox and -Dox groups.

5.3.2 Effects of ZNF414 overexpression on SU.86.86 cell cycle

For the cell cycle analysis, ZNF414-overexpressing SU.86.86 cells were cultured for five days in the presence or absence of doxycycline. On the designed time point on day 5, cells were permeabilized, and the DNA of the induced and uninduced (control) cells was stained with a fluorescence dye, propidium iodide (PI). Differences in the cell cycle were examined by measuring the quantitation of DNA content from the induced and control cells. Representative example of the results is shown in Figure 13. The amount of DNA content was nearly identical at each cell cycle phase between the groups. The majority of cells were in G0/G1 and S phase, covering approximately 80% of the total cell number. No statistical significance was observed in any cell cycle phase between these two groups. Therefore, it was suggested that ZNF414 overexpression does not affect to cell cycle of SU.86.86 cells.

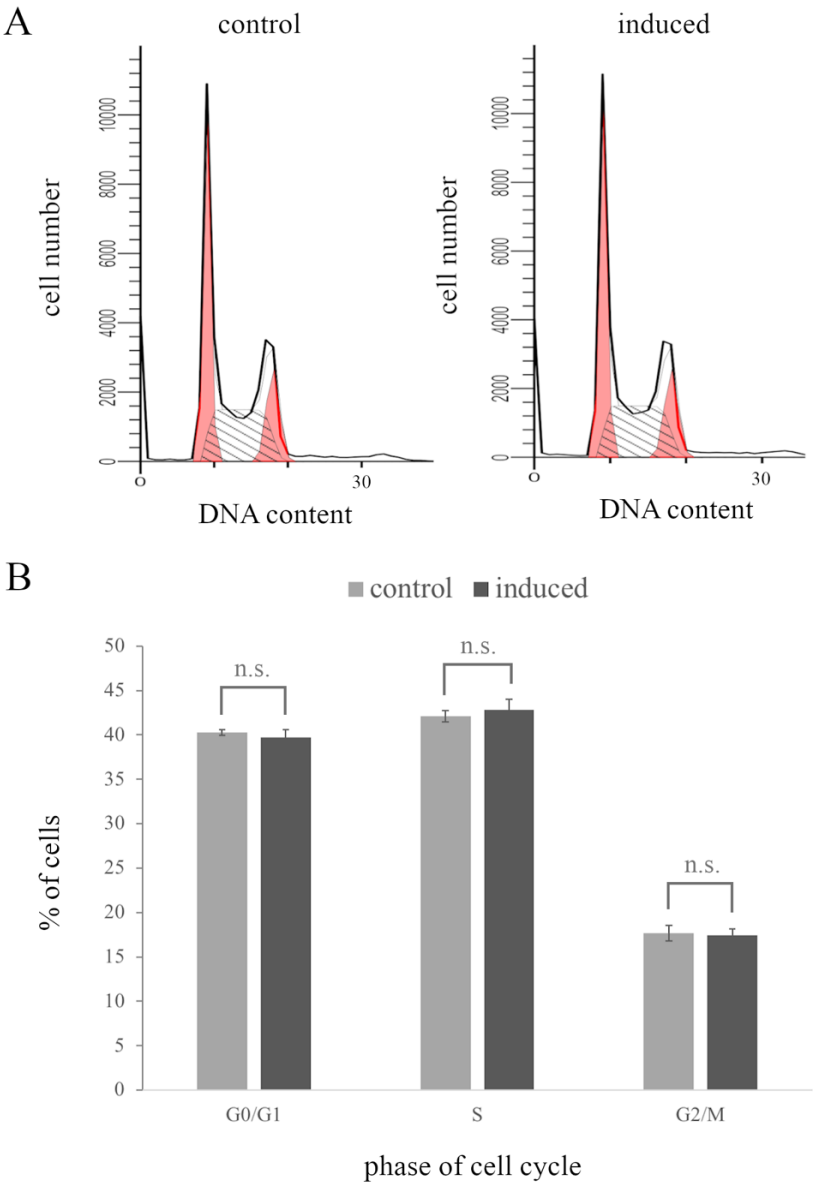

Figure 13. **Representative results of the cell cycle analysis performed for transduced SU.86.86 cells.** Cells were cultured with the presence (induced) or absence (control) of doxycycline for five days in order to determine the effects of ZNF414 overexpression on the SU.86.86 cell cycle. A) Quantitation of the DNA content in each phase of the cell cycle. Highest peak marked in red represents the G0/G1 phase whereas the lower peak in red represents the G2/M phase. The area in the middle of the peaks represents the S phase. B) The percentage of the cells in each cell cycle phase was compared between control and induced groups. Results showed no statistical difference in any of the phases.

6 DISCUSSION

Zinc-finger proteins have been in a focus in cancer studies ever since their transcription factor function was demonstrated to contribute to the development and progression of cancer.[17,56] Still, the functional roles of a majority of annotated zinc-finger proteins are yet to be discovered. Our research group was the first to discover the association of the ZNF414 with pancreatic cancer.[72] Knockdown of the ZNF414 expression by siRNA-based gene silencing resulted in a decreased growth and distinct G1 arrest in pancreatic cancer cells indicating its important role as a regulator of cancer cell growth.[72] While previous research has mainly focused on the effects of oncogenic zinc-finger protein knockdown, the aim of this study was to evaluate the functional consequences of the ZNF414 overexpression in pancreatic cancer cells which could potentially provide new knowledge on how this particular protein contributes to the regulation of cancer cell growth.

6.1 Successful cloning of the ZNF414 into the pLVX-TetOne-Puro

Lentivirus-based Lenti-X™ Tet-One® Puro Inducible Expression System was used in this study to transduce the SU.86.86 and Hs700T cells with pLVX-TetOne-Puro vector. This method enables tightly controlled transgene expression after the vector DNA has integrated into the host cell genome. Since the effects of sustained ZNF414 expression were yet unknown, controlled expression was desirable feature for chosen lentiviral vector. Lentiviruses are derived from human immunodeficiency virus type 1 (HIV-1) and typically pseudotyped with vesicular stomatitis virus G-protein (VSV-G) to broaden the range of transfection targets.[74,75] The use of lentiviruses as vectors has considerably increased during the years, since they can stably and effectively transduce both dividing and non-dividing cells.[76] Moreover, ideal vector should have the genetic capacity to carry the transgene into the host cells without inducing cytotoxicity. Since pLVX-TetOne-Puro meets these criteria, the particular vector was a suitable choice for this study to transduce the pancreatic cancer cells.

The lentiviral vector was originally sent to GeneArt service (Thermo Fisher Scientific, Darmstadt, Germany) where the ZNF414 gene, with V5 tag, was supposed to be cloned into the pLVX-TetOne-Puro. Despite the multiple attempts, the GeneArt was unable to clone the ZNF414 insert into the plasmid. However, they managed to synthetize the ZNF414 gene and

deliver it as part of the pMK-RQ plasmid backbone. Therefore, the first aim of the study was to clone the synthetized ZNF414 from the pMK-RQ plasmid into the pLVX-TetOne-Puro vector.

E. coli cells are most commonly used bacterial species for transformation as part of the cloning process. They were chosen for this study since E. coli provide fast and efficient method for high-copy (pLVX-TetOne-Puro) and low-copy (pMK-RQ) number plasmid replication. In addition, E. coli can stably be transformed even with larger plasmids. The pLVX-TetOne-Puro-ZNF414 is relatively large in size (>10 kb) which could normally pose issues in transformation, since the transformation efficiency tends to decrease when the plasmid size increases.[77] The growth of colonies missing the transformed plasmid was inhibited by using specific antibiotics in LB agar plates: kanamycin with the pMK-RQ-ZNF414 and ampicillin with the pLVX-TetOne-Puro-ZNF414 since their DNA contains the resistance gene for these particular antibiotics. Colony PCR was chosen from the array of methods to verify the presence of ZNF414 in the selected colonies. Colony PCR do not require further culturing of the colonies or excess preparations of the plasmid DNA making it a rapid method for screening the presence of desired plasmid in E. coli cells. After the ZNF414 was cloned into the pLVX-TetOne-Puro, the proper orientation of the ZNF414 was verified by sequencing. Typically, orientation could be examined by restriction enzyme digestion, but the lack of correct restriction enzymes and the time saving issues made it easier to test the orientation in this way. In the end, the ZNF414 was successfully cloned into the lentiviral pLVX-TetOne-Puro.

6.2 ZNF414 expression increases in transduced SU.86.86 cells but not in transduced Hs700T cells

The ZNF414-overexpressing pancreatic cell lines were generated by transducing SU.86.86 and Hs700T cells with the pLVX-TetOne-Puro-ZNF414 vector. Prior to transduction, puromycin kill curve experiment was performed in order to examine the lowest concentration of puromycin which can eliminate the non-transduced cells. The non-transduced cells do not contain the pLVX-TetOne-Puro-ZNF414 vector, and thereby the puromycin resistance gene. In addition, by optimizing the puromycin concentration, non-transduced cells can be removed from the cell population without reaching cytotoxic levels of the antibiotic which could be harmful for successfully transduced cells. In summary, optimal puromycin concentration was found to be the same for both SU.86.86 and Hs700T cells. The results of the kill curve experiment are in

line with other relevant studies regarding the optimal puromycin concentration.[78,79] Therefore, the validity of the results could be confirmed.

Lentiviral supernatants were produced in 293T cells as instructed by the manufacturer. The choice was supported by the other studies in which transient production in 293T cells is confirmed to be a fast and effective method especially when lentiviral vector is enveloped with VSV-G.[80–82] Prior to target cell transduction, virus concentration is typically examined by counting the number of produced titers in a given volume of media. This step can prevent the failure of transduction since lentiviral vectors bearing VSV-G can be cytotoxic for cells when added into cell medium at high concentrations.[83] There is also a possibility that the viral titers are produced in too low quantities due to the unsuccessful transfection of the 293T cells. To prevent the loss of viable virus titers, this study did not include the virus quantification as lentiviral 293T medium was directly replaced with target cell medium after filtering. Instead, the target cells were subjected for two different concentrations of lentiviral medium in case the other of these concentrations would be either insufficient or highly cytotoxic. After transduction, the SU.86.86 cells maintained their viability in both virus concentrations and proliferated normally during the puromycin selection. When compared to the SU.86.86 cells, the number of viable Hs700T cells was distinctively lower in both virus concentrations. However, the cell concentration of Hs700T increased after they were cultured for two weeks.

Prior to the functional studies, relative ZNF414 expression was compared between induced and uninduced cells to evaluate the functionality of the pLVX-TetOne-Puro-ZNF414 vector in transduced cells. The results demonstrate that the transduction was successful in the SU.86.86 cells as they expressed high levels of the ZNF414 when cultured in the presence of doxycycline. However, the ZNF414 expression levels did not increase in the Hs700T cells despite multiple attempts with varying concentrations of doxycycline and the cells were therefore left out of further functional studies. Although the transduction would have been successful in Hs700T cells, the fold induction of ZNF414 can still be low for various reasons, such as poor target cell viability, poor transduction efficiency, or the presence of tetracycline derivatives in used target cell medium. However, the Hs700T cells were cultured with tetracycline-free serum during the entire process and their viability was relatively normal at the time of induction suggesting that the possible reason could only be the insufficient transduction. In future studies, transduction efficiency could be increased by confirming viability of virus titers with a titration, by optimizing density of target cells before transduction, or by using transduction enhancers,

including the polybrene. Polybrene increases the transduction efficiency by enhancing the virus adsorption on target cell membranes both receptor and envelope-independent manner by reducing charge repulsion between cell membrane and the virus.[84]

One possible explanation for inefficient Hs700T transduction could be the VSV-G used to envelope the pLVX-TetOne-Puro vector. The VSV-G has been the most popular glycoprotein for enveloping HIV-1 based lentiviral vectors.[74,81] It interacts with an ubiquitous cellular ''receptor'' on target cells, giving the vector a broad species and tissue tropism.[74,75] Despite the multiple advantages of VSV-G, transduction efficiency can be increased by modifying the cell and species specificity with alternative pseudotypes.[74] For example, HIV-1-based lentiviral vectors transduce insulin-secreting beta cells in human islets more efficiently when pseudotyped with LCMV rather than with VSV-G.[85] In addition, LMCV-WE pseudotypes were found to efficiently transduce solid gliosarcoma cells *in vivo*, whereas the VSV-G transduced only few tumor cells and targeted mostly neurons in the surrounding tumor environment.[86] However, the Hs700T cells have been successfully transduced with VSV-G pseudotyped lentiviral vector in our previous studies indicating that the transduction should be theoretically possible with this particular envelope protein.[72] To properly evaluate the specificity of VSV-G for Hs700T cells and its association with unsuccessful Hs700T transduction, the present study should be repeated with a different pseudotype alongside the VSV-G.

6.3 Proliferation and cell cycle of the SU.86.86 cells are not affected by the ZNF414 overexpression

Transduced SU.86.86 cells were used as a model for studying the cellular phenotype of the ZNF414 overexpression. The ZNF414 overexpression was hypothesized to affect the pancreatic cancer cells by leading to increased cell growth and altered phenotype, especially in SU.86.86 cells with low endogenous ZNF414 expression. Contrary to the hypothesized outcome, overexpression did not result as a statistically significant increase in the proliferation of SU.86.86 cells. In addition, results showed no statistical difference in the cell cycle between the induced and uninduced SU.86.86 cells. These findings may be explained by characteristics of cancer cells as they already grow at an abnormally rapid rate. Increasing the expression of one individual TF may no longer be able to increase the proliferation due to the achieved growth limit.

Theoretically, a possible explanation for the results could be the insertional ‘’mutagenesis‘’ as every gene transfer vector carries a risk of integrating near promoters or other unfavorable regions of DNA.[81] Studies have demonstrated that the HIV-1-based lentiviral vectors typically integrate along the length of transcriptionally active genes.[81] It is possible that lentiviral vectors may directly promote gene transcription either by acting upon a nearby gene enhancer or promoter, or by causing chances to the structure of local chromatin due to its presence in the regulatory region.[81] In this case, the pLVX-TetOne-Puro-ZNF414 may have integrated in a way that caused unforeseen effects on cancer cell growth and cell cycle. However, the probability of such effects remains low since the ZNF414-overexpressing SU.86.86 cell line is mixed population instead of clone suggesting that these undesired phenotypes would more likely be covered under others. To investigate the possibility, the entire genome of the transduced SU.86.86 cells should be sequenced to locate the integration site of the pLVX-TetOne-Puro-ZNF414. However, this would only be possible with clonal cell lines.

Due to the lack of similar ZNF414 studies, the results cannot be reliably compared with previous findings. However, zinc-finger proteins are known to play important roles in many cellular processes, including cell development, differentiation and tumor suppression.[17,56] In general, their role in cancer onset and progression has been widely studied which has led to increasing number of evidence regarding their involvement in cancer progression.[17]

Taken together, the study suggests that the overexpression of ZNF414 does not affect the proliferation and cell cycle of pancreatic cancer cells. The generalizability of the results is limited by the narrow sampling as the functional studies were performed with only one pancreatic cancer cell line. Moreover, this study examined the effects of ZNF414 overexpression only on proliferation and cell cycle from the array of characteristic features for cancer. Thus, the possible effects of ZNF414 overexpression on other features should not be excluded. In addition, the SU.86.86 and Hs700T cell lines were chosen for this study because of their different endogenous expression levels of the ZNF414. Since the transduction was unsuccessful in the Hs700T cells, the impact of endogenous ZNF414 expression level on the study outcome could not be evaluated. Therefore, the study should be repeated with additional pancreatic cell lines in order to confirm the findings and gain a more thorough insight into the role of ZNF414 overexpression in pancreatic cancer.

7 CONCLUSIONS

Aim of this study was to generate ZNF414-overexpressing SU.86.86 and Hs700T pancreatic cancer cell lines and use them as models for studying the functional consequences of ZNF414 overexpression in pancreatic cancer cells. The main findings obtained from this research are the following:

1. As a results of target cell transduction, the SU.86.86 expressed high levels of ZNF414 when cultured in the presence of doxycycline. Similar effect in transduced Hs700T cells was not detected suggesting that the transduction efficiency was poor in Hs700T cells. Future studies should consider using alternative pseudotypes alongside the VSV-G or transduction enhancers to increase the efficiency of target cell transduction.

2. The proliferation and cell cycle of pancreatic cancer cells are not affected by ZNF414 overexpression. Functional studies were performed only with the SU.86.86 pancreatic cancer cell line which limits the generalizability of the result. Therefore, the study should be repeated with additional pancreatic cell lines to confirm the findings and gain a deeper understanding of the ZNF414 role in pancreatic cancer.

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