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The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-7716-1 (Print)
ISBN 978-951-29-7717-8 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy – Turku, Finland 2019

”Noinnikkäästi”
-Mamma

ABSTRACT

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Long non-coding RNAs in cutaneous squamous cell carcinoma

University of Turku, Faculty of Medicine, Dermatology and Venereology, Turku Doctoral Programme of Molecular Medicine (TuDMM), Western Cancer Centre of the Cancer Center Finland (FICAN West), University of Turku and Turku University Hospital, MediCity Research Laboratory, University of Turku, Turku, Finland

Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Finland, 2019

Long-term exposure to the sun UV-radiation is the leading cause for the development of skin cancer. Keratinocyte-derived cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer and its incidence is increasing globally. Although the most frequent mutational targets in cSCC development have been characterized, a comprehensive understanding of the molecular events in cSCC pathogenesis remains incomplete.

There is an obvious need for clinically useful prognostic biomarkers and therapeutic targets for recurrent and metastatic cSCCs. Long non-coding RNAs (lncRNAs) are a largely uncharacterized group of regulatory RNAs involved in various biological processes and their role in cancer progression is emerging. However, their role in cSCC is largely unknown. The main objective of this thesis was to investigate lncRNAs in order to identify new biomarkers for progression of cSCC and characterize novel therapeutic targets for recurrent and metastatic cSCC.

In this study two tumorigenic lncRNAs in cSCC were identified, and based on their expression and function and with the permission of the HUGO Gene Nomenclature Committee they were named PICSAR and PRECSIT. They are specifically upregulated in cSCC cells in culture and *in vivo* and they contribute to cSCC progression by distinct mechanisms. PICSAR promotes cSCC cell growth by activating ERK1/2 *via* suppression of DUSP6 expression. Furthermore, PICSAR regulates adhesion and migration of cSCC cells by regulating integrin expression. PRECSIT expression in cSCC cells was shown to be regulated by the p53 pathway. Additionally, PRECSIT was found to regulate invasion of cSCC cells by regulating STAT3 signaling and expression of MMP-1, MMP-13, MMP-13, and MMP-10.

In conclusion, lncRNAs PICSAR and PRECSIT may serve as novel biomarkers and putative therapeutic targets in cSCC.

KEYWORDS: cutaneous squamous cell carcinoma, long non-coding RNA, cancer

TIIVISTELMÄ

Minna Piipponen

Pitkät ei-koodaavat RNA-molekyylit ihon levyepiteelisyövässä

Turun yliopisto, Lääketieteellinen tiedekunta, Iho- ja sukupuolitautioppi, Turun Molekyylilääketieteen tohtoriohjelma (TuDMM), Läntinen Syöpäkeskus (FICAN West), Turun yliopisto ja Turun yliopistollinen sairaala, MediCity-tutkimuslaboratorio, Turun yliopisto, Turku, Suomi

Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Suomi, 2019

Jatkuva altistuminen auringon UV-säteilylle on suurin syy ihon levyepiteelisyövän (cutaneous squamous cell carcinoma, cSCC) kehittymiseen. Se on yleisin metastaattinen ihosyöpä maailmanlaajuisesti ja syöpätapausten määrä on kasvussa. Useita cSCC:n kehittymiseen liittyviä DNA-mutaatioita on tunnistettu, mutta kokonaisvaltainen käsitys syövän kehittymisen aikana tapahtuvista molekulaarisista muutoksista on yhä selvittämättä.

Uusia merkkiaineita tarvitaan, jotta voitaisiin paremmin tunnistaa kehittyykö varhaisen vaiheen syöpä aggressiiviseksi metastasoivaksi syöväksi. Pitkät ei-koodaavat RNA:t (long non-coding RNA, lncRNA) ovat varsin tuntematon ryhmä molekyylejä, joiden merkitystä syövässä on tutkittu laajalti, mutta niiden yhteyttä cSCC:hen ei juurikaan tunneta. Tämän väitöskirjatyön tavoitteena oli löytää ja karakterisoida lncRNA:ita, joita voitaisiin käyttää merkkiaineina nopeasti etenevän tai leviävän cSCC:n havaitsemisessa sekä uusien hoitomuotojen kehittämisessä.

Tässä työssä havaittiin kaksi lncRNA:ta, jotka liittyvät cSCC:n kehittymiseen. Tutkimustulosten perusteella sekä ihmisen perimän kansainvälistä kartoitus- ja sekvensointihanketta johtavan organisaation nimeämiskomitean luvalla ne nimettiin PICSAR:iksi ja PRECSIT:iksi. Niiden ilmentyminen on koholla cSCC:ssä ja ne vaikuttavat cSCC:n kehittymiseen eri mekanismeilla. PICSAR edistää syövän kasvua aktivoimalla ERK1/2 kinaasia DUSP6:n kautta. Lisäksi se säätelee solujen kiinnittymistä ja liikkumista vaikuttamalla integriini-solureseptorien ilmentymiseen. PRECSIT:in ilmentymistä säädellään p53-signalointireitin kautta. PRECSIT säätelee cSCC-solujen invaasiota STAT3-signalointireitin välityksellä vaikuttamalla MMP-1, MMP-3, MMP-10 ja MMP-13 geenien ilmentymiseen.

PICSAR ja PRECSIT voisivat toimia uusina merkkiaineina syövän diagnostiikassa ja uusien hoitomuotojen kehittämisessä cSCC:lle.

AVAINSANAT: ihon levyepiteelisyöpä, pitkä ei-koodaava RNA

Table of Contents

Abbreviations

AK	Actinic keratosis
BCC	Basal cell carcinoma
circRNA	Circular RNA
cSCC	Cutaneous squamous cell carcinoma
cSCCIS	Cutaneous squamous cell carcinoma *in situ*
DMBA	9,10-dimethyl-1,2-benzanthracene
dsRNA	Double-stranded RNA
DUSP	Dual-specificity phosphatase
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal-transition
ENCODE	Encyclopedia of DNA Elements
ERK1/2	Extracellular signal-regulated kinase 1/2
FGFR3	Fibroblast growth factor receptor 3
FFPE	Formalin fixed paraffin embedded
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GENCODE	Encyclopædia of genes and gene variants
gRNA	Guide RNA
HaCaT	Non-tumorigenic, spontaneously immortalized human keratinocyte cell line
HE	Hematoxylin and eosin
HGNC	The gene nomenclature committee of the human genome organization
HGP	The Human Genome Project
HNSCC	Head and neck squamous cell carcinoma
IHC	Immunohistochemistry
lncRNA	Long non-coding RNA
lincRNA	Long intergenic non-coding RNA
MAPK	Mitogen activated protein kinase
miRNA	Micro-RNA
mRNA	Messenger RNA
MMP	Matrix metalloproteinase

NAT	Natural antisense transcript
NHEK	Normal human epidermal keratinocytes
ncRNA	Non-coding RNA
NMSC	Non-melanoma skin cancer
ORF	Open reading frame
PICSAR	p38 inhibited cutaneous squamous cell carcinoma associated lincRNA
piRNA	Piwi-interacting RNA
PI3K	Phosphoinositide 3-kinase
PRECSIT	p53-regulated carcinoma-associated STAT3 activating long intergenic non-protein coding transcript
pri-miRNA	Precursor miRNA
qRT-PCR	Quantitative real-time polymerase chain reaction
RISC	RNA-induced gene silencing complex
RNA	Ribonucleic acid
RNA-ISH	RNA *in situ* hybridization
RNA-seq	RNA sequencing
RNA Pol	RNA polymerase
RNP	Ribonucleoprotein
rRNA	Ribosomal RNA
RTK	Receptor tyrosine kinase
SCID	Severe combined immunodeficiency
SK	Seborrheic keratosis
scaRNA	Small Cajal-body specific RNA
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
SNP	Single-nucleotide polymorphism
SRP RNA	Signal recognition particle RNA
STAT3	Signal transducer and activator of transcription 3
TERT	Telomerase reverse transcriptase
TPA	Tetradecanoyl-phorbol acetate
tRNA	Transfer RNA
tsncRNA	tRNA-derived small non-coding RNA
UT-SCC	Human cutaneous squamous cell carcinoma cell line
UV	Ultraviolet
vRNA	Vault RNA

List of Original Publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-III.

I. Piipponen M, Nissinen L, Farshchian M, Riihilä P, Kivisaari A, Kallajoki M, Peltonen J, Peltonen S, Kähäri VM. Long noncoding RNA PICSAR promotes growth of cutaneous squamous cell carcinoma by regulating ERK1/2 activity. *J Invest Dermatol* **136**:1701-10, 2016

II. Piipponen M, Heino J, Kähäri VM, Nissinen L. Long non-coding RNA PICSAR decreases adhesion and promotes migration of squamous carcinoma cells by downregulating α2β1 and α5β1 integrin expression. *Biol Open* **7**:pii:bio037044, 2018

III. Piipponen M, Nissinen L, Riihilä P, Farshchian M, Kallajoki M, Peltonen J, Peltonen S, Kähäri VM. p53-regulated long non-coding RNA PRECSIT promotes progression of cutaneous squamous cell carcinoma *via* STAT3 signaling. (*AmJPathol* in revision)

1 Introduction

Long-term exposure to solar UV-radiation is the leading cause for the development of skin cancer. Keratinocyte-derived cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer and its incidence is increasing globally. Primary cSCC arises as a result of malignant transformation of epidermal keratinocytes, which primarily occurs due to accumulation of genomic mutations after chronic exposure to UV-light. Tumor-suppressor p53 is an important factor in protecting the genomic integrity and it is one of the most frequent targets for mutational inactivation in keratinocytes. Mutationally inactivated p53 can be detected already in actinic keratosis (AK), a potentially malignant skin lesion and the first step in cSCC carcinogenesis. However, it is difficult to distinguish which of these lesions have the potential to progress to invasive cSCC. Most often primary cSCCs can be surgically removed but they harbor a tendency for recurrence and there is a poor prognosis for advanced and metastatic cSCCs.

The mutational background of protein-coding genes in cSCC is generally well described, but the role of non-coding RNAs (ncRNA) is largely unknown. In addition to the gene regulation by microRNAs (miRNAs) there are several other types of functional ncRNAs most likely to be involved in cSCC progression. Long non-coding RNAs (lncRNAs) have emerged as important regulatory molecules in normal cells, for instance in development and immune response, and there is increasing evidence for the role of lncRNAs in human diseases. LncRNAs can contribute to the pathogenesis of cancers by mediating the expression of tumor-promoting or -suppressing genes, and the extracellulary delivered lncRNAs can affect gene expression of cells in the surrounding microenvironment.

The strictly regulated temporal and tissue-specific expression of lncRNAs is a major advantage when designing new targeted cancer therapies. In addition, differentially expressed lncRNAs can serve as specific prognostic and diagnostic molecular markers. Here, the role of lncRNAs in cSCC was studied in culture and *in vivo*. These results provide new evidence for two previously uncharacterized lncRNAs, PICSAR and PRECSIT, which are specifically upregulated in cSCC and contribute to cSCC progression by distinct functions.

2 Review of Literature

2.1 Cutaneous squamous cell carcinoma

2.1.1 Overview

Cancer arises from genetic alterations in the genome allowing transformation of normal human cells into malignant (reviewed in Hanahan & Weinberg, 2000). It is well known that a long-term sun ultraviolet (UV) exposure predisposes to accumulation of epidermal DNA-damage and eventually to development of skin cancer (reviewed in Narayanan et al, 2010; Matsumura & Ananthaswamy, 2004). Nevertheless, skin cancers are the most common cancer types globally with increasing incidence, especially among Caucasian population (Lomas et al, 2012; reviewed in Leiter et al, 2014).

Cutaneous squamous cell carcinoma (cSCC) is a non-melanoma skin cancer (NMSC) and the second most common malignancy after basal cell carcinoma (BCC) (Lomas et al, 2012). It is one of the most common cancers globally with a varying annual incidence rate between 13 and 77 cases per 100 000 persons in Europe (Callens et al, 2016; Rubió-Casadevall et al, 2016; Eisemann et al, 2014; Korhonen et al, 2019; Venables et al, 2019) and up to 270 cases per 100 000 persons in Australia (Pandeya et al, 2017; Keim et al, 2015). Collectively, the incidence of cSCC is continuously increasing worldwide (Rogers et al, 2015; Robsahm et al, 2015; Umezono et al, 2019; Birch-Johansen et al, 2010; Lomas et al, 2012). To distinguish cSCCs and BCCs from other less common NMSCs they are often referred to as "keratinocyte carcinomas" as they both originate from keratinocytes (reviewed in Nehal & Bichakjian, 2018). However, they are two distinct types of skin cancers and a careful diagnosis is required to distinguish the low and high risk tumors and to choose the right clinical management (reviewed in Nehal & Bichakjian, 2018). To emphasize their different pathologies, cSCCs have a risk to metastasize and there is a poor prognosis for metastatic and recurrent cSCCs whereas BCCs rarely metastasize (reviewed in Verkouteren et al, 2017; Burton et al, 2016). Even though the mortality rate of cSCC is relatively low, approximately 3 % (Schmults et al, 2013; Czarnecki, 2017; Robsahm et al, 2015), the overall high incidence of cSCC and other

NMSCs poses not only a financial burden to the health care but it has a major impact on the patients' quality of life (reviewed in Gaulin et al, 2015).

2.1.2 The pathogenesis of cSCC

The major risk factor for cSCC is the cumulative exposure to sun UV-light (reviewed in Green & Olsen, 2017). Additionally, indoor tanning is associated with increased risk for cSCC, especially at young age (Wehner et al, 2012). Other risk factors include immunosuppression, chronic non-healing wounds, tobacco smoking, human papillomavirus infection and certain chemical exposures such as pesticides (reviewed in Green & Olsen, 2017). There are also rare hereditary disorders, such as xeroderma pigmentosum and epidermolysis bullosa, which are associated with a risk for cSCC development due to mutations in key signaling factors or genes essential for functional epidermal structure (reviewed in Green & Olsen, 2017).

The development of cSCC is a multistep process that involves accumulation of several molecular and cellular changes before resulting in a visible skin cancer (reviewed in Seebode et al, 2016). Actinic keratoses (AKs) indicate an early sign of cSCC development. They are epidermal keratinocytic dysplasia, usually seen as thickened or scarly skin patches resulting from chronic sun exposure (reviewed in Cockerell, 2000). A more progressed form of skin carcinogenesis is carcinoma *in situ* where atypical keratinocytes extend throughout the whole epidermis (reviewed in Cockerell, 2000). If these lesions are not treated they have a risk to progress into invasive cSCC (Schmults et al, 2013; reviewed in Ratushny et al, 2012).

2.1.2.1 Harmful effects of the sun exposure

The solar UV-radiation is divided into three subgroups based on their wave lengths; UVA (320-400 nm), UVB (290-320 nm) and UVC (100-280 nm), of which UVA is the predominant form that reaches the earth, and it penetrates to the dermal layer of skin (**Figure 1**) (reviewed in Matsumura & Ananthaswamy, 2004). UVA is less carcinogenic than UVB and UVC as it is weakly absorbed by DNA, but it induces indirect DNA-damage by oxidative stress and promotes skin aging and wrinkling (reviewed in Yaar & Gilchrest, 2007). UVC is strongly mutagenic having the highest energy but it does not penetrate the ozone layer, thus the harmful biological effects of the UV-radiation are mainly caused by the UVA and UVB (reviewed in Matsumura & Ananthaswamy, 2004; Valejo Coelho et al, 2016). UVB, even though constituting a minor part of the ambient UV-radiation, is absorbed in the skin epidermis (**Figure 1**) and it is responsible in inducing DNA-damage, oxidative stress and immunosuppression (reviewed in Matsumura & Ananthaswamy, 2004; Valejo Coelho et al, 2016). In contrast to UVA, UVB is directly absorbed by DNA and it

interferes nucleotide base pairing. This results in formation of DNA photoproducts, such as cyclobutane dimers between thymine (T) or cytosine (C) residues, or pyrimidine-pyrimidone (6–4) photoproducts (reviewed in Pfeifer et al, 2005). These lesions can lead to DNA-mutations, also known as "UV-signature mutations" characteristic of C→T transitions and CC→TT mutations, if the DNA repair system fails to correct them (Brash, 2015; reviewed in Matsumura & Ananthaswamy, 2004). This is why the UVB exposure is particularly efficient in promoting skin carcinogenesis. In addition to UV-radiation, a considerable part of the energy that is emitted by the sun is infrared (IR) radiation (760 nm – 1 mm) (**Figure 1**). It is capable of penetrating to the epidermis, dermis and subcutaneous tissue (reviewed in Krutmann et al, 2012). Primarily, it causes a temperature increase in the skin. However, there is increasing evidence that a long-term IR exposure contributes to premature skin aging in addition to UVA (reviewed in Krutmann et al, 2012).

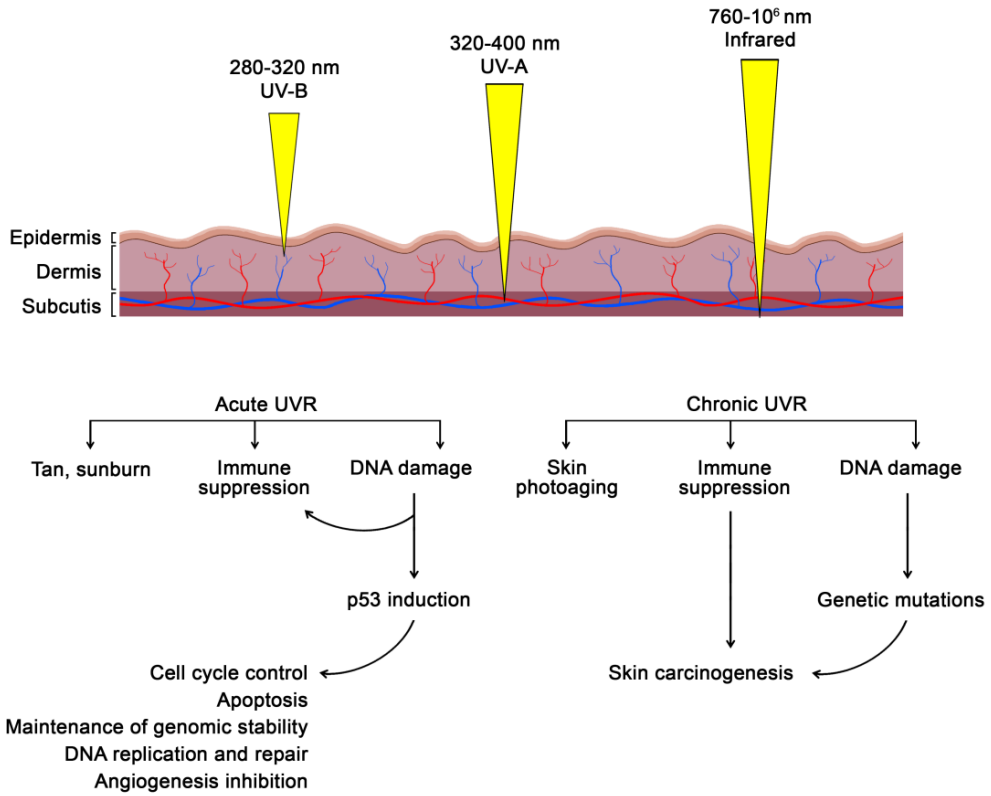

Figure 1. A schematic view of the skin structure and implications of acute and chronic ultraviolet radiation. Adapted from Matsumura & Ananthaswamy, 2004.

2.1.2.2 Molecular changes in cSCC pathogenesis

cSCC is a cancer with a very high mutational burden. Prevalent C→T transitions have been detected in cSCC tumors, which presents a typical mutational signature caused by UVB-irradiation (Pickering et al, 2014; Inman et al, 2018; South et al, 2014; Cho et al, 2018; Li et al, 2015; Mueller et al, 2019). An average mutational frequency of more than 50 mutations per mega base pair of DNA is higher than in any other common tumor types, for instance melanoma, lung or colorectal cancer (Pickering et al, 2014; South et al, 2014). In a genomic point of view cSCC is a very complex disease with a high level of heterogeneity (Inman et al, 2018; South et al, 2014; Li et al, 2015), which makes it a challenging target for cancer therapy.

2.1.2.2.1 Inactivated tumor suppressor genes in cSCC

The consequences of acute UVR (**Figure 1**) include tan or sunburn, immune suppression and DNA-damage (reviewed in Matsumura & Ananthaswamy, 2004). DNA-damage leads to the activation of the **p53** protein which is one of the most central factors in maintaining genomic stability and controlling cellular responses, such as cell proliferation and apoptosis (reviewed in Levine, 1997). Unfortunately, p53 is a common target for genetic alterations in many cancers, especially in UV-induced skin cancers and cSCC (Pickering et al, 2014; Inman et al, 2018; South et al, 2014; Cho et al, 2018; Li et al, 2015). This is the case particularly with metastatic cSCCs with nearly 95 % of samples detected with genetic alterations in the *TP53* gene (Pickering et al, 2014; Li et al, 2015) in comparison to primary cSCCs with a mutational frequency closer to 50-60 % (South et al, 2014; Yilmaz et al, 2017). Moreover, p53 mutations are frequently found already in actinic keratosis (Campbell et al, 1993; Ziegler et al, 1994; Taguchi et al, 1994), highlighting the carcinogenic role of chronic UVR (**Figure 1**) in cancer initiation. Loss of p53 function allows cells to bypass apoptosis resulting in clonal expansion of the mutated cells which further increases the mutation frequency (reviewed in Benjamin & Ananthaswamy, 2007; Brash, 2006). Overall, cSCC harbors a very high mutation rate compared to other common cancers, such as breast cancer or melanoma (Pickering et al, 2014; South et al, 2014; Li et al, 2015; Inman et al, 2018). *In vivo* studies have shown that p53 mutations arise very early on in mouse skin after UVB exposure and the growth of the p53-mutant keratinocytes is driven by UVB (Zhang et al, 2001; Melnikova et al, 2005; Kramata et al, 2005). Consistent with this, p53 mutations are also present in sun-exposed normal human skin among many other cancer driver genes (Martincorena et al, 2015).

In addition to p53, **p16(INK4a)** and **p14(ARF)** are important cell cycle regulators and commonly non-functional in cSCC due to inactivating mutations in the *CDKN2A* gene encoding these proteins (Brown et al, 2004; Pickering et al, 2014;

South et al, 2014; Li et al, 2015; Al-Rohil et al, 2016; Inman et al, 2018; Cho et al 2018). *CDKN2A* mutations are found also in actinic keratosis, although less frequently than in cSCC (Mortier et al, 2002; Kanellou et al, 2008). Interestingly, *CDKN2A* is not mutated in the sun-exposed normal skin (Martincorena et al, 2015), indicating that *CDKN2A* inactivation occurs at a later stage in the progression from actinic keratosis to cSCC. Other less common mutational targets in cSCC include *CCDN1* and *MYC* genes, also responsible in cell cycle regulation (Li et al, 2015; Al-Rohil et al, 2016; Toll et al, 2009), however they may play a more specific role in the progression of oral SCCs (Martín-Ezquerra et al, 2010; Akervall et al, 2003). The inactivation of cell cycle regulators, such as p53 and p16(INK4a), gives cells a growth advantage and it predisposes them to genomic instability, however many other mutations are needed to contribute tumorigenesis (**Figure 2**) (reviewed in Dotto & Rustgi, 2016). When mutated, these target genes further promote uncontrolled cell growth and they may have a preferential role in cSCC progression by interfering cell differentiation.

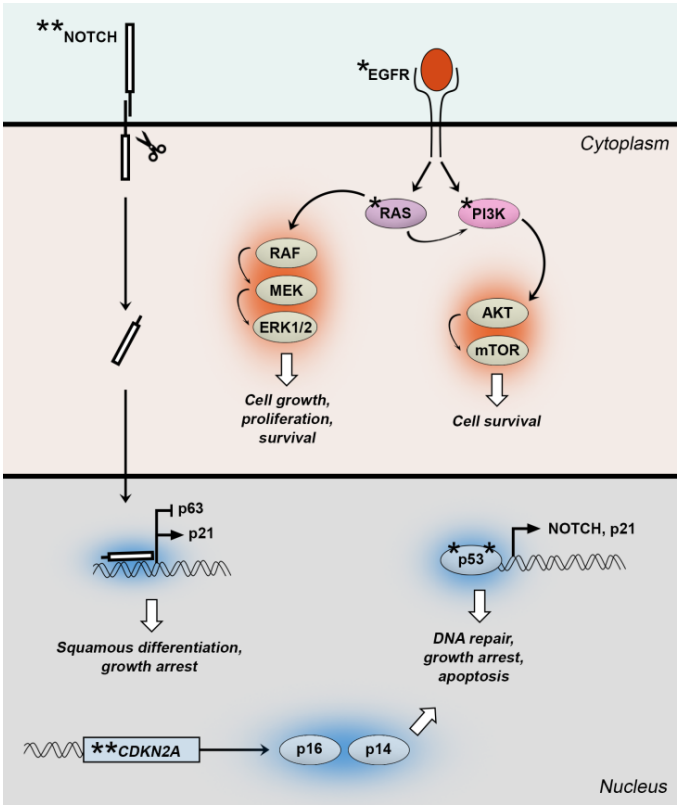

Figure 2. Early genetic alterations and signaling pathways involved in cSCC progression. Activating (*) and inactivating (**) mutations are indicated with one or two asterisks and the consequent activation or inactivation with a red and blue colors, respectively.

Notch signaling is an evolutionary conserved pathway with a key role in regulating cellular development and it plays a pivotal role in controlling normal skin homeostasis and function (reviewed in Nowell & Radtke, 2013). Notch1 promotes keratinocyte differentiation and it is involved in maintaining the functional skin barrier (reviewed in Lefort & Dotto, 2004). Emphasizing its important regulatory role in the skin, loss of Notch1 results in skin tumorigenesis. (**Figure 2**) (reviewed in Nowell & Radtke, 2013). Notch1 has been shown to mediate tumor suppression by reducing p63 expression, leading to inhibition of cell growth and induced differentiation (Nguyen et al, 2006). It can also function *via* p53 to suppress ROCK1/2 and MRCKα kinases or induce tumor suppressor protein p21 expression leading to cell growth inhibition (Lefort et al, 2007; Rangarajan et al, 2001). Additionally, Notch1 can regulate cSCC tumorigenesis by modulating inflammatory response and tumor microenvironment (Demehri et al, 2008 and 2009; Di Piazza et al, 2012). *NOTCH1* and *NOTCH2* are frequently mutated in cSCC, resulting in truncated Notch receptors and abrogated signaling (Wang et al, 2011; Pickering et al, 2014; South et al, 2014; Li et al, 2015; Al- Rohil et al, 2016; Inman et al, 2018; Cho et al, 2018). *NOTCH1* mutations occur early in cSCC progression, which is proposed to be a downstream consequence of mutated p53 as *NOTCH1* is a direct transcriptional target for p53 in keratinocytes (South et al, 2014; Lefort et al, 2007). In addition, *NOTCH1* mutations are considerably common in sun-exposed normal skin, supporting the tumorigenic role for p53 and Notch mutations in early skin carcinogenesis (Martincorena et al, 2015; South et al, 2014).

2.1.2.2.2 Mutationally activated oncogenes in cSCC

HRas is a well-studied member of the Ras GTPase subfamily responsible in controlling a wide range of cellular pathways, such as the mitogen-activated protein kinase (MAPK) signaling, and it acts as a proto-oncogene in many different cancers (reviewed in Pylayeva-Gupta, 2011). Activating mutations in the *HRAS* gene are frequently found in cSCC, in addition to less common *NRAS* and *KRAS* mutations (Pickering et al, 2014; South et al, 2014; Bamford et al, 2004; Li et al, 2015; Al-Rohil et al, 2016; Inman et al, 2018; Cho et al, 2018). The proportion of *HRAS* mutations is particularly high in cSCCs treated with BRAF-inhibitor vemurafenib, which is a commonly used treatment for melanoma (Su et al, 2012; Oberholzer et al, 2012; South et al, 2014). The adverse effect of the drug is caused by a paradoxical MAPK activation and accelerated growth of *HRAS* mutated lesions (Su et al, 2012; reviewed in Wu et al, 2017). Interestingly, the BRAF-inhibitor induced cSCCs with mutated *HRAS* harbor higher number of *NOTCH1* mutations when compared to sporadic cSCCs, implicating Notch1 as an important factor in HRas driven skin carcinogenesis (**Figure 2**) (South et al, 2014). The loss of Notch1 signaling alone is

not sufficient for tumor formation in mice (Lefort et al, 2007). However, when combined with oncogenic Hras expression there is aggressive tumor formation whereas oncogenic Hras expression alone results in only small nodules or no tumors at all (Lefort et al, 2007).

The most commonly used mouse model for cSCC is generated by topically applying a highly carcinogenic agent 9,10-dimethyl-1,2-benzanthracene (DMBA) to mouse skin (Abel et al, 2009). During the first stage of skin carcinogenesis DMBA causes genetic alterations of which the *Hras* gene is the primary target (Abel et al, 2009; Nassar et al, 2015). Second, a treatment with a tumorigenic agent, such as tetradecanoyl-phorbol acetate (TPA) enables the clonal expansion of the cell population carrying Hras mutation, resulting in sustained epidermal hyperplasia and skin tumors (Abel et al, 2009). Overall, the histology and the genomic background of the mouse DMBA/TPA-induced cSCCs are very similar to human cSCCs (Nassar et al, 2015). Sustained activation of Hras results in a marked induction of epidermal growth factor receptor (EGFR) and its ligands in cSCC mouse model (Casanova et al, 2002; Dlugosz et al, 1995). EGFR is also a target for mutational activation in human cSCC (Li et al, 2015; Al-Rohil et al, 2016). EGFR plays an important role in normal epidermal homeostasis, but sustained EGFR activity can further induce Ras signaling and uncontrolled cell growth and survival *via* MAPK and phosphoinositide 3-kinase (PI3K) signaling downstream of Ras (**Figure 2**) (reviewed in Doma et al, 2013).

Another important signaling factor downstream of EGFR is the signal transducer and activator of transcription 3 (STAT3). STAT3 plays an important role in normal epithelia in skin wound healing promoting keratinocyte migration (Sano et al, 1999) but aberrant STAT3 activity contributes to skin carcinogenesis (Chan et al, 2008; Kataoka et al, 2008; reviewed in Macias et al, 2013; Sano et al, 2008). STAT3 activation is also strongly associated with inflammation and development of psoriasis (reviewed in Calautti et al, 2018). Activating mutations in the *STAT3* gene are not found in cSCC but its tumorigenic function is strongly dependent on activated growth factor signaling, for example *via* EGFR (reviewed in Macias et al, 2013; Sano et al, 2008). EGFR-mediated STAT3 activation promotes tumorigenesis in DMBA/TPA-induced mouse cSCCs (Chan et al, 2004). Expression of constitutively active Stat3 in mouse skin drives rapid cSCC progression and these tumors are highly vascularized and poorly differentiated (Chan et al, 2008). By contrast, Stat3-deficiency drives keratinocytes to apoptosis after DMBA-treatment and tumor formation is completely prevented in Stat3-deficient mice after DMBA/TPA-treatment (Chan et al, 2004). Stat3 is rapidly deactivated and downregulated in keratinocytes upon UVB exposure, however repeated UVB exposure results in constitutively active Stat3 in mouse cSCCs (Sano et al, 2005). In consistent with this, expression of constitutively active Stat3 in mouse epidermis

results in epidermal hyperproliferation after UVB exposure, whereas keratinocytes of Stat3 deficient mice are highly sensitive to apoptosis after UVB exposure (Kim et al, 2009). The levels of Stat3 target genes, such as cell cycle regulator cyclin D1 and anti-apoptotic Bcl-xL correlate with Stat3 expression in both mouse models, indicating Stat3 as an important regulator in UVB-induced skin carcinogenesis (Kim et al, 2009).

Receptor tyrosine kinases (RTKs) are cell surface receptors important in a wide range of cellular processes, and RTK gene amplifications and activating mutations are commonly found in cancer (reviewed in Schlessinger, 2000). The majority of activating mutations in cSCC are associated with Ras, RTK and PI3K pathway genes (Li et al, 2015). Most frequently mutated or amplified RTK genes in cSCC include *EGFR*, *FGFR3*, *KIT* and *ERBB4* (Li et al, 2015; Al-Rohil et al, 2016). *ERBB4* and *FGFR3* belong to the same RTK family with *EGFR*. The role of *ERBB4* mutations in non-melanoma skin cancer is not well known but they have been implicated in cutaneous melanoma (Lau et al, 2014; Manca et al, 2013). Nevertheless, it remains controversial whether they act as driver or passenger mutations in melanoma. Activating *FGFR3* mutations are common in seborrheic keratoses (SK), which are benign epidermal hyperplastic papillomas, but the mutationally activated FGFR3 is not sufficient for the development of cSCC, in accordance with a low rate of *FGFR3* mutations in cSCC (Hafner et al, 2010; Duperret et al, 2014).

Overall, skin carcinogenesis is a complex process involving several genetic alterations before cells acquire an invasive phenotype. Based on several genomic analyses of cSCC tumors and cell lines the most profound mutations in driving cSCC tumorigenesis are characterized, such as p53, HRas and Notch1 (**Figure 2**). In addition to these other mutated oncogenes have been identified in cSCC, such as *PIK3CA*, *MTOR* and *KIT,* and mutated tumor suppressors genes *FAT1* and *KMT2C* (Li et al, 2015; Pickering et al, 2014; Al-Rohil et al, 2016; Inman et al, 2018). The question remains, what is the order and combination of these alterations in cancer initiation and progression, as some of them are found already in normal sun-exposed skin without any sign of malignancy. It seems that the number of driver mutations per cell is a major contributor to cancer progression, as these cells are clonally expanding and increasing the genomic instability and mutational burden (Martincorena et al, 2015).

2.1.2.2.3 Epigenetic changes in cSCC

Genomic DNA-alterations are not solely driving carcinogenesis but epigenetic deregulation is a well-recognized phenomenon in cancer, allowing cells to activate or repress gene transcription by chromatin modifications (reviewed in Dawson, 2017). Many cancer types exhibit hypermethylation of gene promoters, which leads

to transcriptional gene repression (reviewed in Clark & Melki, 2002). In cSCC certain tumor-suppressors, such as p16(INK4a), p14(ARF), the inflammasome adaptor ASC and E-cadherin have been detected with hypermethylated gene promoter and gene inactivation (Brown et al, 2004; Murao et al, 2006; Meier et al, 2016; Chiles et al, 2003). Chronic UVA-irradiation has been also shown to induce hypermethylation of the P16(INK4a) gene promoter in HaCaT keratinocytes (Chen et al, 2012). In accordance, UVB- and DMBA/TPA-induced mouse skin tumors exhibit differential epigenetic regulation and some of these changes reflect the aberrant DNA-methylation of human cSCCs (Yang et al, 2014, 2019; Fraga et al, 2004). When cSCC and AK methylation profiles have been examined it is interesting that they show a similar, aberrant methylation pattern with cancer-specific features when compared to healthy epidermis (Rodríguez-Paredes et al, 2018), indicating UV-induced epigenetic deregulation during skin carcinogenesis. In addition, two distinct keratin methylation profiles could be detected in AK and cSCC lesions, suggesting two subclasses of AK and cSCC originating from different keratinocyte differentiation stages (Rodríguez-Paredes et al, 2018).

In contrast to hypermethylation a global decrease in DNA-methylation, or hypomethylation, has been also observed in cancer (reviewed in Ehrlich, 2009). In accordance, hypomethylation is associated with aging and sun exposure of the epidermis (Vandiver et al, 2015). Moreover, the same hypomethylated areas in the non-malignant sun-exposed lesions were shown to be hypomethylated in cSCCs (Vandiver et al, 2015), highlighting the carcinogenic effect of UV-irradiation in skin carcinogenesis.

2.1.2.2.4 Telomerase activity in cSCC

As described in the previous examples, the high burden of UV-induced mutations in cSCCs and sun-exposed skin contribute to deregulation of many important signaling proteins, eventually resulting in skin cancer (**Figure 2**). It is important to note that mutations in the gene regulatory areas, such as gene promoters and enhancers can also have adverse consequences regarding tumorigenesis. Recently, activating mutations in the *TERT* gene promoter have been reported in several cancer types and they seem to be particularly prevalent in cutaneous melanoma, BCC and cSCC (Pópulo et al, 2014; Griewank et al, 2013; Scott et al, 2014). These mutations lead to induced expression of the gene encoding telomerase reverse transcriptase, a catalytically active subunit of telomerase (Sauerwald et al, 2013). Telomerase has a key role in the normal physiological state in protecting the telomeric DNA in the chromosome ends and in preventing the replicative senescence of proliferating cells. However, reactivation of telomerase activity allows telomere elongation and unlimited cell replication, one of the hallmarks of cancer (reviewed in Hanahan & Weinberg, 2000).

Paradoxically, progressive telomerase shortening due to impaired telomerase activity can lead to chromosome instability as the unprotected telomere ends can form chromosome fusions (reviewed in Hanahan & Weinberg, 2000). This would normally lead to cell cycle arrest, however if the cell escapes the cellular checkpoint because of a defective p53 the genomic instability is passed to the next progeny (reviewed in Artandi & DePinho, 2010). This may be an important driver of epithelial carcinogenesis, as p53 is one of the first targets of mutational inactivation in skin cancer. Indeed, two distinct telomere phenotypes are characterized in cSCCs; short/intermediate telomeres with a conserved size in all tumor areas and intermediate/long telomeres with increased variation in size within different tumor areas (Leufke et al, 2014). Similar subgroups are present in immunosuppression induced cSCCs, suggesting two different mechanisms for cancer initiation (Leufke et al, 2014). Interestingly, the short/intermediate telomeres correlate with low p53 expression, *vice versa* high p53 expression is more often detected in the cSCCs with intermediate/long telomeres. Additionally, the number of aberrant cells and telomeric aggregates, a sign of telomeric fusions, is increased with tumor progression from actinic keratosis to cSCC (Leufke et al, 2014). In another study telomere length was shown to be shorter in cSCC compared to actinic keratosis and carcinoma *in situ*, indicating a correlation in telomere shortening and cSCC progression (Yamada-Hishida et al, 2018).

2.1.2.2.5 Molecular changes driving cSCC invasion

In order for epithelial cells to gain a motile phenotype they must undergo a process called epithelial-mesenchymal-transition (EMT), a common strategy for cancer cells to become invasive (reviewed in Skrypek et al, 2017). A subpopulation of migratory cancer cells that have undergone EMT can be found in patient derived cSCC cell lines and the proportion of these cells correlate with tumor progression (Biddle et al, 2011). Additionally, based on *in vivo* studies the cancer cell of origin affects the ability of the tumor cells to undergo EMT. The cSCC tumors originating from mouse hair follicles are more prone to EMT and metastasis when compared to tumors originating from the interfollicular epidermis (Latil et al, 2017). Invasive human cSCC tumors often exhibit low expression of cell-cell adhesion markers, such as E-cadherin, and induced expression of vimentin and podoplanin as a sign of EMT (Barrette et al, 2014; Toll et al, 2013; Hesse et al, 2016).

When comparing actinic keratosis or carcinoma *in situ* with invasive cSCCs, it is evident that expression of matrix metalloproteinases (MMPs) is one key step in order cells to become invasive (Inman et al, 2018; Ra et al, 2011; Lambert et al, 2014; Mitsui et al, 2014; García-Díez et al, 2019; reviewed in Nissinen & Kähäri, 2014). Reorganization of the extracellular matrix is needed so that the cells are able to invade to the surrounding tissue. Together with MMP activation loss of basement

membrane collagen is associated with cSCC progression (reviewed in Kerkelä & Saarialho-Kere, 2003). The tumor microenvironment plays an important role in cSCC progression and MMPs are capable of regulating activity of several chemokines, cytokines and growth factors to promote inflammation and cancer progression (reviewed in Nissinen & Kähäri, 2014). On the other hand, immunosuppression is a major risk factor for cSCC development and MMPs can regulate inflammation in a way that it helps the cancer cells escape the immune surveillance (reviewed in Nissinen & Kähäri, 2014).

2.1.3 The diagnosis, treatment and prognosis of cSCC

In addition to a clinical examination a skin biopsy is required for cSCC diagnosis. Based on several parameters, such as the tumor size, location and histologic subtype tumors can be classified into cSCCs with high or low metastatic potential (reviewed in Nehal & Bichakjian, 2018; Kallini et al, 2015; Burton et al, 2016). For example, poorly differentiated cSCCs with perineural invasion and more than 2 mm thickness possess a high risk for metastasis (reviewed in Kallini et al, 2015; Burton et al, 2016). In general, patients with a primary, low-risk cSCC that can be surgically removed have a good prognosis. However, patients with distant metastases are associated with poor outcome and high mortality (reviewed in Burton et al, 2016). Special attention should be given to immunosuppressed patients in order to prevent cSCC development (reviewed in Burton et al, 2016). Radiation and chemotherapy can be used for advanced and recurrent high-risk tumors that cannot be completely removed surgically, especially for tumors located in the facial area (reviewed in Kallini et al, 2015; Que et al, 2018). Other targeted therapies, such as EGFR and mTOR inhibitors may be beneficial in cSCC treatment, however there is always a risk for cancer cells acquiring drug resistance (reviewed in Harwood et al, 2016). Recently, PD-1 targeting immunotherapy has been shown to be a promising treatment for patients with metastatic or locally advanced cSCCs (Migden et al, 2018).

There is an urgent need for clinically useful prognostic biomarkers for predicting the risk of recurrence and metastatic potential of cSCC from early stage. Several promising cSCC biomarkers have been discovered, such as certain matrix metalloproteinases (reviewed in Kerkelä & Saarialho-Kere, 2003), cell surface tyrosine kinase receptor EphB2 (Farshchian et al, 2015), inflammation related SerpinA1 (Farshchian et al, 2011), AIM2 (Farshchian et al, 2017a) and several complement system proteins (Riihilä et al, 2014; 2015; 2017). Additionally, ATF3 transcription factor (Dziunycz et al, 2014), the IKK kinase complex (Toll et al, 2015), epigenetic regulators p300, PCAF and EZH2 (Bosic et al, 2016; Hernández-Ruiz et al, 2018), and certain miRNAs (reviewed in Yu & Li, 2016) could serve as molecular biomarkers and important therapeutic targets for cSCC.

2.2 Non-coding RNAs

2.2.1 The short history of non-coding RNAs

DNA-transcription is a process where genetic information from genomic DNA is transcribed to RNA. This is carried out mainly by RNA polymerase II (RNA Pol II) that enzymatically converts DNA to messenger RNAs (mRNAs), which are then translated into proteins (reviewed in Hurwitz, 2005). Not all DNA is encoded into proteins, but a significant part is transcribed into so called non-coding RNAs (ncRNAs) (reviewed in Djebali et al, 2012; Jacquier, 2009). The transfer RNA (tRNA) and ribosomal RNA (rRNA) were discovered in the 1950s providing the first evidence of the functional importance of ncRNAs (Hoagland et al, 1958; Palade, 1955). Later on in the 1980s the first RNA enzymes, "ribozymes", were discovered (reviewed in Reymond et al, 2009). RNA polymerase I (RNA Pol I) is mainly responsible for rRNA synthesis and RNA polymerase III (RNA Pol III) for the synthesis of tRNA and 5S rRNA component of the ribosome, which are all necessary for protein translation (Roeder & Rutter 1969). Therefore, there would not be any protein without RNA. In addition to them, many other RNAs are involved in the complex regulation of protein synthesis, including small nuclear RNAs (snRNAs) (Reddy & Busch, 1983) and telomerase RNA (reviewed in Wang & Feigon, 2017).

With the help of an international consortium and an enormous amount of time and money invested in the Human Genome Project (HGP) in the 1990s, followed by new projects such as the 1000 Genomes Project (1KGP), the Encyclopedia of DNA Elements project (ENCODE) and the Encyclopædia of genes and gene variants project (GENCODE), we have broadened our knowledge of the human genomic landscape (Harrow et al, 2012; reviewed in Moraes & Góes, 2016). Based on the current statistics from the GENCODE project the number of genes in the human genome is around 60 000 of which protein coding genes make up almost one third, 19 881 genes (GENCODE version 21). This is quite close to what some of the scientists estimated in the early 2000 before completing the HGP despite the most extreme guesses (Pennisi, 2000). What was not known at the time that the amount of non-protein coding genes would be almost twice as much as protein coding genes (ENCODE Project Consortium, 2012). However, the exact number of protein and non-protein coding transcripts is difficult to determine because of complex expression patterns and variability between tissue types and individuals, and regulation of transcription (reviewed in Pertea, 2012). The current GENCODE estimation for the total number of gene transcripts is nearly 200 000 (GENCODE version 21), but as well as the total number of genes, this is dependent of many factors and will most likely change over time.

2.2.2 Biogenesis and classification of ncRNAs

The biogenesis and molecular functions of ncRNAs differ greatly from each other. They are roughly classified into infrastructural and regulatory ncRNAs (reviewed in Mattick & Makunin, 2006) (**Figure 3**). The infrastructural ncRNAs are so-called housekeeping genes with well-defined cellular functions. They are RNAs mainly involved in protein synthesis including tRNAs, rRNA, telomerase RNA, small nucleolar RNAs (snoRNAs) with small Cajal-body specific RNAs (scaRNAs) and small nuclear RNAs (snRNAs) or commonly referred to as spliceosomal uRNAs. In addition, signal recognition particle RNAs (SRP RNAs) and vault RNAs (vRNAs or vtRNAs) are also considered as infrastructural ncRNAs (reviewed in Mattick & Makunin, 2006). The human SRP RNA, also known as 7SL RNA, is the RNA-component of the signal recognition particle ribonucleoprotein complex and it has an important role in signal peptide binding during protein translocation to the cell membrane (Walter & Blobel, 1982). It has a similar role also in archaea, bacteria and plants (reviewed in Rosenblad et al, 2009). Likewise, the vRNAs are evolutionary conserved and bound to proteins, forming large ribonucleoprotein particles in cells (Kedersha & Rome, 1986) however their exact cellular function still remains unclear. They have been suggested to have a role in intracellular trafficking (reviewed in van Zon et al, 2003) and more recently in immune response (Li et al, 2015), drug resistance (Gopinath et al, 2010; Persson et al, 2009) and apoptosis (Amort et al, 2015). Similar to vRNAs some infrastructural RNAs have additional functional roles beside protein synthesis (reviewed in Hu et al, 2012). For instance, tRNA-derived small non-coding RNAs (tsncRNAs) are newly defined small functional ncRNAs generated from tRNAs by specific endonuclease cleavage and their dysregulation has been detected in cancer and other human diseases (reviewed in Zhu et al, 2018).

Regulatory ncRNAs function mostly by base-pairing with DNA or RNA (reviewed in Quinn & Chang, 2016; Guil & Esteller, 2015), or by interacting directly with proteins (reviewed in Hogg & Collins, 2008). There is also cross-talk between different ncRNAs (reviewed in Yamamura et al, 2018). Conventionally they are classified into small or long non-coding RNAs (lncRNAs) based on their length (**Figure 3**). LncRNAs are considered as ncRNAs larger than 200 nucleotides in size, and everything smaller than that are termed small or short ncRNAs (Kapranov et al, 2007). Some pseudogenes can be also transcribed into lncRNAs (reviewed in Grandér & Johnsson, 2016). This kind of division, however, is not absolute as for instance some lncRNAs are less than 200 nucleotides in size. Also, some lncRNAs can function both as regulatory lncRNAs and they can be processed to yield small ncRNAs (reviewed in Ma et al, 2013). There is also some overlapping between these subgroups as some ncRNAs may have functional duality, such as snRNAs and snoRNAs which have additional functional roles aside their well-known role as infrastuctural RNAs (Li et al, 2012; Chen & Heard, 2013; reviewed in Dupuis-

Sandoval et al, 2015). In **Figure 3** they are included in both groups, as infrastructural and small regulatory ncRNAs.

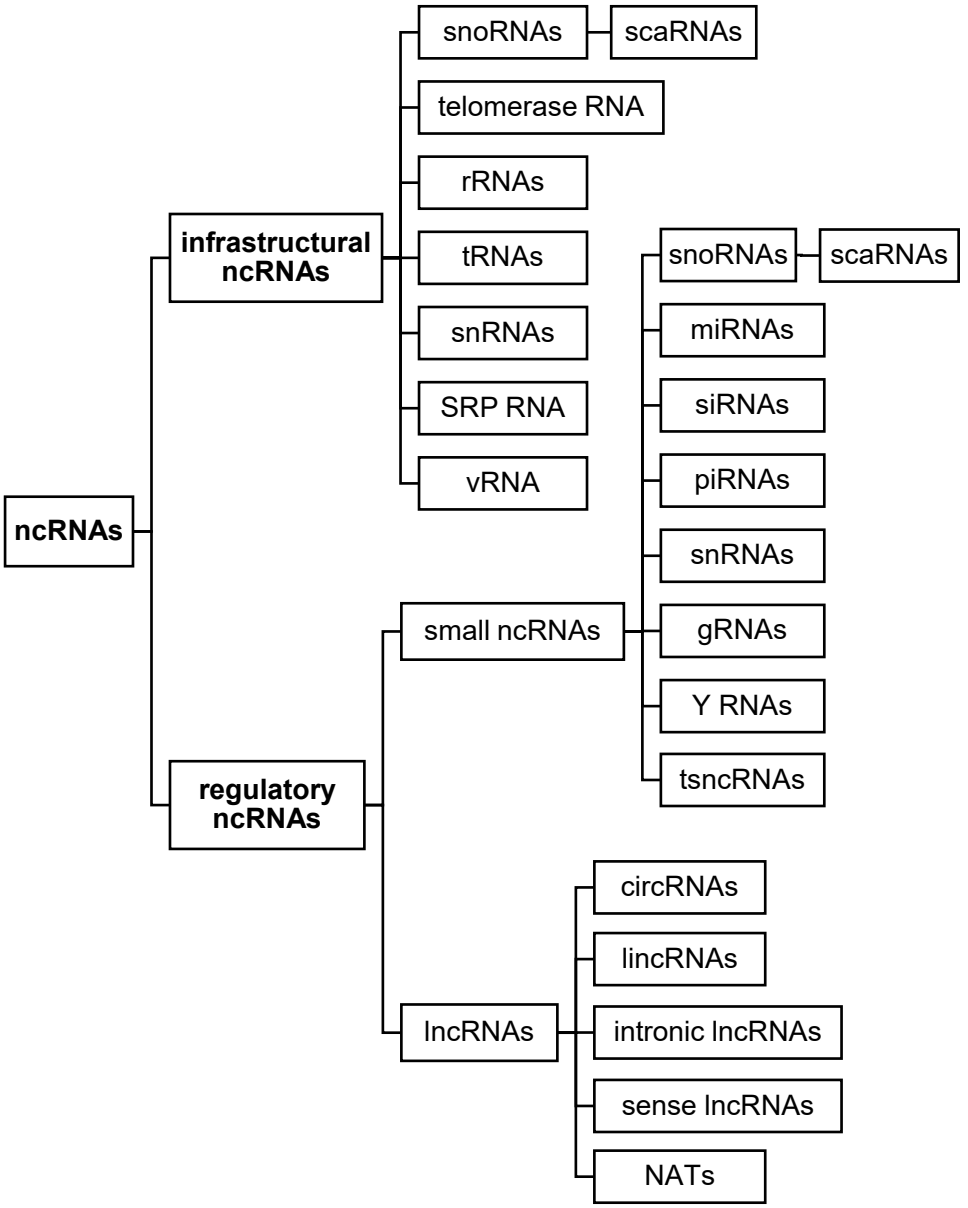

Figure 3. Classification of ncRNAs based on their structure and function.

2.2.3 MicroRNAs and other small ncRNAs

Small ncRNAs are typically divided into three subgroups based on their structure and function, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs) (**Figure 3**). They all share a role in gene silencing. However, their biogenesis and mechanism of action differ from each other. In addition to them, there are also other small non-coding RNAs, such as guide RNAs (gRNAs or sgRNAs) and Y RNAs (**Figure 3**). The *Tryptanosoma* parasite has a unique regulatory system for mitochondrial mRNA editing that involves gRNAs (Seiwert & Stuart, 1994). They are not expressed in humans, but the CRISPR-Cas9 genome editing technology utilizes artificially made gRNAs in directing the nuclease activity to the target loci (reviewed in Nowak et al, 2016). Y RNAs are evolutionary conserved (Wolin et al, 2013) and originally discovered in patients with *lupus erythematosus* complexed with the Ro60 auto-antigen (Lerner et al, 1981). They regulate the subcellular localization of Ro60 but there are also several reports supporting their role in the initiation of DNA replication (reviewed in Kowalski & Krude, 2015).

miRNA genes are transcribed into precursor miRNAs (pri-miRNAs) mainly by RNA Polymerase II (RNA Pol II), but a small subset of miRNAs can be transcribed also by RNA Polymerase III (Lee et al, 2004; Borchert et al, 2006). The pri-miRNAs are further processed in the nucleus by an RNase III nuclease, Drosha-DGCR8 complex into hairpin-shaped pre-miRNAs, which are then exported to the cytoplasm (Han et al, 2004). Finally, the pre-miRNAs are cleaved by another RNase III nuclease, Dicer-TRBD complex and the mature miRNA is incorporated with Dicer-TRBD and Argonaute proteins to form the functional gene silencing complex RISC (RNA-induced gene silencing complex) (Wang et al, 2009). The RISC complex is guided by a miRNA to the specific gene transcript based on sequence complementarity and it inhibits protein translation with or without mRNA degradation (reviewed in Stroynowska-Czerwinska et al, 2014). The miRNA genes are evolutionary conserved, although the animal and plant miRNAs differ in their sequences and processing (reviewed in Millar & Waterhouse, 2005). The mammalian miRNA genes are often clustered and located in the intronic or exonic areas within a protein or non-coding gene, or in rare cases miRNA genes can reside next to their own gene promoter (reviewed in Olena & Patton, 2010).

Endogenous siRNAs are derived from long double-stranded RNAs (dsRNAs) and similarly to miRNAs they are processed in the cytoplasm by the Dicer-TRBD nuclease complex prior to forming the RISC complex (Hammond et al, 2000). In plants siRNAs present a natural defense mechanism against viral mRNAs (Hamilton & Baulcombe, 1999). Studies with *Drosophila* have shown that the endogenous siRNA pathway regulates for instance heterochromatin formation (Fagegaltier et al, 2009) and embryo development (Lucchetta et al, 2009). In mouse oocytes, they have

been shown to regulate meiosis (Stein et al, 2015). The biological role of endogenous siRNAs in humans is not well understood, nevertheless the characterization of the siRNA pathway has led to the development of artificially made siRNAs that are widely used in gene silencing studies *in vitro* (reviewed in Mocellin & Provenzano, 2004). Unlike miRNAs and siRNAs, piRNAs are derived from single-stranded RNA (ssRNA) and they are processed by a mechanism independent of RNAse III enzymes (reviewed in Le Thomas et al, 2014). The piRNA genes are clustered and they arise mainly from DNA regions with repetitive elements, such as retrotransposons (Brennecke et al, 2007). In *Drosophila* they are important mediators of transposable element silencing, a mechanism to protect genome integrity (reviewed in Aravin et al, 2007). In mammals, they are specifically expressed in germ-cells during spermatogenesis (Girard et al, 2006; Pantano et al, 2015), nevertheless their role in somatic cells is not yet well understood.

2.2.4 Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are mainly transcribed by RNA Pol II and they undergo post-transcriptional processing, such as 5'-capping, splicing and polyadenylation (reviewed in Quinn & Chang, 2016). In this way they closely resemble mRNAs with the exception that they are not translated into proteins. As mentioned earlier, to distinguish them from small ncRNAs they are considered as non-coding RNAs larger than 200 nucleotides. Further classification into distinct lncRNA subgroups is commonly based on their structure or genomic location (**Figure 3**). Circular RNAs (circRNA) present a newly discovered group of lncRNAs that are structurally different from most lncRNAs. They are produced by back-splicing of pre-mRNAs, resulting in covalently closed circular RNAs without polyadenylation (reviewed in Barrett & Salzman, 2016). Like many other lncRNAs they show very dynamic expression during differentiation. Despite most lncRNAs, expression of circRNAs seems to be conserved among mammals (reviewed in Barrett & Salzman, 2016). Linear lncRNAs can be categorized into four major types based on their genomic location (**Figure 4**). Long intergenic or intervening non-coding RNAs (lincRNAs) are transcribed from distinct loci, often from their own promoters, whereas intronic lncRNAs are transcribed from intronic region within a protein-coding gene (reviewed in Ma et al, 2013). Sense lncRNAs are transcribed from the sense strand of a protein-coding gene and they contain also exons from protein-coding genes (reviewed in Ma et al, 2013) (**Figure 4**). Natural antisense transcripts (NATs) are transcribed from the antisense strand of a protein-coding gene, overlapping either exonic or intronic regions (reviewed in Ma et al, 2013) (**Figure 4**). LncRNAs can be also transcribed from a bidirectional promoter, meaning that they are produced divergently from a protein-coding gene (**Figure 4**).

There is increasing evidence that divergent transcription is driving generation of new genes, and this could be an important source for emerging lncRNAs (reviewed in Kapusta & Feschotte, 2014). Moreover, divergent lncRNAs can have regulatory functions. Divergent transcription is relatively common for example in human and murine embryonic stem cells (Sigova et al, 2013). Moreover, expression of these lncRNA/mRNA pairs is coordinated during differentiation (Sigova et al, 2013), suggesting an important regulatory function for divergently expressed lncRNAs in embryonic development. There are also few examples of bidirectionally expressed lncRNAs functional in cancer (reviewed in Albrecht & Ørom, 2016).

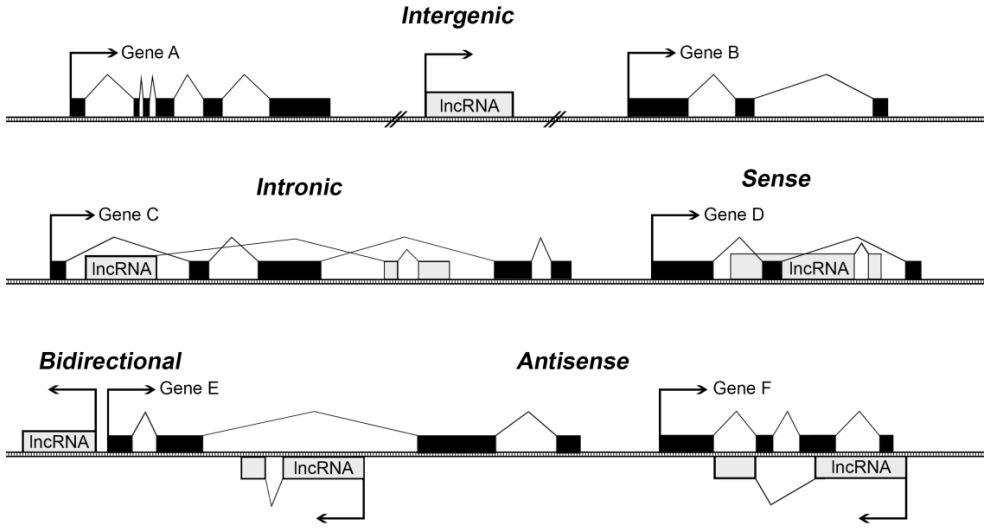

Figure 4. Classification of lncRNAs based on their genomic context.

As the majority of the human genome is transcribed yielding thousands of lncRNA transcripts, it has also drawn a lot of speculation whether a part of the transcription is only "transcriptional noise" producing merely useless transcripts as a by-product of the process. However, the proportion of non-protein coding sequences in the genome grows as a function of developmental complexity, whereas the proportion of protein-coding sequences declines, indicating that the non-coding sequences have evolved for a reason (reviewed in Mattick, 2004). Supporting this hypothesis numerous studies have shown that ncRNAs, especially lncRNAs exhibit very cell and tissue type specific expression (Washietl et al, 2014; Wu et al, 2014; Guo et al, 2014) and sub-cellular localization (Zhang et al, 2014; Cabili e al, 2015). In addition, they are specifically expressed during processes like cellular development (reviewed in Devaux et al, 2015; Hassan et al, 2015), differentiation (reviewed in

Jeong & Goodell, 2016; Flynn & Chang, 2014) and stress response (Chang et al, 2014; reviewed in Place & Noonan, 2014; Turner et al, 2014). Additionally, it is becoming evident, that many lncRNAs are dysregulated in human diseases (Brunner et al, 2012; reviewed in Taft et al, 2010), which make them an attractive target for finding new biomarkers and therapeutic targets (reviewed in Sánchez & Huarte, 2013; Slaby et al, 2017). The focus of the following chapters will be mainly on the regulation of lncRNAs and their cellular functions.

2.2.4.1 Challenges in defining lncRNAs

The research around lncRNAs is relatively young and new lncRNA genes are discovered from transcriptomic profiling, however their detailed characterization is challenging and time consuming. Many have questioned what is the true protein-coding potential of lncRNAs. Frankly, it is not that straightforward to define. The most general approach to search for protein coding transcripts is to analyze the features of their open reading frames (ORFs), for example their length and phylogenetic conservation. Many lncRNAs do have ORFs, however most of them are shorter than ORFs of protein coding genes and they are located very closely upstream of the translation start site, predisposing transcripts to degradation by nonsense-mediated decay (Niazi & Valadkhan, 2012). Overall, it is very unlikely for most lncRNAs to be translated into proteins or even short peptides based on the structure of their ORFs (Niazi & Valadkhan, 2012; Derrien et al, 2012). Another strategy is to look at the conservation of the transcripts and their ORFs by a sequence alignment-based approach, such as Basic Local Alignment Tool (BLAST) (Chen et al, 2015) or phylogenetic assessment of codon substitution frequencies (PhyloCSF) (Lin et al, 2011). Interestingly, lncRNAs are poorly conserved by their nucleotide sequence and they rarely have orthologs within different species but their gene promoters are almost as conserved as protein coding promoters (Pang et al, 2006; Derrien et al, 2012; Washietl et al, 2014). In addition, lncRNAs seem to be under rapid evolutionary turnover (reviewed in Nitsche & Stadler, 2017).

The challenge in using computational analyses when predicting protein coding capacity of a transcript is that they use different algorithms and based on the given criteria the results may vary. Therefore, it is more reliable to use additional methods beside the predictions based on the genomic features. Ribosome profiling is a powerful, deep-sequencing based technique to examine protein translation globally and *in vivo* (reviewed in Ingolia, 2014). In principle, the translating ribosomes are mapped with nucleotide resolution across the transcriptome based on the sequenced fragments that are occupied by ribosomes, so called ribosome footprints (reviewed in Ingolia, 2014). The translated ORFs defined by ribosome profiling can be used to identify new protein coding regions in the genome that may encode alternative

isoforms of known proteins or they might be short ORFs (sORFs) as a source for new peptides (reviewed in Brar & Weissman, 2015). This method can be also combined with mass-spectrometric analysis to evaluate whether the defined ORFs are encoding peptides (Slavoff et al, 2013; Fritsch et al, 2012).

Although most of the ribosome footprints are mapped to known protein coding areas, there are several reports where they have been found outside of these areas, indicating non-coding RNA translation (Ruiz-Orera et al, 2014; Chew et al, 2013; Ingolia et al, 2014). However, this does not always lead to a stable and functionally relevant polypeptide. Instead, the translational process itself might be regulatory. For example, translation of an upstream ORF (uORF) can inhibit translation of the downstream protein-coding ORF (reviewed in Somers et al, 2013). It has been also proposed that translated ncRNAs could be a source for new protein-coding genes through evolutionary selection (reviewed in McLysaght & Guerzoni, 2015). A large fraction of lncRNAs have been shown to associate with ribosomes in human and mouse, however their protein coding potential remains relatively low, indicating that ribosome association does not necessarily mean translation (Zeng et al, 2018). Some of these proteins are also misannotated as lncRNAs (Zeng et al, 2018). Sole lncRNA interaction with the ribosome can also have a regulatory function (reviewed in Pircher et al, 2014), as shown for ZNFX1 antisense RNA 1 (Hansji et al, 2016). Overall, it is challenging to study ncRNA translation and at least for now the debate around lncRNA translation remains (Ji et al, 2015; Guttman et al, 2013).

2.2.4.2 Molecular mechanisms of lncRNAs

There is a broad spectrum of functional mechanisms for lncRNAs. As single-stranded RNA-molecules they can interact with DNA, RNA or proteins, making them very versatile regulators in the cell (reviewed in Wang & Chang, 2011). They have also unique secondary and tertiary structures important for mediating these interactions (reviewed in Blythe et al, 2016). Without the need for translation they can rapidly carry out their regulatory function, after which they can be quickly degraded. Their mechanism of action could be divided into four main types; ***signals, guides, decoys and scaffolds*** (**Figure 5**) (reviewed in Wang & Chang, 2011). Simply, they could be also grouped as lncRNAs functional either in the nucleus, mainly in mediating transcription (reviewed in Kaikkonen & Adelman, 2016) or in the cytoplasm in regulating post-transcriptional events (reviewed in Noh et al, 2018) (**Figure 5**).

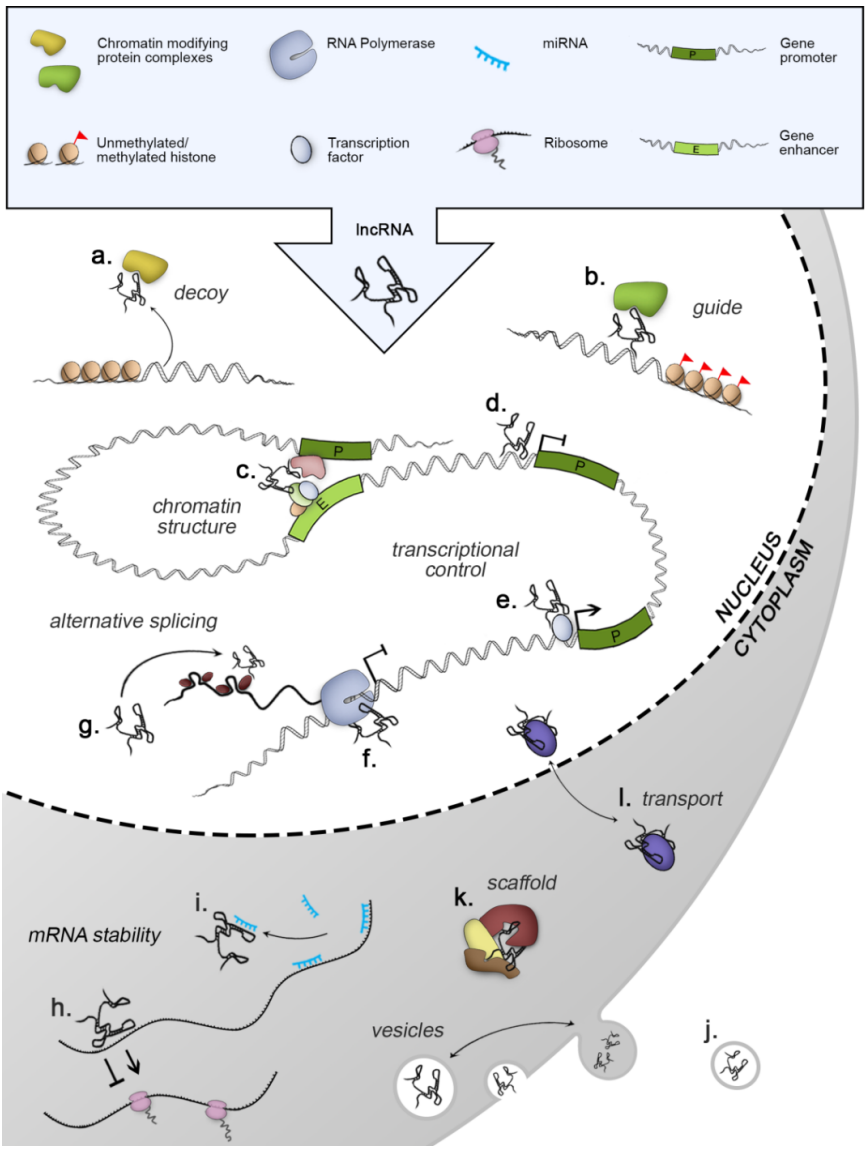

Figure 5. Molecular functions of lncRNAs. In the nucleus lncRNAs can regulate epigenetic changes by decoying (**a**) or guiding (**b**) chromatin modifying complexes to specific genomic loci. LncRNAs can mediate chromosomal looping (**c**) by simultaneously binding to protein complexes or specific DNA-elements and bringing them to close proximity. LncRNAs can inhibit gene transcription by binding to DNA and blocking the transcription factor binding site (**d**). Additionally, gene transcription can be activated or inhibited by lncRNAs guiding or decoying transcription factors (**e**). LncRNA binding to RNA polymerase can inhibit transcription (**f**). Alternative splicing can occur by lncRNA binding to mRNA and blocking the splice-site. LncRNAs can also recruit and guide splicing factors to the sites of transcription (**g**). In the cytoplasm lncRNAs can regulate mRNA stability directly by binding to mRNAs (**h**) or indirectly by binding to miRNAs (**i**) by complementary base-pairing. LncRNAs can be secreted to extracellular vesicles and exosomes allowing them to mediate intercellular signaling (**j**). Scaffold lncRNAs function by flexible lncRNA-protein binding and promote the assembly of active ribonucleoprotein complexes (**k**). Protein-binding lncRNAs can also mediate intracellular translocation of proteins (**l**).

LncRNA expression is very dynamic, allowing a fast response to external stimuli. Specific lncRNA expression has been noted for instance in response to DNA-damage (Sánchez et al, 2014), growth factors (Li et al, 2013; Kambara et al, 2014), immune response (Carpenter et al, 2013) and circadian changes (Coon et al, 2012). Particularly in plants lncRNAs serve as specific signals for environmental changes (reviewed in Liu et al, 2015). Additionally, lncRNAs can be secreted into extracellular vesicles and exosomes, and delivered outside of the cell where they can exert their functions even at long-distance (Huang et al, 2013). Exosomal lncRNAs function as signals in cell-cell communication and they have been implicated in cancer as potential regulators of drug resistance, for instance (reviewed in Zhou et al, 2018). A recent study illustrates a mechanism for lncRNA DINOL (damage induced long noncoding RNA) as ***a signal*** for DNA damage (Schmitt et al, 2016). It is transcribed divergently from p53-responsive *CDKN1A* gene upon DNA damage and it binds to and stabilizes p53, promoting p53-dependent gene expression, cell cycle arrest and apoptosis in response to DNA damage (Schmitt et al, 2016). In addition to DINOL, many other DNA damage inducible lncRNAs are characterized, such as NORAD (non-coding RNA activated by DNA damage; LINC00657) and PANDAR (promoter of CDKN1A antisense DNA damage activated RNA) (Lee et al, 2016; Hung et al, 2011).

Several lncRNAs have been shown to regulate gene expression by epigenetic mechanisms (reviewed in Marchese & Huarte, 2014). In principle, a lncRNA can bind to and guide gene regulatory complexes, such as gene silencing PRC2-complex, or gene activating complexes to a specific loci in the genome and bind to DNA by RNA:DNA heteroduplex or by forming a stable RNA:DNA:DNA triplex (reviewed in Wang & Chang, 2011). ***Guide*** lncRNAs can function in *cis* or *trans*, meaning that they can mediate changes in gene expression of the neighboring gene (*cis*) or distantly located gene (*trans*) (reviewed in Roberts et al, 2014).

In contrast to a guide lncRNA the functional mechanism of ***a decoy*** involves lncRNA binding to a gene regulator in order to negatively affect its function. In other words it prevents the effector molecule from binding to its target (reviewed in Wang & Chang, 2011). An interesting example of a decoy lncRNA is growth arrest-specific 5 (GAS5; NCRNA00030). It accumulates in growth-arrested cells, for example after serum starvation and it functions as a repressor for glucocorticoid receptor (GR) (Kino et al, 2010). Mechanistically it interacts with the DNA-binding domain of the GR and inhibits the receptor from binding to its target regulatory regions of the glucocorticoid-responsive genes (Kino et al, 2010). Another example of a decoy lncRNA is so called miRNA sponge. The competitive endogenous RNA (ceRNA) hypothesis suggests that RNA-transcripts can decoy miRNAs in case they have complementary miRNA-binding sites with the target mRNA (reviewed in Thomson & Dinger, 2016). Therefore, lncRNAs can regulate for example mRNA stability by

competing miRNA targeting (reviewed in Bayoumi et al, 2016). This seems to be a central function especially for circRNAs, however it remains to be elucidated how the endogenously expressed miRNA sponges work in the physiological context (reviewed in Thomson & Dinger, 2016). In addition to miRNA targeting there are also lncRNAs which can bind directly to mRNA and modulate its stability (Gong & Maquat 2011; Gong et al, 2015; Faghihi et al, 2008). The previous examples of guides and decoys demonstrate how lncRNAs can regulate gene transcription indirectly by interacting with gene regulatory complexes or miRNAs. It is important to note that lncRNAs can inhibit transcription directly as well. A few lncRNAs have been shown to directly bind to RNA Pol II leading to transcription inhibition (Espinoza et al, 2004; Mariner et al, 2008). Additionally, lncRNA binding to genomic DNA may inhibit pre-initiation complex assembly and gene transcription (Martianov et al, 2007).

Scaffold lncRNAs are lncRNAs that are interacting with proteins or RNP complexes bringing together different effectors and allowing proper organization of a functionally active RNP complex (reviewed in Wang & Chang, 2011). In this way lncRNAs are able to modulate a variety of different regulatory functions in the cell (reviewed in Geisler & Coller, 2013). One well-studied example of a scaffold lncRNA is HOX antisense transcript RNA (NCRNA00072), better known as HOTAIR. It simultaneously binds to PRC2-complex and another chromatin modifying complex, LSD1/coREST/REST, to mediate gene silencing (Tsai et al, 2010). Another scaffolding function for HOTAIR has been shown, where it associates with E3 ubiquitin ligases and their respective substrates, facilitating degradation of the target transcripts (Yoon et al, 2013). There is also evidence for lncRNAs in organizing the nuclear structure. Nuclear Enriched Abundant Transcript 1 (NEAT1; LINC00084) is a classic example of a lncRNA working as an assembly platform required for nuclear paraspeckle formation and maintenance (Mao et al, 2011). In this case NEAT1 works as a scaffold for recruiting proteins to assemble paraspeckles at NEAT1 gene loci. It has been also proposed that there might be several other lncRNAs whose transcription may serve as a mark for nuclear proteins to pull the genomic DNA into new positions, therefore changing the 3D organization of the genome (Melé & Rinn, 2016).

2.2.4.3 LncRNAs in cancer

A lot of effort has been done in characterizing the driver mutations in the genes that are regulating fundamental signaling pathways, such as p53 and Myc (reviewed in Garraway & Lander, 2013). For example, even a small change in the DNA such as a point mutation or single-nucleotide polymorphism (SNP) can alter the amino acid composition of a signaling protein disrupting its function. Similarly, SNPs can affect

lncRNA structure and functionality (Lai et al, 2013; Tan et al, 2017; reviewed in Gao & Wei, 2017). Moreover, a significant part of mutations reside in the non-coding regions of the genome which may affect gene expression due to alterations in gene regulatory areas, but lncRNA expression can be affected as well (reviewed in Khurana et al, 2016; Schmitt & Chang, 2016; Gao & Wei, 2017). In this regard it is not surprising that several cancer-related lncRNAs are transcribed from loci that are associated with increased cancer risk (reviewed in Cheetham et al, 2013; Gao & Wei, 2017).

Deregulation of lncRNAs has been noted in many human cancers and their functional importance in cancer is emerging (reviewed in Gutschner & Diederichs, 2012; Schmitt & Chang, 2016; Lin & Yang, 2018). Similarly to proteins, lncRNAs are found to be important regulators at different stages of tumorigenesis; regulators of proliferative signaling, angiogenesis, invasion, epigenetics, *et cetera* (reviewed in Gutschner & Diederichs). Differential lncRNA expression in cancer could be utilized by using them as prognostic and diagnostic markers (reviewed in Qi et al, 2016; Silva et al, 2015). LncRNAs have been implicated in drug resistance of cancer cells (reviewed in Deng et al, 2016). Therefore a specific lncRNA expression signature could be used to help to distinguish patients with good chemo-response and drug resistance. Moreover, a deeper understanding of their functional mechanisms may provide novel lncRNA-targeted therapies for cancer treatment (reviewed in Leucci, 2018; Slaby et al, 2017; Sánchez & Huarte, 2013). Here, I will describe a few examples of functionally relevant lncRNAs in cancer.

H19 (LINC00008) is one of the most extensively studied lncRNAs today, originally found in the 1990s and it presents one the earliest clues of functional lncRNAs, even though the definition did not exist at the time (Brannan et al, 1990). It was first identified as a highly expressed RNA in mouse embryo, mutually imprinted with its neighboring insulin-like growth factor 2 gene, *IGF2* (Brannan et al, 1990). Several lines of evidence support a tumorigenic role for H19 (reviewed in Yoshimura et al, 2018), however it may act differentially depending on the cell developmental stage (reviewed in Raveh et al, 2015). Firstly, it is a source for miR-675, which is expressed from the first exon of H19 gene (Cai & Cullen, 2007). This miRNA functions as oncogene in many malignancies by targeting important regulators, such as tumor-suppressive retinoblastoma protein (Rb) (reviewed in Raveh et al, 2015). It has been also implicated in EMT by targeting several EMT-markers (Matouk et al, 2014; Kim et al, 2014). H19 can also decoy miRNAs, such as the let-7 miRNAs (Kallen et al, 2013). Secondly, there seems to be a link between H19 and p53 regulation. H19 expression is repressed by p53 (Dugimont et al, 1998), but under hypoxic stress H19 expression is induced in p53-impaired cells (Matouk et al, 2010). Moreover, H19 has been shown to regulate polyploidy (Shoshani et al, 2012). For example, when cells are in unstable state due to impaired p53 function H19

expression is induced, which prevents cells becoming polyploid (Ravid et al, 2014). By resisting polyploidy-mediated growth arrest H19 has an advantage to accelerate uncontrolled cell proliferation, which further increases accumulation of DNA-mutations (reviewed in Raveh et al, 2015). Overall, H19 is regulating tumor progression on many different levels in a complex but coordinated manner (reviewed in Raveh et al, 2015).

MALAT1 (metastasis associated lung adenocarcinoma transcript 1) is another well characterized lncRNA involved in various cellular processes and implicated as a tumor promoting factor in many cancers (reviewed in Zhang et al, 2017). MALAT1, also known as NEAT2 (LINC00047), is transcribed from the same gene locus as NEAT1, a functional scaffolding lncRNA in the nuclear paraspeckles (Mao et al, 2011). However, there is no significant homology between them (Hutchinson et al, 2007). In fact, despite their similar localization in the nuclear speckles, they have distinct functions. As indicated by its name, MALAT1 was found to be highly enriched in metastasis associated non-small cell lung cancer (NSCLC) tumors when compared to non-metastasizing tumors (Ji et al, 2003). Later on, its elevated expression was noted in hepatocellular carcinoma (Lin et al, 2007), endometrial stromal sarcomas (Yamada et al, 2006) and many other human cancers (reviewed in Gutschner et al, 2013). The primary, nuclear-localizing MALAT1 transcript is processed so that the poly(A) tail is cleaved, generating a very stable triple-helix structure to the 3'end of the RNA (Wilusz et al, 2008; Brown et al, 2014). This structure protects MALAT1 from exonucleolytic degradation, but it may have additional functional role in nuclear transport and translation (reviewed in Wilusz, 2016). MALAT1 has shown to regulate alternative splicing by interacting with pre-mRNA splicing factors, such as SRSF1, and controlling their spatial distribution to nuclear speckles (Tripathi et al, 2010). MALAT1 knockdown was shown to reduce alternative splicing of oncogenic isoforms of previously reported SRSF1 target genes (Malakar et al, 2017). In addition, a recent study elucidates how MALAT1 is aberrantly delocalized from the nuclear speckles by a SRSF1-ID4-mutp53 protein complex (Pruszko et al, 2017). This enables MALAT1 recruitment on vascular endothelial growth factor A (VEGFA) pre-mRNAs and production of pro-angiogenic VEGFA isoforms in breast cancer, supporting the role of MALAT1 in alternative splicing (Pruszko et al, 2017). Interestingly, *Malat1* gene depletion results in perfectly viable mice with no apparent abnormalities, but the pre-mRNA splicing or the localization of nuclear speckle components is not affected in these mice (Nakagawa et al, 2012; Zhang et al, 2012). It seems that MALAT1 may not be regulated similarly in mice and humans, especially in a normal physiological stage. Nevertheless, it may acquire a specific function during tumorigenesis.

MEG3 (maternally expressed 3; LINC00023) is a lncRNA functioning as tumor suppressor (reviewed in Balas & Johnson, 2018; Zhou et al, 2012). It is widely

expressed in normal human tissues and the loss of MEG3 expression due to epigenetic silencing of *MEG3* gene promoter is reported in several cancer types (Gao et al, 2017; Zhang et al, 2010; Anwar et al, 2012; Benetatos et al, 2008; Sheng et al, 2014). Similarly to H19, MEG3 has been shown to bind to chromatin modifying protein complexes, such as PRC2 to regulate gene expression (Mondal et al, 2015). One of these targets is transforming growth factor beta (TGF-β) pathway, which is strongly related to EMT regulation (Mondal et al, 2015). Additionally, MEG3 has been shown to bind and stabilize p53 (Zhu et al, 2015). It also activates p53 by repressing expression of MDM2 proto-oncogene, which is a known E3 ubiquitin ligase mediating p53 degradation (Zhou et al, 2007). In several studies MEG3 re-expression has been shown to inhibit tumor growth *in vitro* and *in vivo* (Jin et al, 2018; Xu et al, 2018; Zhang et al, 2017). MEG3 gene deletion results in perinatal death in mice, indicating a crucial role for MEG3 in embryonic development (Takahashi et al, 2009). Therefore, studying MEG3 function in tumorigenesis *in vivo* seems challenging. By studying mouse embryos Takahashi and colleagues showed that teratomas derived from $meg^{-/+}$ embryos exhibited hypertrophic growth compared to the control group (Takahashi et al, 2015). Additionally, a study by Gordon and colleagues indicates that MEG3 has a regulatory function in brain vascularization and it may inhibit tumorigenesis by suppressing angiogenesis (Gordon et al, 2010). Overall, these results implicate a pivotal role for MEG3 functioning as a tumor suppressor.

2.2.5 Importance of lncRNAs in cutaneous biology

2.2.5.1 Functionally relevant lncRNAs in the skin

Several lncRNAs have been characterized in different skin malignancies, which will be discussed more closely in the next chapters. However, not much is known about the role of lncRNA in normal skin homeostasis. Environmental stress can specifically induce or repress lncRNA expression, shown in several human cell lines (Sánchez et al, 2014; Chang et al, 2014; Place & Noonan, 2014; Turner et al, 2014) and the same applies to skin. UV-irradiation, especially the UVB with higher tendency for skin cancer formation, has shown to affect the transcriptional profile of lncRNAs in normal keratinocytes (Kim et al, 2017), fibroblasts (Li et al, 2018) and melanocytes (Zeng et al, 2016). Moreover, UVA and UVB regulate expression of different set of lncRNAs in keratinocytes (Yo & Rünger, 2017), which highlights the differential cellular response to UVA and UVB. It is well known that the most effective way to prevent skin cancer is to avoid exposure to sun UV-light. However, recent findings strongly support cancer protecting role also for vitamin D (reviewed in Reichrath et al, 2017). Vitamin D signaling affects lncRNA expression as well (Jiang & Bikle,

2014). Interestingly, keratinocytes lacking vitamin D receptor show a distinct lncRNA expression pattern with increased expression of oncogenic lncRNAs and decreased expression of tumor-suppressive lncRNAs (Jiang & Bikle, 2014), supporting the cancer protecting role for vitamin D signaling in skin. Overall, these expression profiling studies may give important clues of the protective roles of certain lncRNA against skin cancer progression. On the other hand, some of these lncRNAs may function as tumor-promoters.

To provide more insight to the functional mechanisms for lncRNAs in epidermal regulation, a few examples will be discussed here in detail. DANCR (differentiation antagonizing non-protein coding RNA; KIAA0114), as implied by its name it is a differentiation antagonizing lncRNA. In the study by Kretz and colleagues (Kretz et al, 2012) primary human keratinocytes were treated with calcium to induce differentiation and cells were subjected to RNA sequencing. Differentially expressed transcripts were compared with chromatin signatures to explore their transcription dynamics during differentiation. They found DANCR to be suppressed during terminal differentiation and it was also downregulated in the basal layer of mature human epidermis compared to the suprabasal layer (Kretz et al, 2012). Silencing of DANCR expression in progenitor keratinocytes led to induction of genes associated with epidermal differentiation, such as filaggrin, loricrin, involucrin, and S100 calcium-binding proteins A8 and A9 (Kretz et al, 2012). Moreover, silencing of DANCR in organotypic epidermal tissue resulted in expression of differentiation proteins in the basal layer, where they are not normally expressed. These results implicate that DANCR is required to maintain epidermal progenitor cells in undifferentiated state. Further study by the same group (Lopez-Pajares et al, 2015) found DANCR to be a negative regulator of MAF and MAFB transcription factors, which are important regulators of differentiation in various cell types. DANCR represses their expression epigenetically by guiding a chromatin modifying protein complex to their gene promoters. Subsequent studies have shown similar function for DANCR for instance in osteoblast differentiation (Zhu & Xu, 2013). In addition to DANCR, two other lncRNAs, LINC00941 and BLNCR (beta1-adjacent long non-coding RNA), have been recently implicated in keratinocyte differentiation (Ziegler et al, 2019; Tanis et al, 2019). LINC00941 represses keratinocyte differentiation by antagonizing the function of SPRR5, a previously uncharacterized molecule which is a positive regulator of keratinocyte differentiation (Ziegler et al, 2019). Downregulation of BLNCR expression was noted during keratinocyte differentiation and transcription of BLNCR was shown to be activated by p63 and AP-1 transcription factors (Tanis et al, 2019). BLNCR expression was co-regulated with ITGB1 (integrin beta 1), which is located adjacent to *BLNCR* and transcribed in the opposite direction. The authors of this study propose that the loss of BLNCR

expression is an early event when keratinocytes lose their proliferative capacity and proceed to terminal differentiation (Tanis et al, 2019).

In contrast to DANCR, TINCR (terminal differentiation-induced ncRNA; LINC00036) promotes epidermal differentiation. Kretz and colleagues identified TINCR among the most highly expressed lncRNAs in differentiating keratinocytes and specifically enriched in the differentiated layers of human epidermis (Kretz et al, 2013). They noted that formation of the epidermal barrier was disturbed in TINCR-depleted organotypic human epidermal tissue (Kretz et al, 2013). To support this observation, transcriptome profiling revealed several epidermal barrier formation – related genes to be regulated by TINCR. The functional mechanism of TINCR was examined in two ways. First, TINCR-binding RNAs were detected by hybridizing biotinylated TINCR transcripts in keratinocytes, followed by RNA pull-down and deep sequencing. TINCR-interacting genes were enriched with differentiation-related genes, suggesting a post-transcriptional mechanism for regulation by TINCR. A specific 25-nucleotide motif was found to be necessary for the RNA-mRNA interaction. Next, TINCR protein targets were examined by using a microarray analysis with fluorescently labeled TINCR transcripts (Kretz et al, 2013). The strongest signal for TINCR binding was detected by staufen double-stranded RNA binding protein 1 (STAU1), a well-studied RNA-binding protein. Interestingly, there was a significant overlap between gene expression profiles of TINCR- and STAU1-depleted epidermis, indicating that they are both required for epidermal differentiation. In addition, they seem to function by stabilizing mRNAs rather than targeting them to degradation. In contrast to DANCR, TINCR was identified as a positive upstream regulator for MAF and MAFB transcription factors by stabilizing their mRNA stability (Lopez-Pajares et al, 2015). In another study TINCR was shown to stabilize mRNA of calmodulin like 5 (CALML5), an epidermal differentiation promoting protein enriched in the differentiated layers of human epidermis (Sun et al, 2015). Altogether, these observations implicate TINCR as a key mediator in driving epidermal differentiation. Similarly to TINCR, a recently identified novel lncRNA SMRT-2 (SCC misregulated transcript 2) was shown to be induced during keratinocyte differentiation and SMRT-2 knockdown resulted in altered gene expression signature, which may occur *via* transcriptional regulators KLF4 (Kruppel Like Factor 4) and ZNF750 (Zinc Finger Protein 750) (Lee et al, 2018).

2.2.5.2 Involvement of lncRNAs in skin disorders

LncRNA expression profiles are explored in several skin disorders, for instance in abnormal scarring (Liang et al, 2015; Li et al, 2015; Sun et al, 2017; Tu et al, 2018), psoriasis (Ahn et al, 2016; Tsoi et al, 2015; Gupta et al, 2016) and actinic dermatitis (Lei et al, 2017). Interestingly, there are also few lncRNAs associated with rare genetic

syndromes with cutaneous defects, such as Beckwith–Wiedemann and McCune–Albright syndromes (reviewed in Wan & Wang, 2014). There is still a lack of detailed characterization of the functionally relevant lncRNAs in many of these disorders. However, one good example of a psoriasis associated lncRNA is described here in more detail.

LncRNA PRINS (psoriasis susceptibility-related RNA gene induced by stress; NCRNA00074) is the first lncRNA characterized in psoriasis (Sonkoly et al, 2005). Its expression was higher in the uninvolved epidermis of psoriatic patients compared with healthy epidermis. It was specifically induced in HaCaT cells after serum starvation, UVB-irradiation, viral infection or translational inhibition, suggesting a protective role for PRINS in cellular stress response (Sonkoly et al, 2005). Further studies by the same group revealed two target genes for PRINS – G1P3 (or interferon alpha inducible protein 6, IFI6) and nucleophosmin 1 (NPM1) (Szegedi et al, 2010 and 2012). PRINS was shown to positively regulate G1P3, which was found remarkably abundant in psoriatic lesional tissue, indicating a possible role for G1P3 in the pathogenesis of the disease (Szegedi et al, 2010). NMP1 was found to physically interact with PRINS, which was shown by *in vitro* pull-down assay and by examining their expression patterns in the epidermis (Szegedi et al, 2012). Induction of PRINS by UVB was shown in the previous study (Sonkoly et al, 2005). Interestingly, NPM1 is also known to be regulated by UVB in fibroblasts and cancer cells, resulting in nucleoplasmic translocation of the nucleolar NPM1. Here, NPM1 translocation was inhibited in PRINS-depleted keratinocytes after UVB treatment, indicating that by physically interacting with NPM1 PRINS is modulating its intracellular shuttling (Szegedi et al, 2012). In addition to these functional findings for PRINS, a recent study shows that PRINS has an important role in keratinocyte innate immune response in regulating cytokine expression (Danis et al, 2017), highlighting the association with PRINS in psoriasis susceptibility.

The role of lncRNAs in skin wound healing is largely unknown, even though functions of miRNAs are widely studied in wound healing (reviewed in Herter & Xu Landén, 2017). LOC100130476 and LOC105372576 are recently identified lncRNAs, both observed with reduced expression levels in chronic non-healing wounds compared to normal skin (Herter et al, 2018; Li et al, 2019). Induced expression of LOC100130476 led to restriction of inflammatory chemokine production and improved wound healing and based on these findings it was named WAKMAR2, wound and keratinocyte migration associated lncRNA 2 (Herter et al, 2018). LOC105372576, named WAKMAR1 (wound and keratinocyte migration-associated lncRNA 1) was shown to regulate keratinocyte motility through E2F1 transcription factor (Li et al, 2019). Similarly to WAKMAR2, induced expression of WAKMAR1 resulted in increased keratinocyte migration and re-epitelization of human *ex vivo* wounds (Li et al, 2019). Together, WAKMAR1 and WAKMAR2 deficiency may be

associated with the pathogenesis of chronic wounds. In another study MALAT1 was shown to stimulate healing of ischemic wounds *in vivo* by treating the wounds with conditioned media enriched in MALAT1-containing exosomes (Cooper et al, 2018). The same treatment increased migration of human dermal fibroblasts *in vitro*, whereas treatment with MALAT1-depleted exosomes decreased their migration (Cooper et al, 2018). Similarly to MALAT1, exosomal delivery of lncRNA H19 was shown to improve angiogenesis and healing of diabetic wounds in a diabetic rat model (Tao et al, 2018). Interestingly, even though MALAT1 and H19 are mainly linked to cancer progression, they may be used therapeutically to improve chronic wound closure. Topically applied mevastatin, a cholesterol-reducing agent, has been shown to promote wound healing *in vivo* by inducing epithelialization and keratinocyte-driven angiogenesis (Sawaya et al, 2018). Cholesterol is synthetized in the epidermis and it is a precursor for cortisol, which is a negative regulator of wound healing acting *via* glucocorticoid receptor (GR) (Vukelic et al, 2011). Here, mevastatin was shown to block GR activation and cortisol synthesis by inhibiting c-myc (Sawaya et al, 2018). GAS5 lncRNA, a known GR repressor (Kino et al, 2010) was strongly induced by mevastatin in diabetic foot ulcers *in vivo* and GAS5 overexpression in spontaneously immortalized keratinocyte cells (HaCaT) led to c-myc inhibition (Sawaya et al, 2018). Collectively, these lncRNAs may work as potential therapeutic targets in treating chronic non-healing wounds.

2.2.5.3 Emerging roles of lncRNAs in skin cancers

As mentioned previously, lncRNA expression is altered in the skin after UV-irradiation (Kim et al, 2017; Li et al, 2018; Zeng et al, 2016; Yo & Rünger, 2017), indicating that some of them might have a protective role in skin cancer formation. In some instances, lncRNA expression or regulation may be disturbed for example due to genetic alteration in cancer. This is very likely particularly in skin cancers, as they are highly susceptible for UV-induced mutations that arise early in skin carcinogenesis (reviewed in Chen et al, 2014). Many skin cancer associated gene mutations are characterized, such as p53, however the impact on lncRNA regulation by UV-induced mutations is not known.

BRAF is a well-studied serine/threonine kinase which is mutationally activated in many human cancers, including melanoma (reviewed in Holderfield et al, 2014). Paradoxically, the majority of BRAF mutations arise in skin areas without chronic sun-induced damage (Curtin et al, 2005). In an intriguing study by Flockhart and colleagues transcriptome profiles between normal melanocytes and BRAF-mutant melanomas were compared to identify new BRAF-oncogene targeted genes in melanoma progression (Flockhart et al, 2012). They discovered lncRNA BANCR (BRAF-activated non-protein coding RNA) as one of the most highly induced

transcripts by oncogenic BRAF, and it was shown to regulate melanoma cell migration (Flockhart et al, 2012). Another fine example of a lncRNA deregulated by genetic alteration is SAMMSON, survival associated mitochondrial melanoma specific oncogenic non-coding RNA (LINC01212). Leucci and colleagues discovered the SAMMSON gene co-amplified with a focal amplification of chromosome 3p13-3p14, a known gene alteration which is associated with a poor prognosis in a subset of melanomas (Leucci et al, 2016). This area encompasses MITF gene (microphthalmia-associated transcription factor) approximately 30 kilobases upstream of SAMMSON and it is an important regulator of melanocyte survival and pigment production. However, when amplified or mutated, MITF has an oncogenic role in melanoma progression (Garraway et al, 2005; Yokoyama et al, 2011). SAMMSON expression was positively correlated with melanoma malignancy and copy number gain (Leucci et al, 2016). Furthermore, it was shown to promote melanoma growth and survival *via* mitochondrial p32 protein (Leucci et al, 2016). There was no correlation in MITF and SAMMSON expression levels in clinical melanoma samples, and MITF expression was not affected in *cis* by SAMMSON (Leucci et al, 2016). Therefore, even though SAMMSON and MITF are not co-regulated, the MITF-SAMMSON amplicon may be beneficial for transforming melanocytes due to their combined, oncogenic regulatory role in melanoma.

While many lncRNAs have been characterized in melanoma (reviewed in Richtig et al, 2017) the role of lncRNAs in non-melanoma skin cancers is not well understood. A literature search revealed a few lncRNAs implicated in non-melanoma skin cancer, such as TINCR and SMRT-2 (**Table 1**). They are both differentiation promoting lncRNA in keratinocytes and decreased expression of TINCR and SMRT-2 was noted in human cSCC specimens (Kretz et al, 2013; Lee et al, 2018). Additionally, a notable decrease in TINCR expression was reported in murine DMBA/TPA-induced cSCC tumors compared to normal skin (Ponzio et al, 2017). SMRT-2 expression was shown to be markedly suppressed also in Ras-driven human organotypic neoplasia, suggesting a tumor-suppressive role for SMRT-2 in cSCC (Lee et al, 2018). TINCR and SMRT-2 may function as tumor-suppressive lncRNAs in cSCC, especially as lack of differentiation is associated with poor prognosis and a risk factor for metastatic cSCC (reviewed in Kallini et al, 2015). Based on the literature LINC00319, LINC00520, LINC01048 and MALAT1 are the only functionally studied lncRNAs in human cSCC (Li et al, 2018; Mei & Zhong, 2019; Chen et al, 2019; Zhang et al, 2019). In addition, a novel lncRNA transcript, AK144841 found in mouse DMBA/TPA-induced cSCC may present a novel tumor promoter (Ponzio et al, 2017), however its contribution to human cSCC remains to be elucidated. Of note, none functionally characterized lncRNAs in basal cell carcinoma (BCC) were found. LincRNA-p21 is highly inducible by UVB in human and mouse keratinocytes and in mouse skin *in vivo* (Hall et al, 2015). Moreover, the

transcriptional response was shown to be p53-dependent. LincRNA-p21 is an important regulator of UVB-induced apoptosis in keratinocytes (Hall et al, 2015). Interestingly, both lincRNA-p21 expression and UVB-induced apoptosis were significantly inhibited in mouse epidermis harboring a mutation in a single p53 allele (Hall et al, 2015). These results indicate a protective role for lincRNA-p21 in skin carcinogenesis.

Table 1. Long non-coding RNAs implicated in keratinocyte cancers.

LncRNA	Expression	Function	Reference
TINCR	Downregulated in cSCC	Controls human epidermal differentiation by stabilization of differentiation mRNAs	Kretz et al, 2013
SMRT-2	Downregulated in cSCC	Mediates keratinocyte differentiation	Lee et al, 2018
LINC00319	Upregulated in cSCC	Regulates cell growth, migration, invasion and apoptosis by miR-1207-5p-mediated regulation of cyclin-dependent kinase 3	Li et al, 2018
LINC00520	Downregulated in cSCC	Inhibits cSCC progression by targeting EGFR and PI3K/AKT signaling pathways	Mei & Zhong, 2019
LINC01048	Upregulated in cSCC	Interacts with TAF15 transcription factor to induce YAP1 transcription and tumorigenic function *via* Hippo signaling pathway	Chen et al, 2019
MALAT1	Upregulated in cSCC	Positively regulates EGFR protein expression *via* c-MYC and KTN1	Zhang et al, 2019
lincRNA-p21	Induced in mouse and human keratinocytes by UVB	Tumor suppressive role in triggering UVB-induced apoptotic death	Hall et al, 2015
AK144841	Induced in mouse DMBA/TPA-induced cSCC	Downregulates several anticancer and cell differentiation genes in mouse	Ponzio et al, 2017
H19, Hottip, Nespas, mHOTAIR, Malat1, SRA	Upregulated in vitamin D receptor (VDR) deleted mouse keratinocytes and epidermis	Potential oncogenes in skin cancer progression	Jiang & Bikle, 2014
Kcnq1ot1, lincRNA-p21, Foxn2-as, Gtl2-as, H19-as	Inhibited in VDR-deleted mouse keratinocytes and epidermis	Potential tumor suppressors in skin cancer formation	Jiang & Bikle, 2014
H19, CASC15, SPRY4-IT	Upregulated in BCC	Potential oncogenes in BCC	Sand et al, 2016

3 Aims of the Study

1. To investigate the role of deregulated lncRNAs in cSCC and to elucidate their role in cSCC progression. The main objective is to find and characterize new biomarkers for evaluating the risk of progression and metastasis of cSCC and identify novel therapeutic targets for recurrent and metastatic cSCC. (I)
2. To investigate the functional role of lncRNA PICSAR in cSCC progression. (II)
3. To investigate the role of lncRNA PRECSIT in cSCC progression. (III)

4 Materials and Methods

4.1 Ethical issues (I, II, III)

The use of normal skin and tumor samples was approved by the Ethics Committee of the Hospital District of Southwest Finland. A written informed consent was given from all the participants and the study was performed with the permission of Turku University Hospital according to the Declaration of Helsinki. The experiments with mice were carried out with the permission of the State Provincial Office of Southern Finland.

4.2 Cells and tumor samples

4.2.1 Normal human epidermal keratinocytes (I, III)

Normal human epidermal keratinocytes (NHEKs) were established from skin of healthy individuals undergoing mammoplasty (Farshchian et al, 2011). NHEK-PC was purchased from PromoCell (Heidelberg, Germany). The HaCaT cell line was kindly provided by Dr. Norbert E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). NHEKs and HaCaT cells were cultured as previously described (Riihilä et al, 2014; Farshchian et al, 2015).

4.2.2 Human cSCC cell lines (I, II, III)

Human primary (n = 5; UT-SCC-12A, UT-SCC91, UT-SCC105, UT-SCC111 and UT-SCC118) and metastatic (n = 3; UT-SCC7, UT-SCC59A and UT-SCC115) cSCC cell lines were established from surgically removed SCCs of the skin in Turku University Hospital and cultured as previously described (Riihilä et al, 2014). The authenticity of the cell lines has been verified by short tandem repeat profiling (Farshchian et al, 2017b). To determine the p53 mutational status of cSCC cells *TP53* single-nucleotide variants were examined with the Integrative Genomics Viewer 2.3 software (https://software.broadinstitute.org/software/igv/) (Robinson et al, 2011) using the RNA-seq data of cSCC cells (Farshchian et al, 2017a; GSE66412) (III, Supplementary Figure S1 and Supplementary Table S1).

4.2.3 Tissue samples of cSCC tumors and normal skin (I)

Tissue samples from primary cSCC tumors were collected from surgically removed tumors in the Turku University Hospital (Farshchian et al, 2011). Normal skin tissue samples were collected from the upper arm of healthy volunteers during a mammoplasty operation. Total RNA was extracted from tissue samples using RNeasy mini kit (Qiagen, Germantown, MD) and subjected for qRT-PCR analysis.

4.2.4 Tissue microarrays (I, III)

Tissue microarrays consisting of paraffin-embedded and formalin-fixed samples from normal sun-protected skin (n = 10), seborrheic keratosis (n = 26), actinic keratosis (n = 50), cSCC *in situ* (n = 25), and invasive cSCC (n = 81) were generated from the archival paraffin blocks from the Department of Pathology, Turku University Hospital (Riihilä et al, 2015; Farshchian et al, 2015).

4.3 Antibodies

The following antibodies used in the original publications (I-III) are listed in **Table 2** (WB, Western blot; IHC, immunohistochemistry; FC, flow cytometry; IFM, immunofluorescence microscopy).

Table 2. Antibodies used in the original publications.

	Antigen	Catalog no.	Supplier	Method
I	pERK1/2	9101	Cell Signaling Technology, Beverly, MA	WB
I	ERK1/2	9102	Cell Signaling Technology	WB
I	DUSP6	sc-28902	Santa Cruz Biotechnology, Dallas, TX	WB
I	p38α	05-454	Thermo Fisher Scientific, Fremont, CA	WB
I	p38δ	sc-7585	Santa Cruz Biotechnology	WB
I	pCREB	9191	Cell Signaling Technology	WB
I	Ki-67 (MIB-1)	M7240	Agilent Technologies, Santa Clara, CA	IHC, WB
II	Integrin α2	555668	BD Biosciences, San Jose, CA	FC
II	Integrin α5	555615	BD Biosciences	FC
II	Integrin β1	553715	BD Biosciences	FC
II	Integrin α2	MCA2025	Bio-Rad, Hercules, CA	IFM
II	Integrin α5	AB1949	Merck Millipore, Burlington, MA	IFM

	Antigen	Catalog no.	Supplier	Method
II	Alexa Fluor® 488 conjugated phalloidin	A12379	Invitrogen, Carlsbad, CA, USA	IFM
II	pSrc	2101	Cell Signaling Technology	WB
II	Src	2108	Cell Signaling Technology	WB
III	p53	DO-1	Santa Cruz Biotechnology	WB
III	p53	DO-7	Sigma-Aldrich, St Louis, MO	IHC
III	p21	2G12	BD Biosciences	WB
III	pSTAT3	D3A7	Cell Signaling Technology	WB, IHC
III	STAT3	124H6	Cell Signaling Technology	WB
III	MMP-1	IM35L	CalbioChem, La Jolla, CA	WB
III	MMP-3	HPA007875	Sigma-Aldrich	WB
III	MMP-10	MA5-14233	Thermo Fisher Scientific	WB
III	MMP-13	IM64L	CalbioChem	WB
III	TIMP-1	IM32	CalbioChem	WB
III	CD34	sc-18917	Santa Cruz Biotechnology	IHC
I-III	β-actin	A-1978	Sigma-Aldrich	WB

4.4 Quantitative real-time PCR

Total RNA was extracted from the cultured cSCC cells and tissue samples using RNeasy mini kit (Qiagen) and cDNA was synthetized. RealTimeDesign Software (https://www.biosearchtech.com) was used to design specific primers and probes (Oligomer, Helsinki, Finland). All the primers and probes used in the original publications (I-III) are listed in **Table 3**. All qRT-PCR reactions were performed in triplicate using the ABI 7900 (Applied Biosystems, Carlsbad, CA) or QuantStudio 12K Flex (Thermo Fisher Scientific) system at the Finnish Functional Genomics Centre (FFGC) in Turku, Finland. The standard curve method was used to analyze the results (Larionov et al, 2005).

Table 3. Primers and probes used in the original publications in qRT-PCR.

	Primer or probe	Sequence
I, II	PICSAR forw	5′-TGC CTG GAC TTT CAA GAG GTA A-3′
	PICSAR rev	5′-GCT CTC AGT CAG CAG ACA CTT-3′
	PICSAR probe	5′-Fam CCG AGC TCT GCT CTG AGG CCT BHQ1-3′
I	DUSP6 forw	5′-GCC GCA GGA GCT ATA CGA G-3′
	DUSP6 rev	5′-ACC GGC AGG TTA CCC TTC T-3′
	DUSP6 probe	5′-Fam TCG TCG CAC ATC GAG TCG GC BHQ1-3′
II	Integrin α2 forw	5′-TGG ATT TGC GTG TGG ACA TC-3′
	Integrin α2 rev	5′-GGC AGT TCT AGA ATA GGC TTC AA-3′
	Integrin α2 probe	5′-Fam-TCT GGA AAA CCC TGG CAC TAG CCC TG-BHQ1-3′
II	Integrin α5 forw	5′-GGG TGG CCT TCG GTT TAC AG-3′
	Integrin α5 rev	5′-GCT TTG CGA GTT GTT GAG ATT C-3′
	Integrin α5 probe	5′-Fam TCC CTC ATC TCC GGG ACA CTA A BHQ1-3′
II	Integrin β1 forw	5′-CAA GGG CAA ACG TGT GAG A-3′
	Integrin β1 rev	5′-TGA AGG CTC TGC ACT GAA CA-3′
	Integrin β1 probe	5′-Fam TGT GTC AGA CCT GCC TTG GTG TC BHQ1-3′
III	MALAT1	*Proprietary (cat. Hs00273907_s1, Thermo Fischer Scientific)*
III	MMP-1 forw	5′-AAG ATG AAA CGT GGA CCA ACA ATT-3′
	MMP-1 rev	5′-CCA AGA GAA TGG AAG AGT TC-3′
	MMP-1 probe	5′-Fam CAG AGA GTA CAA CTT ACA TCG TGT TGC GGC TC Tamra-3′
III	MMP-3	*Proprietary (cat. Hs00968305_m1, Thermo Fischer Scientific)*
III	MMP-10 forw	5′-GGA CCT GGG CTT TAT GGA GAT AT-3′
	MMP-10 rev	5′-CCC AGG GAG TGG CCA AGT-3′
	MMP-10 probe	5′-Fam CAT CAG GCA CCA ATT TAT TCC TCG TTG CT Tamra-3′
III	MMP-13 forw	5′-AAA TTA TGG AGG AGA TGC CCA TT-3′
	MMP-13 rev	5′-TCC TTG GAG TGG TCA AGA CCT AA-3′
	MMP-13 probe	5′-Fam CTA CAA CTT GTT TCT TGT TGC TGC GCA TGA Tamra-3′
I-III	β-actin forw	5′-TCA CCC ACA CTG TGC CCA TCT ACG C-3′
	β-actin rev	5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′
	β-actin probe	5′-Fam CAG CGG AAC CGC TCA TTG CCA ATG G BHQ1-3′
I, II	GAPDH forw	5′-ACC CAC TCC TCC ACC TTT GA-3′
	GAPDH rev	5′-TTG CTG TAG CCA AAT TCG TTG-3′
	GAPDH probe	5′-Fam ACG ACC ACT TTG TCA AGC TCA TTT CCT GGT BHQ1-3′

4.5 RNA sequencing and bioinformatic analyses

RNA isolation for all RNA-seq analyses was done using the miRNeasy Mini Kit (Qiagen). The SOLiD™ Whole Transcriptome Analysis Kit (Applied Biosystems) was used to prepare the whole transcriptome libraries for cSCC cells (n = 8) and NHEKs (n = 4). The gene expression analysis was performed using the SOLiD 3Plus system at the FFGC. The reads were aligned against the human reference genome hg19. The quantile-to-quantile adjustment (R/Bioconductor package edgeR) was used in data normalization. LncRNA genes were identified based on annotated

lncRNAs by the HGNC (http://www.genenames.org). RNA-seq data of cSCC cell lines and NHEKs (GSE66412) is available online.

The RNA-seq analyses for cSCC cells (UT-SCC12A, UT-SCC59A and UT-SCC118) after PICSAR knockdown and for cSCC cells (UT-SCC7, UT-SCC59A, UT-SCC105) after PRECSIT knockdown were performed using the Illumina HiSeq2500 system (Illumina, San Diego, CA) at the FFGC. The reads were aligned against the human reference genome hg19 for PICSAR knockdown RNA-seq and hg38 for PRECSIT knockdown RNA-seq. TMM normalization method was used for data normalization (R/Bioconductor package edgeR). Morpheus software was used to generate heatmaps (https://software.broadinstitute.org/morpheus). RNA-seq data was analyzed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA; FC log2 > 0.5, $P < 0.05$), the Gene Ontology Enrichment Analysis (http://www.geneontology.org/), Kyoto Encyclopedia of Genes and Genomes Pathway Analysis (http://www.genome.jp/kegg/) and the Reactome Pathway Knowledgebase (https://reactome.org/). RNA-seq data of PICSAR knockdown cSCC cells (GSE77950) is available online.

Following computational programs were used to evaluate potential miRNA binding sites in PICSAR; TargetScan (http://www.targetscan.org) (Agarwal et al, 2015), miRWalk (http://mirwalk.umm.uni-heidelberg.de/) (Dweep and Gretz, 2015) and DIANA–lncBase v2 (www.microrna.gr/LncBase) (Paraskevopoulou et al, 2015).

4.6 Western blot analysis

Samples of cSCC cultures were analyzed by western blotting with antibodies listed in **Table 2**. Protein expression was quantitated using the LI-COR Odyssey® CLx fluorescent imaging system with fluorescently-labeled secondary antibodies (LI-COR Biosciences, Lincoln, NE).

4.7 Flow cytometry (II)

To quantitate integrin expression on the cell surface cell cultures were trypsinized and 700 000 cells per sample was suspended in 1 % FCS-PBS and incubated on ice for 30 minutes. Cells were stained with monoclonal antibodies listed in **Table 2** for 1 hour in +4°C in agitation and washed with PBS. Alexa Fluor® 488 conjugated goat anti-mouse antibody was used as a secondary antibody (**Table 2**). FACSCalibur flow cytometer (BD Biosciences) was used for cell analysis at the FFGC. Flowing Software 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland, www.flowingsoftware.com) was used for data analysis.

To analyze cell cycle cSCC cultures were trypsinized 72 hours after siRNA transfection, followed by washing with PBS and suspension in sodium citrate buffer (40 mM Na-Citrate, 0.3% Triton X-100, 0.05 mg/ml propidium iodide, PBS). After

20 minutes of incubation cell cycle analysis was performed using BD LSR FortessaTM cell analyzer (BD Biosciences) at the Cell Imaging Core of the Turku Centre for Biotechnology, Turku, Finland. The FlowJo software, v.10 (Tree Star Inc., Ashland, OR) was used to analyze the results.

4.8 Protein and RNA detection in fixed cells and tissues

4.8.1 Immunofluorescence (II)

Cell cultures were fixed with 4% paraformaldehyde-PBS for 10 minutes, blocked with 3 % BSA-PBS for 30 minutes and permeabilized with 0.2 % Triton X-100-PBS for 5 minutes. Cells were labeled with primary antibodies listed in **Table 2** in 3 % BSA-PBS for 3 hours. Alexa Fluor® 568 conjugated goat anti-mouse or anti-rabbit antibody (Invitrogen) was used as secondary antibody in 1:200 dilution together with Alexa Fluor® 488 conjugated phalloidin in 1:50 dilution in 3 % BSA-PBS. Hoechst 33342 (Thermo Fisher Scientific) was used to visualize nuclei. Cells were mounted with Mowiol-DABCO (Sigma-Aldrich) and samples were examined with Zeiss AxioVert 200M (Carl Zeiss, Jena, Germany).

4.8.2 RNA *in situ* hybridization (I, III)

Formalin-fixed paraffin-embedded tissue samples were subjected for RNA *in situ* hybridization (RNA-ISH) analysis using QuantiGene® ViewRNA™ ISH Tissue Assay kit (Affymetrix, Inc., Santa Clara, USA) with TYPE 1 probe set for PICSAR and TYPE 6 probe set for MMP13 (both from Affymetrix) according to the manufacturer's instructions. Fast Red and Fast Blue substrates (Affymetrix) were used to detect alkaline phosphatase activity in TYPE 1 probe set for PICSAR and TYPE 6 probe set for MMP13, respectively. Tissue sections were visualized using a brightfield microscope Olympus BX60 (Olympus Optical Co., Ltd., Tokyo, Japan). For the negative control hybridization the probe sets were omitted.

For PRECSIT detection *in situ* formalin-fixed paraffin-embedded tissue samples were subjected for RNA-ISH analysis using RNAscope® ISH Assay (Advanced Cell Diagnostics, Newark, CA) in cooperation with Bioneer (Bioneer A/S, Hørsholm, Denmark, http://www.bioneer.dk). The assay was performed by using automated Ventana Discovery Ultra slide-staining system (Roche, Basel, Switzerland) as previously described (Anderson et al, 2016). PRECSIT-specific probe (cat. 559389) was designed and purchased at Advanced Cell Diagnostics (ACD, Newark, CA). Human PPIB (Cyclophilin B) and bacterial DapB (4-hydroxy-tetrahydrodipicolinate reductase) mRNAs were used as positive and negative controls for RNA-ISH (III,

Supplementary Figure S3). PRECSIT expression was visualized using the Pannoramic Midi FL slide scanner (3DHISTECH Ltd., Budapest, Hungary) and quantitated by counting PRECSIT positive cells and compared with the number of total cell count in a section at 40x magnification using QuPath bioimage analysis software (Bankhead et al, 2017).

RNA-ISH analysis of cultured cSCC cells and NHEKs was performed with QuantiGene® ViewRNA™ ISH Cell Assay kit according to the manufacturer's instructions (Affymetrix). Cells were fixed in 4% paraformaldehyde for 30 minutes treated with protease (1:2 000) and hybridized with PICSAR specific probe set. For nuclear staining 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) or Hoechst (Thermo Fisher Scientific) was used. Fluorescence signal of PICSAR was detected using Zeiss LSM 510 META or Zeiss AxioVert 200M microscopes.

4.8.3 Immunohistochemistry (I, III)

To evaluate tumor histology tissue samples from paraffin-embedded formalin-fixed human cSCC xenograft tumors were stained with Mayer's hematoxylin (Sigma-Aldrich) and or eosin (Junttila et al, 2007). Proliferating cells were identified by immunohistochemistry using monoclonal antibody for Ki-67 (**Table 2**) with hematoxylin as counterstain. The relative number of Ki-67-positive cells was determined by counting 500-2 600 cells at 20x magnification in all tumor sections with ImageJ Software (Schneider et al, 2012).

Tissue microarrays from NS, SK, AK, cSCCIS and invasive cSCC were stained with mouse monoclonal p53 antibody (**Table 2**) at the Histology Core of the Institute of Biomedicine at the University of Turku and visualized using the Pannoramic Midi FL slide scanner (3DHISTECH Ltd.). This antibody reacts with the mutant and the wild-type p53. A semiquantitative analysis was performed to evaluate the p53 expression and mutational status based on the staining intensity of p53; - negative (no expression, likely a missense mutation in p53 resulting in no protein expression), + weak (very weak expression in the basal layer of the skin, presumably functional wild-type p53), ++ moderate (moderate nuclear p53 expression throughout the epidermis, p53 may be functional and activated), +++ strong (strong nuclear accumulation of p53 in the majority of cells, p53 is likely mutated and non-functional).

4.9 Construction of expression vectors (II)

Primer-BLAST was used for designing specific primers for PICSAR (Oligomer): forward primer 5'-CTGGCTCACCCTGGCACTG-3', reverse primer 5'-CACCTAAGCAATGCAGAGAGG-3'. Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) was used to amplify the DNA by PCR using cSCC

cell derived cDNA as template. The PCR reaction was cycled as follows; 98°C 30s; 98°C 10s, 66°C 30s and 72°C 30s (40 cycles); 72°C 10 minutes. The DNA fragment was cloned into pcDNA3.1(-) (Invitrogen) containing the neomycin resistance gene. The construct was further sequenced to verify the orientation and integrity of the ligated PICSAR insert.

4.10 Functional assays in cell culture

4.10.1 Cell transfections (I, II, III)

4.10.1.1 Gene knockdown using small interfering RNAs

Cells were cultured to 50 % confluence and transfected with commercially available small interfering RNAs (siRNAs) (**Table 4**) at 75 nM concentration (Qiagen) in serum-free medium using siLentFect™ Lipid Reagent (Bio-Rad). After 6 h, the medium was equilibrated to 10% FCS. To obtain p38α/δ double knockdown, both siRNAs were added in a final concentration of 75 nM and control cells were transfected using control siRNA in final concentration of 150 nM.

4.10.1.2 Cell transfection with a gene expression construct (II)

Lipofectamine 3000 transfection reagent (Invitrogen) was used to transfect cSCC cells with an empty vector (pcDNA3.1) or PICSAR expression construct (pcDNA3.1_PICSAR) and transfected cells were selected with 1 μg/ml Geneticin (G418 sulfate, Invitrogen). Selective pressure of PICSAR expression in cSCC cells was maintained using 500 μg/ml Geneticin.

Table 4. Commercially available siRNAs used in the original publications.

	Target gene	Catalog no.	Sequence
I-III	AllStars Negative Control siRNA	SI03650318	*proprietary*
I, II	PICSAR	SI04916219 (PICSAR siRNA1)	5′-CACGGCCAACGTGGAGCTCTA-3′
I, II	PICSAR	SI04916233 (PICSAR siRNA2)	5′-CTGCAGTCACTTCACAGTGAA-3′
I	p38α	SI00605157	5′-CAGAGAACTGCGGTTACTTAA-3′
I	p38δ	SI02222941	5′-CCGGAGTGGCATGAAGCTGTA-3′
III	PRECSIT	SI04765145 (PRECSIT siRNA1)	5′-GAGGTTCGGGAAGGAAAGGAAA-3′
III	PRECSIT	SI04765159 (PRECSIT siRNA2)	5′-CAGGGATGGTGACAAGCGGAA-3′

4.10.2 Inhibitor assays (I, III)

For a specific inhibition of MAPKs, cSCC cells were treated with p38 inhibitors BIRB796 and SB203580 (10 μM), and MEK1/2 inhibitor PD98059 (30 μM) (all from Calbiochem) for 24 hours. For DUSP6 inhibition cSCC cells were treated with (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI) (5 μM, Calbiochem) for 6 hours and incubation was continued for 18 hours with 1 μM BCI. For STAT3 inhibition cSCC cells were treated with 100 μM STAT3 inhibitor S31-201 (Cayman Chemical, Ann Arbor, MI).

4.10.3 Adenoviral infection (I, III)

UT-SCC12A cells were infected with control (RAdLacZ) and dominant negative MKK3bcontaining (RAdMKK3bA) adenoviral vectors and incubated for 6 hours in 0.5 % FCS at MOI 600. UT-SCC7, UT-SCC12A and HaCaT cells were infected with RAdp53 containing the p53 wild-type (Katayose et al, 1995) or with empty virus RAd66 (Wilkinson and Akrigg, 1992) and incubated as previously. Recombinant adenovirus for dominant negative MKK3b (RAdMKK3bA) (Wang et al, 1998) was kindly provided by Dr. Jiahuai Han (Scripps Research Institute, La Jolla, CA, USA). Escherichia coli β-galactosidase containing recombinant adenovirus (RAdLacZ) (Wilkinson and Akrigg, 1992) was kindly provided by Dr. Gavin W.G. Wilkinson (University of Cardiff).

4.10.4 Analysis of cell viability and proliferation (I)

Viability of PICSAR and control siRNA treated cSCC cells was determined using WST-1 cell proliferation reagent (Roche). After 48 and 72 hours of siRNA transfection cells were seeded in a 96-well plate and absorbance was measured in 450 nm (Multiskan FC microplate reader, Thermo Fisher Scientific) after 1 hour of incubation with the WST-1 reagent in +37°C.

4.10.5 Cell adhesion assays (II)

To study real-time cell adhesion cSCC cells were seeded on collagen I (PureCol, Advanced BioMatrix, Carlsbad, CA) or fibronectin (Merck Millipore, both 5 μg/cm^2) coated electronic microtiter plate (E-Plate 96, Roche) and cell adhesion was measured using the xCELLigence real-time cell analyzer (RTCA; Roche).

The formation of lamellipodia was studied by microscopic quantitation of cSCC cells. Cells were plated on collagen I or fibronectin coated 96-wells (both 5 μg/cm^2) and fixed 4 hours after plating (8% formaldehyde, 10% sucrose in PBS supplemented with 1 mM $MgSO_4$ and 1 mM $CaCl_2$) for 30 minutes in room temperature. The

number of cells with lamellipodia or spread cells was compared to the total cell number in each well from three parallel wells (three images per well) at 20x magnification.

4.10.6 Cell migration assays (I, II)

Cell migration was studied in wound healing assay either manually or using the the IncuCyte ZOOM® real-time cell imaging system. Cultures of cSCC cells were grown to confluency and hydroxyurea was added in 1 mM final concentration in DMEM with 10 % FCS to prevent proliferation of cells. After 6 hours cell monolayer was scratched and incubation was continued in 1% FCS with 0.5 mM hydroxyurea. Cells were imaged either using the Olympus IX70 inverted microscope (Olympus Optical Co.) or the IncuCyte ZOOM®. The data analysis was done either manually by measuring the mean area of the cell deprived scratch zone and comparing it to the 0h time point using ImageJ software (Schneider et al, 2012) or using the IncuCyte ZOOM 2014A software.

To study the migration of individual cSCC cells they were plated on ImageLock™ 96-wells coated with fibronectin or collagen I (both 5 μg/cm^2) and imaged every 10 minutes using the IncuCyte ZOOM® real-time cell imaging system. Individual migrating cells were tracked using the ImageJ software.

4.10.7 Cell invasion assay (III)

To study cell invasion cSCC cells were plated on collagen type I coated (5 μg/cm^2; PureCol, Advanced BioMatrix) ImageLock 96-well plate (Essen Bioscience) 24 hours after transfection with PRECSIT siRNAs or negative control siRNA and cells were let to adhere overnight. Cell monolayer was scratched and cells were overlaid with collagen type I solution by mixing type I collagen (PureCol) with 5X Dulbecco's Modified Eagle Medium (DMEM) and 0.2 M HEPES buffer (pH 7.4) at a ratio of 7:2:1, respectively. Finally, 1 M NaOH was added to obtain the final pH at 7.4. The collagen I solution (2.2 mg/ml) was let to polymerize for 2 hours at +37°C and cell culture medium with 0.5 % fetal calf serum was added on top. The gap closure was imaged and the relative cell invasion was quantitated using the IncuCyte ZOOM® 2016B software.

4.11 Human cSCC xenograft model (I, III)

For human cSCC xenograft model UT-SCC12A cells were transfected with PICSAR or control siRNA. UT-SCC7 cells were transfected with PRECSIT or control siRNA. Cells were detached 72 hours after transfection and injected subcutaneously in 100

µl of phosphate buffered saline (7×10^6 UT-SCC12A cells and 5×10^6 UT-SCC7 cells) into the back of 6 weeks old female SCID mice (CB17/Icr-*Prkdcscid*/IcrIcoCrl) (Charles River Laboratories, Wilmington, MA) (n = 7 for control and PICSAR knockdown tumors; n = 6 for control and n = 8 for PRECSIT knockdown tumors). Tumor size was measured twice a week and the volume of tumors was calculated as $V = (\text{length} \times \text{width}^2)/2$ (Euhus et al, 1986). Mice were sacrificed 18 or 16 days after tumor implantation and tumors were excised, weighed and fixed in fresh phosphate-buffered 4 % paraformaldehyde for 24 hours at +4°C. Fixed tumors were embedded in paraffin and sectioned for 5-µm-thick slices for immunostaining (Junttila et al, 2007).

4.12 Statistical analysis

The SPSS Statistics software, v. 20.0 and v. 25 (IBM, Armonk, NY) was used for statistical analyses. Two-tailed Student's *t*-test and two-tailed Mann-Whitney *U*-test were used for group comparisons. Fisher's exact test was used for statistical analysis with immunohistochemistry stainings.

5 Results

5.1 Expression of several lncRNAs is altered in cSCC cells compared to NHEKs (I, III)

A whole-genome-wide expression profiling was performed for cSCC cell lines (primary n=5, metastatic n=3) and NHEKs (n=4) using the SOLiD 3Plus system. The analysis revealed several differentially regulated lncRNA genes in cSCC cells compared to NHEKs (I, Figure 1a) (**Figure 6**). Among these, LINC00162 (PICSAR) was the most upregulated lncRNA in cSCC cells compared to NHEKs. As the second most upregulated lncRNA was LINC00346 (PRECSIT). These two lncRNAs were selected for further investigation.

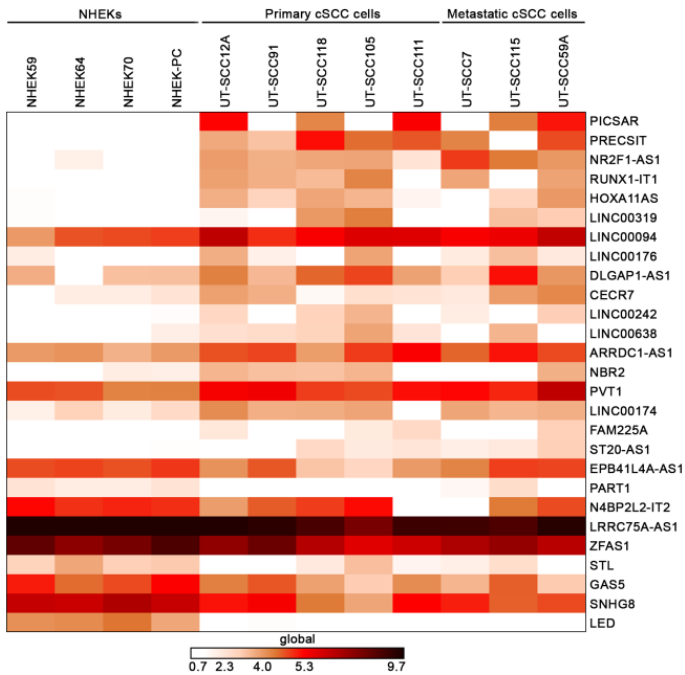

Figure 6. Expression profile of lncRNAs in cSCC cells and NHEKs. A heatmap showing significantly (P<0.05) regulated lncRNAs in cSCC cell lines compared to NHEKs. The Mann-Whitney U-test was used for statistical analysis.

5.2 LncRNAs PICSAR and PRECSIT are specifically upregulated in cSCC (I, III)

Analysis with qRT-PCR verified overexpression of PICSAR and PRECSIT in cSCC cell lines (n=6–8) compared to NHEKs (n=7–8) (I, Figure 1b; III, Figure 1a). PRECSIT localization was noted mostly in the nuclear cell fraction (III, Figure 1b), whereas PICSAR was enriched in the cytoplasmic cell fraction by qRT-PCR analysis (**Figure 7**). PICSAR was upregulated also in cSCC tissues *in vivo* by qRT-PRC analysis (n=6), whereas its expression in normal skin (n=7) was very low (I, Figure 1c).

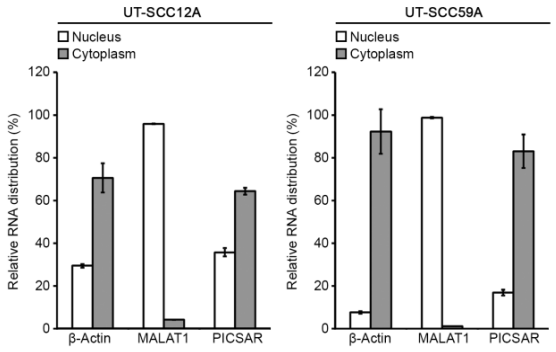

Figure 7. PICSAR is preferentially expressed in the cytoplasm of cSCC cells. qRT-PCR analysis of PICSAR expression in the nuclear and cytoplasmic cSCC cell fractions. β-Actin and MALAT1 were used as controls for cytoplasmic and nuclear RNA expression. Mean±SD is shown (n=3).

Mainly cytoplasmic signal was noted for PICSAR in cSCC cells using RNA *in situ* hybridization (RNA-ISH) but no PICSAR expression was noted in NHEKs in culture (I, Figure 1d, Supplementary Figure S2). Specific expression of PICSAR was noted also in tumor cells in tissue sections of xenografts established with human cSCC cells (UT-SCC12A) (I, Figure 1e). MMP-13 mRNA expression was detected in cSCC cells in the same xenografts as a positive control (I, Figure 1e) (Airola et al, 1997, Stokes et al, 2010). PRECSIT expression *in situ* was noted in xenografts established with human cSCC cells (UT-SCC7) showing the highest expression level at the edge of the tumors whereas in the inner tumor mass the expression of PRECSIT was relatively low (III, Figure 4e). Localization of PRECSIT was noted in the cytoplasm and nucleus of the tumor cells, with a prevalent appearance in the perinuclear region (III, Figure 4e).

To investigate PICSAR and PRECSIT expression *in vivo* in NS, SK, AK, cSCCIS and invasive cSCC formalin-fixed paraffin-embedded tissue samples were subjected to RNA-ISH analysis. Specific PICSAR expression was noted in tumor cells in AK (n=26), cSCCIS (n=20), and cSCC (n=21), whereas no signal was detected in normal skin (n=9) (I, Figure 2a–d). The number of PICSAR positive tissue sections was the highest in invasive cSCC (43 %), whereas all NS sections were negative for PICSAR (I, Figure 2e). The number of PICSAR positive sections was also higher in cSCCIS (35 %) when compared to AK (23 %) (I, Figure 2e). Primarily nuclear localization was noted for PRECSIT *in vivo* (III, Figure 1c-g). The percentage of PRECSIT

positive cells was significantly higher in invasive cSCC when compared with NS, SK, AK and cSCCIS (III, Figure 1h). PRECSIT expression was also higher in AK and cSCCIS when compared with SK (III, Figure 1h). Nearly half (49 %) of the invasive cSCCs showed strong PRECSIT expression (>30 % PRECSIT positive cells) whereas mostly weak (<15 % PRECSIT positive cells) or moderate (15-30 % PRECSIT positive cells) PRECSIT expression was noted in NS and SK (III, Figure 1i). PRECSIT copy number was quantified by counting the number of PRECSIT particles per cell. There was significantly higher percentage of cells expressing high level of PRECSIT (≥5 particles per cell) in invasive cSCC (III, Figure 1d) when compared to NS, SK, AK or cSCCIS (III, Figure 1j). In addition, the relative number of tumor cells detected with ≥5 PRECSIT particles correlated with the overall percentage of PRECSIT positive cells in tissue sections of SK, AK, cSCCIS and invasive cSCC (III, Supplementary Figure S2).

5.3 Regulation of lncRNAs in cSCC (I, III)

5.3.1 Regulation of PICSAR expression by the p38 MAPK pathway (I)

The regulation of PICSAR basal expression was investigated by specifically inhibiting p38 and ERK1/2 MAPKs in cSCC cells. By inhibiting all p38 isoforms (p38α/β/δ) by BIRB796 PICSAR expression was significantly upregulated (I, Figure 3a). Treatment with SB203580, inhibiting p38α and p38β isoforms, or with PD98059 inhibiting MEK1/2 upstream of ERK1/2 had no effect on the expression of PICSAR (I, Figure 3a). Next, expression of p38α and p38δ was inhibited in cSCC cell by siRNA-mediated knockdown or by delivering a dominant negative mutant of MAP kinase kinase 3b (RAdMKK3bA), an upstream activator of p38α and p38δ, into cSCC cells. Inhibition of p38α and p38δ expression in cSCC cells resulted in a significant upregulation of PICSAR (I, Figure 3b and c), in consistent with the findings with the p38 inhibitor assay.

5.3.2 Regulation of PRECSIT expression by the p53 pathway (III)

Most of the cSCC cells used in this study harbor mutations in *TP53* gene resulting in a nonfunctional p53 protein (III, Supplementary Table S1). The expression of p53 *in vivo* was examined by IHC analysis using an antibody that recognizes both the wild-type and mutated p53 and based on the p53 staining intensity the tissues were categorized in four groups; weak (+), moderate (++), strong (+++) and negative (-) (III, Figure 2a, Supplementary Table S1). Strong p53 staining or absence of p53 was

detected only in malignant tissues, cSCC, cSCCIS, and AK, indicating mutationally inactivated p53 in these tissues (III, Figure 2a and b). All NS lesions showed weak nuclear p53 staining in the basal epidermal keratinocytes (III, Figure 2a and b).

The p53 expression level was compared with PRECSIT expression in parallel tissue sections *in vivo*. High level of PRECSIT expression (>30 % PRECSIT positive cells) was noted in AK, cSCCIS and cSCC tissues within all p53 staining groups (weak, moderate, strong and negative), but there was no correlation between the relative number of PRECSIT positive cells and p53 intensity (III, Figure 2c). However, 56% of all cSCC tissue sections showed PRECSIT accumulation (≥5 PRECSIT particles per cell) and the majority of these tissue sections (45%) showed negative p53 immunoreactivity (III, Figure 2d and e, Supplementary Table S3). Interestingly, this represents 84% of all p53 negative cSCC tissue samples, indicating that accumulation of PRECSIT correlates with the absence of p53 expression in cSCC. PRECSIT accumulation was correlated with negative p53 immunoreactivity also in AK and cSCCIS, where PRECSIT accumulation was noted in 75 % of all p53 negative lesions (III, Figure 2e, Supplementary Table S3). In addition, the fraction of lesions with strong p53 staining intensity detected with PRECSIT accumulation was higher in cSCC tissues (41 %) when compared with AK and cSCCIS (10 %) (III, Figure 2e, Supplementary Table S3). In contrast, minority of the AK, cSCCIS (10 %) and cSCC tissue sections (10 %) detected with PRECSIT accumulation correlated with weak (+) p53 staining, as an indicative of a functional p53 *in vivo* (III, Figure 2e, Supplementary Table S3).

To support these observations, the qRT-PCR analysis of the p53 mutation bearing HaCaT cell line (Boukamp et al, 1988) showed significantly higher PRECSIT expression compared to NHEKs (III, Figure 2f). Furthermore, adenoviral delivery of a wild-type p53 into HaCaT or cSCC cells resulted in downregulation of PRECSIT expression, showing that the expression of PRECSIT is regulated by p53 (III, Figure 2g and h).

5.4 Molecular functions of lncRNAs in cSCC (I, II, III)

5.4.1 Regulation of cSCC cell migration and adhesion by PICSAR (I, II)

5.4.1.1 Alteration of the gene expression profile of cSCC cells after PICSAR knockdown (I, II)

To investigate the molecular mechanisms of PICSAR, RNA-seq was performed with PICSAR knockdown cSCC cells (UT-SCC12A, UT-SCC59A, and UT-SCC118) (I,

Figure 4a). Differentially expressed genes (DEG) after PICSAR knockdown were significantly associated with specific biofunctions *M-phase of tumor cell lines*, *transformation of tumor cell lines*, and *phosphorylation of L-serine* by the ingenuity pathway analysis (I, Figure 5a, upper panel). In addition, *ERK/MAPK signaling* was significantly associated ($P<0.0001$) with DEGs in PICSAR knockdown cSCC cells (I, Supplementary Figure S5).

The analysis of DEGs after PICSAR knockdown revealed significantly enriched GO terms such as *cell proliferation* ($P=5.5\times10^{-7}$), *response to wounding* ($P=4.0\times10^{-6}$), and *regulation of cell migration* ($P=1.4\times10^{-5}$) (I, Figure 5a, lower panel). GO terms *laminin binding* ($P=4.1\times10^{-4}$), *extracellular matrix binding* ($P=9.0\times10^{-4}$), and *peptidase regulator activity* ($P=1.1\times10^{-3}$) were also associated with DEGs after PICSAR knockdown in cSCC cells (I, Figure 5a, lower panel). Significantly enriched KEGG pathways associated with DEGs after PICSAR knockdown included *complement and coagulation cascades* ($P=6.6\times10^{-3}$), *hematopoietic cell lineage* ($P=2.3\times10^{-2}$), and *extracellular matrix-receptor interaction* ($P=2.4\times10^{-2}$) (I, Figure 5a, lower panel). The 50 most up- and downregulated genes ($P<0.05$, FC log2>0.7) after PICSAR knockdown are shown in Figure 5b (I). The full list of the top regulated genes in PICSAR knockdown cSCC cells is included in the Supplementary Tables S1 and S2 (I).

5.4.1.2 PICSAR regulates cSCC cell migration on collagen I and fibronectin (I, II)

To study the cellular functions of PICSAR cSCC cells were transfected with two PICSAR targeted siRNAs and control siRNA and harvested 72 hours after transfection. Effective PICSAR knockdown in cSCC cells was detected using RNA-ISH (I, Figure 4a, lower panel; Supplementary Figure S3) and qRT-PCR analysis (I, Figure 4a, upper panel; Supplementary Figure S4a). Analysis of cell migration after PICSAR knockdown was performed with two cSCC cell lines (UT-SCC12A and UT-SCC118). The wound healing assay showed that knockdown of PICSAR decreased cell migration significantly in comparison with the control siRNA transfected cells (I, Figure 4d and e, Supplementary Figures S4d and S4e).

To examine cell motility in more detail real-time cell migration assay was performed to track individual cell migration on fibronectin and collagen I. Decreased cell migration was noted in cSCC cells (UT-SCC12A and UT-SCC59A) after PICSAR knockdown on fibronectin and collagen I (II, Figure 1a; Supplementary Figure S1a).

Stably PICSAR overexpressing cSCC cells were generated by transfecting UT-SCC59A with PICSAR expression construct (pcDNA3.1_PICSAR) or empty vector (pcDNA3.1) and transfected cells were selected with Geneticin. PICSAR expression

level was confirmed by qRT-PCR of the transfected UT-SCC59A cells (II, Figure 3a). Migration of PICSAR overexpressing cSCC cells was studied in a wound healing assay. PICSAR overexpression increased migration of cSCC cells compared to control cells (II, Figure 3f).

5.4.1.3 PICSAR regulates cSCC cell adhesion and spreading on collagen I and fibronectin (II)

To quantitate real-time cell adhesion of cSCC cells after PICSAR knockdown cSCC cells (UT-SCC12A and UT-SCC59A) were transfected with PICSAR siRNA or control siRNA and plated on fibronectin or collagen I and cell adhesion was measured using the xCELLigence system. PICSAR knockdown significantly increased cSCC cell adhesion on collagen I and fibronectin compared to the control siRNA transfected cells (II, Figure 1b; Supplementary Figures S1b and S2a). In addition to dynamic monitoring of cell adhesion cells were fixed to quantitate cell spreading on fibronectin and collagen I. cSCC cells (UT-SCC12A and UT-SCC59A) were transfected with PICSAR siRNA or control siRNA and plated on fibronectin or collagen I. The morphology of PICSAR knockdown cSCC cells was less spherical compared to the control cells and the number of cells with lamellipodia was significantly higher in PICSAR knockdown cSCC cells (UT-SCC12A and UT-SCC59A) compared to the control siRNA transfected cells on fibronectin and collagen I (II, Figure 1c, Supplementary Figures S1c and S2b).

Adhesion of stably PICSAR overexpressing cSCC cells was significantly decreased on fibronectin and collagen I compared to the control cells (II, Figure 3e). Furthermore, PICSAR overexpression resulted in impaired cell spreading (II, Figure 3c). The relative number of spread cells was significantly decreased in PICSAR overexpressing cells compared to the control cells (II, Figure 3c).

5.4.1.4 PICSAR regulates integrin and Src expression in cSCC cells (I, II)

The bioinformatic analysis of the cSCC cell gene expression profile after PICSAR knockdown revealed several differentially expressed genes associated with specific biological processes, such as *extracellular matrix binding* and *extracellular matrix-receptor interaction* (I, Figure 5a), suggesting that PICSAR is regulating genes involved with cell adhesion. To study this in detail, DEGs specifically functioning in cell adhesion (Winograd-Katz et al., 2014) were examined in the PICSAR knockdown RNA-seq (II, Figure 2a). Several of them were significantly regulated, including genes coding for proto-oncogene *SRC* and integrins, particularly *ITGA2*, *ITGA5* and *ITGB1* encoding the α2, α5 and β1 integrin subunits.

Elevated levels of α2, α5 and β1 integrin mRNAs were noted in cSCC cells after PICSAR knockdown (II, Figure 2b; Supplementary Figure S3a). Furthermore, increased expression of α2, α5 and β1 integrins on the surface of cSCC cells was noted after PICSAR knockdown by flow cytometry analysis compared to the control siRNA transfected cells (II, Figure 2c; Supplementary Figure S3b). Immunofluorescence staining for α2 and α5 integrins showed similar localization to the cell surface and adhesion sites both in control siRNA and PICSAR siRNA transfected cSCC cells, indicating that the localization of α2 and α5 integrins is not affected by PICSAR knockdown (II, Figure 2d). In addition to integrin regulation, decreased Src protein level in cSCC cells was noted after PICSAR knockdown (II, Figure 3g; Supplementary Figures S4a and b).

PICSAR overexpression in cSCC cells resulted in significant decrease in α2, α5 and β1 integrin mRNA and protein levels on the cell surface, determined by qRT-PCR and flow cytometry analysis (II, Figures 3a and 3b). Immunofluorescence staining of PICSAR overexpressing cSCC cells revealed expression of α2 and α5 integrins on collagen I and fibronectin, however when compared to the control cells no distinct adhesion sites were detected likely due to decreased cell spreading (II, Figure 3d). In consistent with these results, a significant induction of Src expression was noted in PICSAR overexpressing cSCC cells (II, Figure 3g; Supplementary Figure S4c), suggesting a regulatory link between PICSAR, Src and integrins in mediating cSCC cells adhesion and spreading.

By complementary base pairing lncRNAs can bind to miRNAs and regulate mRNA stability indirectly and several miRNAs have been shown to regulate integrin expression (Chen et al, 2013). However, no potential miRNA-binding sites were found for PICSAR by using bioinformatic analyses, which could explain the regulation of integrin expression by PICSAR.

5.4.2 PICSAR promotes growth of cSCC cells by activating ERK1/2 (I)

5.4.2.1 Knockdown of PICSAR decreases cSCC cell viability (I)

Viability of cSCC cells was studied by determining the number of viable cells 48 and 72 hours after PICSAR knockdown. The number of viable cSCC cells (UT-SCC12A and UT-SCC118) was significantly decreased after PICSAR knockdown compared with control cells (I, Figure 4b; Supplementary Figure S4b). In addition, a marked decrease of expression levels for activated ERK1/2 and the Ki-67 proliferation marker were noted after PICSAR knockdown by analyzing the corresponding cell lysates (I, Figure 4c; Supplementary Figure S4c).

5.4.2.2 PICSAR increases activity of ERK1/2 pathway *via* inhibition of MAPK phosphatase DUSP6 (I)

The RNA-seq of PICSAR knockdown cSCC cells revealed *DUSP1* (dual-specificity phosphatase 1) among the most upregulated genes ($P<0.05$) (I, Figure 5b; Supplementary Table S1). It belongs to the MAPK phosphatase family and it dephosphorylates p38, JNK, and ERK (reviewed in Patterson et al, 2009). In addition to *DUSP1, DUSP6,* a specific negative regulator of ERK2 (reviewed in Patterson et al, 2009) was also noted to be upregulated after PICSAR knockdown (FC log2=1.09) (I, Figure 5c). Increased levels of DUSP6 mRNA and protein was noted after PICSAR knockdown in cSCC cells (I, Figures 5d and e; Supplementary Figure S6). When cSCC cells were treated with a DUSP6-specific inhibitor, phosphorylation of ERK1/2 was potently blocked (I, Figure 5f). However, PICSAR knockdown had no effect on the expression level of activated ERK1/2 in the presence of DUSP6 inhibitor, indicating DUSP6 as a regulatory link between PICSAR and ERK1/2 (I, Figure 5f). These results indicate that PICSAR promotes growth of cSCC cells *via* activation of ERK1/2 signaling pathway by downregulating DUSP6 expression.

5.4.2.3 Knockdown of PICSAR suppresses growth of cSCC xenografts *in vivo* (I)

A cSCC xenograft model using cSCC cells (UT-SCC12A) was used to investigate the role of PICSAR in cSCC progression *in vivo*. PICSAR siRNA1 and control siRNA transfected cSCC cells were incubated for 72 hours and injected subcutaneously into the back of the SCID mice. Tumor growth was measured twice a week. PICSAR knockdown resulted in a significant decrease in tumor growth already at the day 4 when compared with control tumors (I, Figure 6a). Tumors were harvested 18 days after implantation and weighed. The mass of PICSAR knockdown tumors was significantly lower compared with the control tumors (I, Figure 6b). PICSAR knockdown resulted in a significant reduction in the relative number of proliferating tumor cells in cSCC xenografts when compared with the control tumors (I, Figure 6c and d).

Taken together, based on these findings and with the approval of the HUGO Gene Nomenclature Committee we named this lncRNA (LINC00162) as p38 inhibited cutaneous squamous cell carcinoma associated lincRNA, PICSAR.

5.4.3 PRECSIT promotes invasion of cSCC cells by regulating STAT3 and matrix metalloproteinase expression (III)

5.4.3.1 Alteration of the gene expression profile of cSCC cells after PRECSIT knockdown (III)

Expression profiling of cSCC cells (UT-SCC7, UT-SCC59A and UT-SCC105) transfected with PRECSIT siRNA1 and control siRNA (III, Figure 3a) was performed with RNA-seq. The most significant GO terms associated with differentially expressed genes (DEG) in PRECSIT knockdown cSCC cells were biological processes such as *negative regulation of MAPK cascade* (P=8.2×10^{-5}) and *regulation of JAK-STAT cascade* (P=9.7×10^{-4}) (III, Figure 3b). *Jak-STAT signaling* (P=1.1×10^{-4}) was also associated with DEGs after PRECSIT knockdown by the KEGG pathway analysis (III, Figure 3b). The Reactome pathway analysis revealed *signaling by interleukins* (P=2.8×10^{-5}) and *cytokine signaling in immune system* (P=2.6×10^{-3}) as the most significantly associated pathways with DEGs after PRECSIT knockdown (III, Figure 3b).

5.4.3.2 Expression of STAT3 and MMPs are decreased in cSCC cells after PRECSIT knockdown (III)

One of the most frequent DEGs after PRECSIT knockdown was STAT3 (III, Figure 3c), among the top 40 most downregulated genes (P<0.05, FC log2=-1.05) (III, Figure 3d). In addition, several matrix metalloproteinase genes were downregulated (III, Figure 3d). Decreased STAT3 protein expression was confirmed by Western blot analysis of PRECSIT knockdown cells (III, Figure 3e). Also, decreased mRNA (III, Figure 3f) and protein levels (III, Figure 3g) of MMP-1, MMP-3, MMP-10 and MMP-13 were confirmed in UT-SCC7 cells after PRECSIT knockdown.

5.4.3.3 Knockdown of PRECSIT inhibits invasion of cSCC cells (III)

MMP-1 and MMP-13 have an important role in cancer invasion (reviewed in Ala-aho & Kähäri, 2005). In addition, STAT3 has been shown to regulate cell invasion by controlling expression of several MMP genes in cancer cells (Itoh et al., 2006; Zugowski et al, 2011; Xuan et al, 2015; Jia et al, 2017; Yuan et al, 2008). To investigate the cellular functions of PRECSIT, cSCC cells (UT-SCC7 and UT-SCC59A) were transfected with two PRECSIT targeted siRNAs and negative control siRNA and plated on collagen I. Cell invasion through a three-dimensional type I collagen matrix was followed by examining the gap closure for indicated time. Cell invasion was significantly reduced by PRECSIT knockdown in cSCC cells (III, Figure 4a).

Next, cSCC cells were treated with a specific STAT3 inhibitor S31-201, resulting in decreased levels of MMP-1, MMP3, MMP-10 and MMP-13 in cSCC cells (III, Figure 4b). In consistent with this, decreased invasion of cSCC cells was noted after treatment with STAT3 inhibitor (III, Figure 4c).

5.4.3.4 Knockdown of PRECSIT decreases growth of cSCC xenografts *in vivo* (III)

The role of PRECSIT in cSCC progression was studied *in vivo* using a cSCC xenograft model established with cSCC cells. PRECSIT knockdown resulted in a significant decrease in tumor growth compared with control tumors (III, Figure 4d). Histologic analysis did not reveal a notable difference in the tumor morphology between the control and PRECSIT knockdown groups (III, Figure 4d). However, the highest PRECSIT expression was noted at the invasive edge of the xenograft tumors, whereas it was expressed at a markedly lower level in the inner tumor mass (III, Figure 4e). In addition, active STAT3 was also localized at the invasive edge of the xenograft tumor (III, Figure 4e). Taken together, based on these findings and with the approval of the HUGO Gene Nomenclature Committee we named this lncRNA (LINC00346) as p53 regulated carcinoma-associated STAT3 activating long intergenic non-protein coding transcript, PRECSIT.

6 Discussion

6.1 LncRNAs as cancer biomarkers and therapeutic targets

6.1.1 The potential of lncRNA biomarkers in clinical diagnostics

A cancer biomarker can be used as a diagnostic indicator of the disease and some of them can be also used to estimate drug response or cancer prognosis. It may be for instance a gene mutation, a secreted molecule found in the serum or aberrant expression of a certain gene detected in the tumor tissue. With the current technology the cancer genomics is no longer restricted to studying the regulation of the cancer proteome but we are very aware that a lot is happening outside of the protein-coding genes, in the non-coding regions of the genome. The emerging evidence of non-coding RNA deregulation in cancer has encouraged the scientists to search for novel non-coding RNA markers with the help of high-throughput RNA sequencing technology. Long non-coding RNAs (lncRNAs) are excellent candidates for biomarkers as they are generally stable in body fluids, especially when delivered within extracellular vesicles and exosomes (Umu et al, 2018; Yuan et al, 2016). Currently there is at least one diagnostic test utilizing lncRNA detection in the clinical use (Deras et al, 2008) and many potential lncRNA-biomarkers are under development. When delivered outside of the cell lncRNAs can contribute to tumor progression for instance by manipulating the tumor microenvironment (Sang et al, 2018; Conigliaro et al, 2015) or inducing drug resistance (Qu et al, 2016; Lei et al, 2018). By detecting these kinds of regulators in combination with other cancer biomarkers they could bring valuable information to aid cancer diagnosis and treatment.

6.1.2 Therapeutic targeting of lncRNAs

The molecular background of cSCC is extensively investigated and based on several independent studies the mutational landscape and the gene expression profile of cSCC is fairly well described (Pickering et al, 2014; South et al, 2014; Li et al, 2015;

Inman et al, 2018). This is essential in order to identify better predictive and prognostic markers for cSCC. Moreover, by therapeutically targeting a specific cancer driver gene the oncogenic cell signaling cascade and tumor growth could be effectively inhibited. The massive burden of single-nucleotide mutations in cSCC (Pickering et al, 2014; South et al, 2014; Martincorena et al, 2015) poses a challenge to the clinicians to choose the right targeted therapy as it is difficult to distinguish between the prominent drug targets from the passenger mutations that may have little or no impact at all on the tumor growth. This is a serious issue particularly for patients with advanced cSCCs which have a high risk for recurrence and metastasis. Lastly, despite finding an effective treatment there is often a problem with drug resistance and the possibility of a crosstalk between different signaling pathways that may be dependent on a certain mutational combination, such as the unfortunate example of induced growth of cSCC tumors when using BRAF-inhibitors (reviewed in Wu et al, 2017).

The inherent genomic instability of cancer cells enables them to develop different ways to overcome host defense. In addition to the conventionally targeted oncogenic factors also RNAs have been included into the drug development. There has been a huge improvement in generating feasible delivery methods for RNA-based therapies, targeting not only cancer but other diseases as well, and while many of them are under development (reviewed in Kaczmarek et al, 2017) a few RNA-based medicines are already in clinical use (Finkel et al, 2017; Adams et al, 2018). By targeting lncRNAs it may be possible to specifically regulate, increase (Zucchelli et al, 2015; Indrieri et al, 2016) or decrease protein expression (Modarresi et al, 2012; Gong & Maquat, 2011) without the undesirable side-effects of conventional cancer drugs. This is particularly interesting with certain undruggable proteins which are challenging for designing small-molecule inhibitors. LncRNA targeting allows very tissue- and cell-specific treatment. Encouraging results have been gained from clinical trials testing a novel DNA-based therapy which exploits the use of a lncRNA promoter. In this case, H19 gene promoter is incorporated into a DNA-vector carrying a diphtheria toxin-A gene and the cancer cells with induced H19 expression are selectively killed by the toxin they produce from the DNA-vector (Gofrit et al, 2014; Lavie et al, 2017). H19 is a widely studied tumorigenic lncRNA (reviewed in Yoshimura et al, 2018) and this approach may be applicable for various cancers with active H19 expression. There are several lncRNAs with a tumorigenic function and targeting them by antisense oligonucleotides (Katsushima et al, 2016; Amodio et al, 2018) or synthetic ribozymes (Kim et al, 2017; Kharma et al, 2016) delivered in extracellular vesicles would be a favorable approach as a cancer treatment (reviewed in Jiang et al, 2017). Another strategy is to inhibit lncRNA interaction with a protein or protein complex and this kind of steric hindrance would prevent the undesired function of the lncRNA (Finkel et al, 2017; Sarma et al, 2010; Mahmoudi et al, 2009).

Currently, there are several potential lncRNA cancer markers and treatment approaches under investigation (reviewed in Chandra Gupta & Nandan Tripathi, 2017) and it seems that it is only a matter of time before lncRNAs can be utilized in clinical use.

6.2 The role of lncRNAs in cSCC progression

6.2.1 Evaluation of differentially expressed lncRNAs in cSCC

The functional role of lncRNAs in cSCC development is not well known. Valuable clues about functionally relevant lncRNAs can be obtained from transcriptomic profiling studies. However, the detailed characterization of lncRNAs *in vitro* and *in vivo* is needed to evaluate their potential use as cancer biomarkers or therapeutic targets. In this work two lncRNAs, PICSAR and PRECSIT, were identified and characterized, and they may have functional relevance in cSCC progression. Based on the transcriptome profiling PICSAR and PRECSIT were noted among the most differentially expressed lncRNAs in cSCC cells compared to NHEKs. Localization of PICSAR was noted primarily in the cytoplasm, whereas PRECSIT was mainly localized in the nucleus by analyzing cellular fractions of cSCC cells. Their expression was further evaluated *in situ* in a panel of tissue samples from NS, AK, SK, cSCCIS and invasive cSCC. Expression of both lncRNAs was significantly induced in cSCC when compared to NS. Expression of PICSAR was absent in NS, whereas PRECSIT was expressed at very low level, suggesting that both may be prominent markers in early cSCC development.

There are several gene expression profiling studies of cSCC available, however the lncRNA expression data of cSCC is very limited. This is partly due to the fact that most of the newly annotated lncRNA transcripts are not included in these gene expression microarrays. To our knowledge, we are the first ones to demonstrate lncRNA expression *in situ* in cSCC tissues, indicating the lack of detailed characterization of these molecules in cSCC. Very likely there are several functional lncRNAs yet to be discovered. While most of the transcriptional profiling studies of cSCC are focused on protein coding mRNAs, currently only one lncRNA expression profiling study of cSCC has been published (Sand et al, 2016). Supporting our work, PICSAR was reported also as one of the top induced lncRNAs in cSCC in that study (Sand et al, 2016).

Interestingly, comparison of other known lncRNAs with our RNA-seq analysis of cSCC cells and NHEKs (GSE66412) revealed that lncRNAs TINCR and DANCR were also regulated, even though they did not reach the statistical significance. TINCR is induced during epidermal differentiation and its downregulation may be associated with cSCC (Kretz et al, 2013; Ponzio et al, 2017). In consistent with this,

TINCR was downregulated in cSCC cells compared to NHEKs based on our RNA-seq analysis (GSE66412). Conversely, DANCR expression was induced in four out of eight cSCC cell lines compared to NHEKs, showing a similar trend with the lncRNA expression profiling study by Sand and colleagues (Sand et al, 2016). DANCR is not implicated with cSCC in the literature, however its role in suppressing epidermal differentiation may suggest a tumorigenic link for DANCR in cSCC progression. Additionally, LINC00319 which was shown to be induced in cSCC (Li et al, 2018) was also upregulated in five out of eight cSCC cell lines when compared to NHEKs in our RNA-seq data (GSE66412). Similarly, LINC00520 which was recently shown to be downregulated in cSCC (Mei & Zhong, 2019) showed analogous downregulation in our RNA-seq analysis (GSE66412). Two known tumor suppressor lncRNAs, MEG3 and GAS5, were downregulated in cSCC cells compared to NHEKs based on our RNA-seq analysis (GSE66412). Interestingly, MEG3 expression was noted in only two out of six cSCC cell lines, indicating epigenetic MEG3 gene silencing in these cells, which is frequently reported in many cancers (Gao et al, 2017; Zhang et al, 2010; Anwar et al, 2012).

6.2.2 LncRNA PICSAR promotes cSCC growth by regulating ERK1/2 activity

The molecular pathogenesis of cSCC is widely studied and aberrant activation of the MAPK pathway is one of the central drivers of the disease (Lambert et al, 2014; Su et al, 2012; Einspahr et al, 2012). The p38 MAPK is an important factor in regulating cellular stress response, such as the solar UV-radiation, however activation of p38 is strongly implicated in skin carcinogenesis (Schindler et al, 2009; Liu et al, 2013; Junttila et al, 2009), in particular the p38α and p38δ MAPK isoforms that are predominantly expressed by cSCC cells (Junttila et al, 2009). To study the role of p38 in PICSAR expression, p38 activity was blocked by using specific p38 inhibitors, siRNAs and adenoviral delivery of dominant negative mutant of MKK3b, an upstream activator of p38α and p38δ. Inhibition of the p38α and p38δ resulted in PICSAR upregulation. However, inhibition of ERK1/2 MAPK did not affect PICSAR expression, indicating PICSAR as a target for p38 signaling pathway in cSCC cells.

The functional studies revealed that PICSAR knockdown suppressed the proliferation and migration of cSCC cells and growth of cSCC tumors *in vivo*. Knockdown of PICSAR significantly decreased the levels of activated ERK1/2, a well-studied transcription factor controlling cell proliferation. ERK1/2 is a downstream effector on the MAPK pathway and it is shown to be activated by UVA radiation (He et al, 2004; Bachelor & Bowden, 2004). Moreover, mutational activation of EGFR leads to sustained activation of the RAS-RAF-MEK-ERK MAPK pathway, supporting the role of ERK1/2 activation in cutaneous carcinogenesis (reviewed in

Wee & Wang, 2017; Uribe & Gonzalez, 2011). These results suggest that the functional effect of PICSAR involves regulation of the ERK1/2 pathway. RNA-seq for PICSAR knockdown cSCC cells was performed and among the most upregulated genes after PICSAR knockdown was DUSP1 (dual specificity phosphatase 1) encoding a phosphatase with a wide substrate specificity for various MAP kinases (reviewed in Patterson et al, 2009). In addition to DUSP1, DUSP6 was also upregulated in PICSAR knockdown cSCC cells. DUSP6 is localized both in the cytoplasm and nucleus, and it is activated on binding to ERK2 and it specifically dephosphorylates and inactivates ERK2, but not JNK or p38 MAP kinases (Groom et al, 1996; Muda et al, 1998). In this context, a possible regulatory link between PICSAR, DUSP6 and ERK1/2 was further investigated. In the presence of DUSP6 inhibitor PICSAR knockdown had no effect on ERK1/2 activation, suggesting that PICSAR is regulating ERK1/2 activation *via* DUSP6. PICSAR may inhibit DUSP6 expression in cSCC cells by regulating its mRNA stability, directly or indirectly, indicated by the cytoplasmic localization of PICSAR. Together, these findings identify PICSAR as a tumorigenic lncRNA in cSCC by activating ERK1/2 and inducing cSCC cell growth *via* DUSP6 suppression (**Figure 8**).

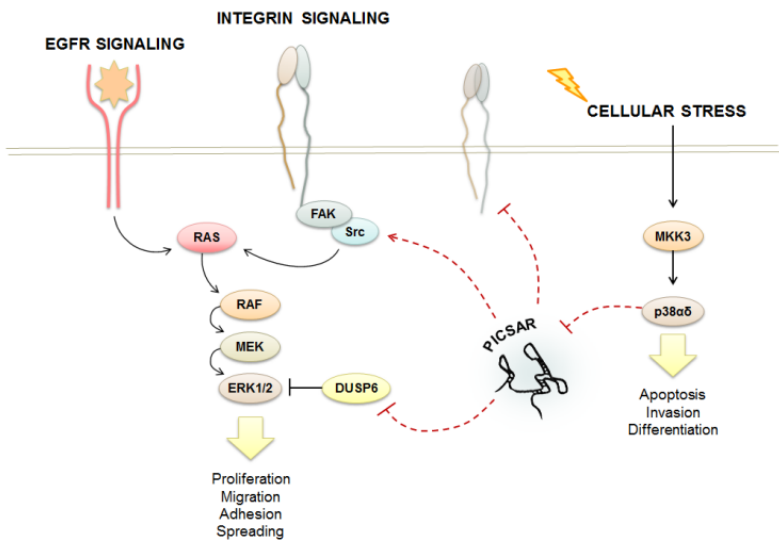

Figure 8. Proposed molecular function for PICSAR in cSCC.

Very little is currently known about PICSAR in cancer. PICSAR has been shown to be upregulated in MDA-MB-231 cells when compared to MCF-7 cells (Shi et al, 2017). These are both commonly used breast cancer cell lines but they differ greatly in their molecular background. The MCF-7 cells present a luminal ephitelial phenotype with oestrogen receptor expression whereas the MDA-MB-231 cells are

highly aggressive, lacking oestrogen and progesterone receptor expression, and they present a mesenchymal phenotype (reviewed in Holliday & Speirs, 2011). PICSAR expression was detected in both cell lines but it was substantially induced in MDA-MB-231 compared to MCF-7 cells, indicating an association with breast cancer progression (Shi et al, 2017). Conversely, PICSAR has been shown to be downregulated in stage I lung adenocarcinoma, suggesting its tumorigenic function may be specific for only certain cancer types (Tian et al, 2017). PICSAR upregulation in cSCC cells and breast cancer cell lines (Shi et al, 2017) may tell something about its functional role in the epithelial cells. Lung adenocarcinomas originate from the lung epithelium as well, however their molecular background is different from lung squamous cell carcinomas and they arise from different anatomical locations (reviewed in Kadara et al, 2016). PICSAR has been reported to be expressed in the brain tissue, specifically in the white matter (Mills et al, 2013). In addition, PICSAR downregulation is associated with male infertility and defective spermatogenesis (Lü et al, 2015). This supports a hypothesis that PICSAR may have an important functional role in maintaining normal homeostasis in certain cell types, however if induced in the inappropriate tissue it may promote cancer progression.

6.2.3 The functional role of PICSAR in cSCC cell migration and adhesion

To further characterize the functional mechanism of PICSAR its role in cell migration was investigated. Decreased migration of individual cSCC cells was noted on collagen I and fibronectin after PICSAR knockdown together with a significant increase in cell adhesion on these matrices. Also, the morphology of PICSAR knockdown cells was less spherical and the cells had more lamellipodia compared to control cells. The RNA-seq data of PICSAR knockdown cells was explored to find differentially expressed genes specially mediating cell adhesion. Several of these genes were regulated, particularly ITGA2, ITGA5 and ITGB1 genes encoding α2, α5 and β1 integrin subunits. They form the functional heterodimeric α2β1 and α5β1 membrane proteins, which function as cell surface receptors for type I collagen and fibronectin. Their mRNA expression was increased after PICSAR knockdown and the flow cytometry analysis confirmed increased expression of the α2, α5 and β1 integrins also on the cell membrane. These observations indicate that PICSAR is regulating cell adhesion and migration by mediating integrin expression in cSCC cells. Indeed, cell migration is dependent on the optimal balance in integrin expression and the changes in the affinity of integrin-ligand binding (Palecek et al, 1997). With increased integrin expression cell adhesion is also increased due to higher number of integrin bonds to the extracellular matrix, resulting in decreased migration rate (Palecek et al, 1997).

To support our findings, stably PICSAR overexpressing cSCC cells were generated to study how this affects cell adhesion and migration. The mRNA levels of α2, α5 and β1 integrins were significantly decreased in PICSAR overexpressing cells. Also, their expression on the cell surface was decreased based on the flow cytometry analysis. PICSAR overexpression resulted in impaired cell spreading and the number of spread cells was significantly decreased in PICSAR overexpressing cells. In addition, adhesion of PICSAR overexpressing cSCC cells was decreased together with an increase in cell migration. These observations are in accordance with our findings with PICSAR knockdown cSCC cells, supporting the role for PICSAR in cSCC cell adhesion and migration.

The exact molecular mechanism how PICSAR controls integrin expression remains unanswered. The level of Src expression was noted to correlate with PICSAR expression. Src expression was decreased after PICSAR knockdown and increased in PICSAR overexpressing cells, indicating a regulatory link between these molecules. Src has an important role in regulating integrin-mediated cell spreading and adhesion by a direct interaction with integrins, but it also regulates cell motility by controlling focal adhesion turnover (Fincham & Frame, 1998; reviewed in Playford & Schaller, 2004). The morphological effects that were observed in cSCC cells after PICSAR knockdown or overexpression are supported by the previous reports where the introduction of a constitutively active variant of cellular Src into normal cells leads to a disruption of cell-matrix adhesions and a rounded phenotype (Wang & Goldberg, 1976; Fincham et al, 1995). In contrast, the catalytically inactive c-Src-mutant increases actin stability and formation of enlarged focal adhesions, which eventually leads to decreased cell motility (Fincham & Frame, 1998). These findings suggest that PICSAR mediates adhesion and migration of cSCC cells by regulating integrin expression on the cell surface. PICSAR regulates also the expression of Src, which may contribute to regulating cell adhesion by mediating integrin affinity (Li et al, 2002) and actin stability (Fincham & Frame, 1998) (**Figure 8**).

6.2.4 LncRNA PRECSIT promotes cSCC progression by regulating STAT3 and MMP expression

LINC00346, which was later named PRECSIT, was found among the most upregulated lncRNAs in cSCC cells compared to NHEKs, suggesting a tumorigenic role in cSCC progression. PRECSIT (LINC00346) upregulation has been reported in breast, bladder, pancreatic, non-small cell lung and hepatocellular cancers (Liu et al, 2016; Ye et al, 2017; Zhang et al, 2018; Wang et al, 2017; Zhang et al, 2015), however its subcellular localization, expression *in situ* or the detailed functional mechanism in these cancers have not been investigated.

The low level of PRECSIT expression in NHEKs and normal skin suggested a correlation between the p53 mutational status and PRECSIT, as the p53 is frequently mutated early in cSCC carcinogenesis (Pickering et al, 2014; South et al, 2014). The mutational status of the p53 protein is routinely assessed in many cancer types by visualizing the nuclear accumulation of the mutant p53 by immunohistochemical analysis (reviewed in Soussi & Béroud, 2001). Total absence of the protein may also imply nonsense mutation in *TP53* resulting in a lack of functional p53 (Sakatani et al, 1998). The p53 staining intensities in our panel of tissue sections of normal skin, seborrheic keratosis, actinic keratosis, cSCC *in situ* and invasive cSCC were in accordance with the previous reports, where the strong or moderate nuclear accumulation of p53 is noted early in cSCC carcinogenesis (Einspahr et al, 1999; Bukhari et al, 2009; Kim et al, 2006; Onodera et al, 1996). Interestingly, *TP53* mutations are not seen in seborrheic keratoses, which are benign skin tumors with a clear UV mutation signature (Hafner et al, 2010; Duperret et al, 2014). In accordance with this, a very low level of nuclear accumulation of mutated p53 was noted in seborrheic keratosis tissue sections, indicating a functional p53 *in vivo* in these lesions.

PRECSIT expression was examined together with p53 expression levels *in vivo* in parallel tissue sections. Low or moderate PRECSIT expression was noted in most of the NS and SK tissues with low p53 staining intensity, suggesting that PRECSIT is suppressed by functional p53 expressed *in vivo* in these lesions. A high level of variation in PRECSIT expression level and p53 staining intensities was noted in AK, cSCCIS and invasive cSCC tissues and it seems that the percentage of PRECSIT positive cells is not directly correlated with p53 mutational status. However, when the p53 staining intensities was compared in tissue samples detected with PRECSIT accumulation (≥ 5 PRECSIT particles per cell) the majority of these cSCC lesions were negative for p53, suggesting that a high PRECSIT copy number per cell could be related to the absence of p53 expression *in vivo*. It remains to be elucidated whether a very low basal level of PRECSIT is actually important for normal regulation of keratinocytes, whereas a significant induction of PRECSIT due to mutationally inactivated p53 could be associated with cSCC progression. Significantly higher expression of PRECSIT was noted in HaCaT cells with homozygously mutated p53 compared with NHEKs with functionally active p53. Moreover, adenoviral delivery of the wild-type p53 into HaCaT or cSCC cells with mutated p53 resulted in downregulation of PRECSIT expression in cSCC cells. These results show for the first time that PRECSIT is a p53 regulated lncRNA in cSCC cells.

The regulatory network between lncRNAs and p53 has been actively studied and several p53-regulated lncRNAs have been identified so far (Sánchez et al, 2014; Léveillé et al, 2015). *Vice versa*, lncRNAs such as MALAT1, RoR and MEG3 have been shown to regulate p53 (Tripathi et al, 2013; Zhang et al, 2013; Zhou et al, 2007).

However, their cellular mechanisms of action differ from one another. A recent study shows that MALAT1 can regulate acetylation of p53 by binding to nuclear protein deleted in breast cancer 1 (DBC1) thus inhibiting the interaction of DBC1 with sirtuin 1 (SIRT1), a histone deacetylase known to deactivate p53 (Chen et al, 2017). MEG3 instead promotes p53 stability indirectly by downregulating the mouse double minute 2 homolog (MDM2) (Zhou et al, 2007). MEG3 has been also shown to inhibit cell proliferation in the absence of p53, suggesting a tumor suppressive function for MEG3 (Zhou et al, 2007). The functional mechanism how p53 regulates PRECSIT expression remains unanswered. It may be that PRECSIT is transcriptionally inhibited by a specific transcription factor which is induced by p53 activation, or PRECSIT may be also epigenetically regulated.

To date, only few studies have reported about functionally relevant lncRNAs in cSCC, in addition to our studies. A recent study reveals MALAT1 as a pro-carcinogenic lncRNA in cSCC (Zhang et al, 2019). The authors demonstrate a functional mechanism for MALAT1 in interacting with c-MYC and transcriptionally activating kinectin 1 (KTN1) which results in positive regulation of EGFR protein expression (Zhang et al, 2019). In addition to MALAT1, LINC00319 was shown to be upregulated in cSCC and its expression was correlated with tumor size and lymphovascular invasion (Li et al, 2018). Supporting this finding, in our RNA-seq analysis of NHEKs and cSCC cells (GSE66412) LINC00319 expression is very low in NHEKs but induced in five out of eight cSCC cell lines. LINC00319 was shown to promote cell proliferation, migration and invasion (Li et al, 2018). Interestingly, induction of MMP-2 and MMP-9 was shown to correlate with LINC00319 upregulation and cell invasion. LINC00319 regulatory mechanism was demonstrated by regulation of cyclin-dependent kinase 3 expression *via* miRNA binding (Li et al, 2018), however the exact way how this affects MMP expression is not clear.

Marked downregulation of MMP-1, MMP-3, MMP-10 and MMP-13 expression was noted both at mRNA and protein levels after PRECSIT knockdown in cSCC cells and this was demonstrated to be a result of decreased levels of activated STAT3 after PRECSIT knockdown. STAT3 functions as a transcription factor for several MMPs, including MMP-1 and MMP-3 (Itoh et al, 2006; Tsareva et al, 2007), and it has shown to mediate invasion signaling *via* MMP induction. MMP-3 and MMP-10 are stromelysins, capable of degrading several ECM-components and they can promote cSCC progression by activating latent MMPs, including MMP-1 and MMP-13 (reviewed in Ala-aho & Kähäri, 2005). Accordingly, elevated expression of MMP-3 and MMP-10 has been reported in cSCC (Boyd et al, 2009; Kerkelä et al, 2001). Additionally, MMP-13 is specifically expressed in head and neck SCCs (HNSCC) (Stokes et al, 2010) and in malignant squamous epithelium of the skin (Airola et al, 1997) and it is potently inhibited by p53 (Ala-aho et al, 2002). Therefore, these results

identify a mechanism how p53 inhibits invasion of cSCC cells by suppressing the expression of PRECSIT (**Figure 9**).

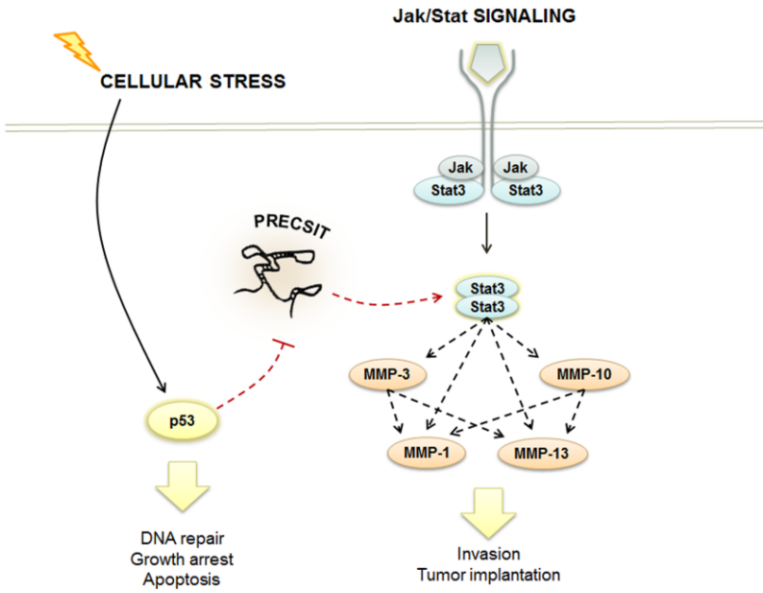

Figure 9. Proposed molecular function for PRECSIT in cSCC.

Decreased invasion of cSCC cells through collagen type I matrix was noted after PRECSIT knockdown. Additionally, inhibition of STAT3 by a small-molecule inhibitor resulted in decreased levels of MMPs and cell invasion, supporting the role for STAT3 in controlling invasion of cSCC cells. Overall, these observations indicate that the decreased invasion of cSCC cells noted after PRECSIT knockdown is due to decreased levels of MMPs, which in turn are dependent on transcriptional activation by STAT3. Decreased level of activated STAT3 was noted after PRECSIT knockdown, indicating that PRECSIT specifically regulates invasion of cSCC cells *via* STAT3 signaling (**Figure 9**).

The *in vivo* xenograft assay revealed that PRECSIT knockdown results in decreased tumor growth. When looking at PRECSIT expression in the control xenograft tumors the highest level of PRECSIT expression was noted at the tumor edges with a similar localization with active STAT3 when compared with the inner tumor mass, indicating that PRECSIT may be associated with tumor invasion and proteolytic activity of MMPs at the invading edge of tumors (reviewed in Kessenbrock et al, 2010). It may be that the decreased tumor growth after PRECSIT knockdown is a result of impaired tumor cell implantation and organization of the ECM at the early stage of tumor formation. Together, these findings indicate that

PRECSIT promotes progression of cSCC by specifically regulating invasion of cSCC cells *via* STAT3 signaling (**Figure 9**).

7 Summary and Conclusion

In this study the role of lncRNAs in cSCC progression was studied in culture and *in vivo*. Two lncRNAs, LINC00162 and LINC00346, were selected for investigation as they were the top most induced lncRNAs in cSCC cells when compared to NHEKs, suggesting them as prominent tumor promoting factors.

Induced expression of LINC00162 and LINC00346 was noted in cSCC cells in culture and in tissues of AK, cSCCIS and invasive cSCC *in vivo* when compared to NHEKs and normal skin. Primarily cytoplasmic localization was noted for LINC00162, whereas LINC00346 was mainly localized to the nucleus.

LINC00162 expression was shown to be inhibited by the p38 signaling pathway. Knockdown of LINC00162 expression resulted in decreased ERK1/2 activation, inhibition of cell proliferation and migration, and inhibition of growth of cSCC xenografts *in vivo*. RNA-seq of cSCC cells after LINC00162 knockdown revealed altered expression of several genes, including DUSP6, an ERK2 specific dual-specificity phosphatase. In the presence of DUSP6 inhibitor LINC00162 knockdown did not lead to decreased ERK1/2 activation, indicating DUSP6 as a regulatory link between LINC00162 and ERK1/2. Based on these observations, this lncRNA was named PICSAR (p38 inhibited cutaneous squamous cell carcinoma associated lincRNA). PICSAR was also shown to mediate cSCC cell adhesion and migration on fibronectin and collagen I by regulating the expression of cell surface receptor integrins.

Expression of LINC00346 was shown to be regulated by the p53 pathway. RNA-seq of cSCC cells after LINC00346 knockdown revealed STAT3 as one of the top downregulated genes together with MMP-1, MMP-3, MMP-10 and MMP-13. Knockdown of LINC00346 suppressed cSCC cell invasion in culture and growth of human cSCC xenografts *in vivo*. Decreased invasion was demonstrated to be a result of decreased level of activated STAT3 and production of MMPs. Based on these findings it was named PRECSIT (p53 regulated carcinoma-associated STAT3-activating long intergenic non-protein coding transcript).

In conclusion, PICSAR and PRECSIT are lncRNAs with a tumorigenic role in cSCC. They may serve as novel biomarkers and putative therapeutic targets for cSCC progression.

Acknowledgements

This work was carried out in the Department of Dermatology and Venereology, MediCity Research Laboratory, and the Cancer Research Laboratory of the Western Cancer Centre of the Cancer Center Finland (FICAN West) in the University of Turku and Turku University Hospital. I thank the directors of the MediCity Research Laboratory and the Cancer Research Laboratory, Professor Sirpa Jalkanen and Docent Pia Vihinen, respectively, for providing excellent working facilities for performing high-quality research.

I would like to express my deepest gratitude to my supervisor Professor Veli-Matti Kähäri for his invaluable guidance and support throughout the PhD process. I highly appreciate your expertise and commitment to this work. Moreover, I am truly grateful to you for believing in me and giving me the best possible training how to stand on my own two feet in the scientific world.

I am very fortunate to have Docent Liisa Nissinen as my other supervisor and I sincerely thank her for the infinite patience and encouragement during these years. I am very grateful for your helpful guidance and all the insightful brainstorming sessions and discussions about science and non-science. Your passion for research is contagious, and your great attitude and optimism is something I will try to hold on to when things do not work as planned.

I want to thank the members of my doctoral thesis committee, Professor Jyrki Heino and Professor Klaus Elenius for their expert advise and contribution to my work. I would also like to extend my appreciation to Professor Tuula Salo and Professor Tapio Visakorpi for reviewing my thesis and providing valuable feedback.

I am grateful to all co-authors Docent Liisa Nissinen, Doctors Pilvi Riihilä, Mehdi Farshchian, Atte Kivisaari, Docent Markku Kallajoki, Professor Juha Peltonen, Adjunct Professor Sirkku Peltonen and Professor Jyrki Heino for excellent collaboration. Collectively, the extensive knowledge of all of you has been crucial to this work. Personnel of the Turku Bioscience services, Jouko Sandholm, Markku Saari, Ketlin Adel, Asta Laiho and personnel of the Histology Core of the Institute of Bioscience, Sinikka Collanus and Erica Nyman are warmly acknowledged for their expert technical assistance. Last but not least, I want to thank all the helpful people

in the MediCity Research Laboratory and Medisiina D Cancer Research Laboratory for their kind assistance.

I warmly thank the director of the Turku Doctoral Programme of Molecular Medicine (TuDMM) Adjunct Professor Kati Elima and the coordinator Eeva Valve, as well as the members of the TuDMM student council for organizing first-rate scientific events, training and also non-scientific activities within the programme. Through the doctoral programme I have met a number of wonderful new people and I am very delighted for all the good times and unforgettable moments spent with fellow students.

None of this would have been possible without the strong support from the current and past members of *the Skin Cancer and Proteinases* (SkiCap) group, Liisa Nissinen, Pilvi Riihilä, Mehdi Farshchian, Mervi Toriseva, Niina Hieta, Kristina Viiklepp, Pegah Rahmati Nezhad, Jaakko Knuutila, Lea Toikka, Kiira Houtsonen, Riitta Yrjönkoski, Sari Pitkänen and Johanna Markola. Special thanks to Sari and Johanna for your skillful technical assistance in the lab. Moreover, I want to thank you all for your friendship and all the joyful moments spent together – thanks for a pleasant journey!

”The more the merrier” applies when a companionship is extended to the neighboring labs. I want to warmly thank the members of the Professor Heino’s and Professor Elenius’ group not just for their help and mutual sharing of resources on a critical moment, but also for all the cheerful times during these years.

I am grateful to all my wonderful friends and partners in crime, who have been there for me. Kiitos Erkku, Ilana ja Pihla ystävyydestä ja virkistävistä hetkistä työn ulkopuolella! Rakkaat ystävät ympäri Suomea, Ville, Mirka, Jani, Sandy, Mikko, Sanna T., Johanna, Jesse, Vesa, Sanna M., kiitos ihanista juttutuokioista, illanvietoista, laskettelureissuista sekä muista seikkailuista! Thanks, Mario, for enjoyable dinner parties and insightful discussions about the miraculous life of a grad student! Lämmin kiitos JepJepJep:ille, Laipolle, Hannulle, Punkulle, Karviselle, Lealle ja Lauralle sunnuntai-iltojen vireyttävästä kuntoutustoiminnasta homeisella treenikämpällä sekä upeasta musiikkiterapiasta! Kiitokset torstai-iltojen vakiosulkkispoppoolle, Matille, Sofialle ja Vesa-Matille sekä muille tähtivieraille hyvistä peleistä ja muusta oheistoiminnasta! How you spend your spare time correlates directly to your overall well-being and motivation on the workplace, therefore I thank you all for sharing good times together.

I am honored to have such a loving and supporting family and I cannot thank them enough for getting where I am now. Kiitos kaikesta, äiskä ja iskä! Sisarusten välillä pätee aina kirjoittamaton sopimus keskinäisestä avunannosta, mutta siitäkin huolimatta olette maailman parhaita ja rakkaimpia ihmisiä, Outi ja Vesku, vilpittömät kiitokset siitä! Kiitos, ihanat kummivanhemmat Siiri ja Pekka kannatuksesta ja rentouttavista Koivulan peli-illoista, Peppi ja Lasu arvokkaasta

kulttuurikasvatuksesta. Sydämelliset kiitokset omille Turun äiskälle ja iskälle, Helille ja Ekille kaikesta avusta ja kannustuksesta – minulla on onni olla osa tätä perhettä. Kiitos, että olette kasvattaneet niin hyväluonteisen ja rakastavan pojan (kaksikin!) tähän maailmaan.

Finally, I wish to thank my dear husband, Aleksi, for everything. I am so grateful for your patience and loving support throughout these years. You mean the world to me.

University of Turku Graduate School Funding, Turku University Foundation, The Finnish Cancer Research Foundation, The Cancer Society of South-West Finland, The state research funding of the Turku University Hospital, The Kymenlaakso Regional Fund of the Finnish Cultural Foundation, Instrumentarium Science Foundation, Ida Montin Foundation, The Paulo Foundation and The Maud Kuistila Memorial Foundation are gratefully acknowledged for the financial support of this work.

Turku, June 18, 2019

Minna Piipponen

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Annales Universitatis Turkuensis

UNIVERSITY OF TURKU

Painosalama Oy, Turku , Finland 2019

ISBN 978-951-29-7716-1 (PRINT)
ISBN 978-951-29-7717-8 (PDF)
ISSN 0355-9483 (Print) ISSN 2343-3213 (Online)