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Non-coding RNAs and Extracellular Vesicles in Cutaneous Squamous Cell Carcinoma

CHEN LI

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Abstract

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Cutaneous squamous cell carcinoma (cSCC) ranks among the most widespread malignancies with metastatic potential. Investigating the molecular mechanism of tumorigenesis will enhance our understanding of cSCC. Aberrant expression of non-coding RNAs has been extensively reported in human cancers. Here, we summarize our work exploring the role of a microRNA (miRNA) (Paper I) and a long non-coding RNA (lncRNA) (Paper II and III) in cSCC. Additionally, we discuss the role of cSCC-derived extracellular vesicles (EVs) in tumor formation (Paper IV).

In Paper I, we explored the function of miR-130a in cSCC. We reported that miR-130a expression was downregulated in cSCC under the regulation of the MAPK pathway. We demonstrated a tumor suppressor role of miR-130a in cSCC: ectopic overexpression of miR-130a suppressed malignant behaviors of human cSCC cells and inhibited primary tumor growth in cSCC xenograft models. Mechanistically, we revealed a link between MAPK and BMP/SMAD signaling pathways, which was mediated by the direct target of miR-130a, ACVR1.

In Paper II, we investigated the role of lncRNA PVT1 in cSCC. Elevated PVT1 expression in cSCC, under MYC regulation, suggested it may contribute to keratinocyte transformation. Subsequently, we revealed that PVT1 exerted an oncogenic role in cSCC through regulating CDKN1A/p21 expression and preventing cellular senescence. We identified exon 2 as a crucial element for maintaining PVT1's oncogenic role. **In Paper III**, we further investigated the underlying mechanism for the oncogenic role of PVT1 in cSCC. Our data revealed that PVT1 is mainly distributed in the nuclei of cSCC cells and the exon 2 is essential for nuclear localization of PVT1. Furthermore, we identified several subunits of the transcription-export (TREX) complex as interacting partners of PVT1 and demonstrated that PVT1 modulated the function of the TREX complex in nuclear export of poly $(A)^+$ RNAs.

In Paper IV, we found that cSCC cells secreted more EVs than primary keratinocytes. Blocking cSCC EV production suppressed xenograft growth, indicating a crucial role of cSCC cell-derived EVs in tumor development. Transcriptome analysis on xenograft tissues suggested that cSCC cell-derived EVs contribute to extracellular matrix organization. Further experiments indicated that metastatic cSCC cell-derived EVs efficiently educated dermal fibroblasts into cancer-associated fibroblasts. Additionally, metastatic cSCC cell-derived EVs activated the TGFβ signaling pathway in dermal fibroblasts. Collectively, our study suggested that cSCC cell-derived EVs play a key role in regulating cSCC development through modulating cancer-stroma communication.

Keywords: cutaneous squamous cell carcinoma, microRNA, long non-coding RNA, extracellular vesicle

Chen Li, Department of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden.

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To my family, whose unconditional love and encouragement kept me going throughout this journey.

List of Papers

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

I. Lohcharoenkal, Warangkana, **Chen Li**, Kunal Das Mahapatra, Jan Lapins, Bernhard Homey, Enikő Sonkoly, and Andor Pivarcsi. (2021) MiR-130a acts as a tumor suppressor microRNA in cutaneous squamous cell carcinoma and regulates the activity of the BMP/SMAD pathway by suppressing ACVR1. *Journal of Investigative Dermatology*. 141, no. 8: 1922-1931. DOI: https://doi.org/10.1016/j.jid.2021.01.028

II. **Chen Li**, Chengxi Sun, Kunal Das Mahapatra, Pilvi Riihilä, Jaakko Knuutila, Liisa Nissinen, Veli-Matti Kähäri, Enikö Sonkoly, Andor Pivarcsi. (2023) Long non-coding RNA PVT1 is overexpressed in cutaneous squamous cell carcinoma and exon 2 is critical for its oncogenicity. *British Journal of Dermatology*. *Under Revision*.

III. **Chen Li**, Chengxi Sun, Kunal Das Mahapatra, Enikö Sonkoly, Andor Pivarcsi. (2023) PVT1 regulates the nuclear export of polyadenylated RNAs through interacting with TREX complex. *Manuscript Under Preparation*.

IV. **Chen Li**, Chengxi Sun, Warangkana Lohcharoenkal, Moham-ad Moustafa Ali, Pengwei Xing, Wenyi Zheng, André Görgens, Manuela O. Gustafsson, Samir EL Andaloussi, Enikö Sonkoly and Andor Pivarcsi. (2023) Cutaneous squamous cell carcinoma-derived extracellular vesicles exert an oncogenic role by activating cancer-associated fibroblasts. *Cell Death Discovery*. 9, 260. DOI: https://doi.org/10.1038/s41420-023-01555-2

Scientific papers not included in this thesis

1. Sun, Chengxi, Kunal Das Mahapatra, Jonathan Elton, **Chen Li**, Winnie Fernando, Warangkana Lohcharoenkal, Jan Lapins, Bernhard Homey, Enikö Sonkoly, and Andor Pivarcsi. (2023) MicroRNA-23b plays a tumor suppressive role in Cutaneous Squamous Cell Carcinoma and targets Ras-related protein RRAS2. *Journal of Investigative Dermatology*. *In Press*. DOI: https://doi.org/10.1016/j.jid.2023.05.026

2. **Chen Li**, et al. Investigation into miRNA Involvement in Skin Ageing. *Manuscript Under Preparation*. Owing to confidentiality agreements with the collaborating party, more comprehensive information is not incorporated in this thesis.

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Abbreviations

UV	ultraviolet
UVR	ultraviolet radiation
ECM	extracellular matrix
cSCC	cutaneous squamous cell carcinoma
BCC	basal cell carcinoma
DDR	DNA damage response
XP	xeroderma pigmentosum
EB	epidermolysis bullosa
RDEB	recessive dystrophic epidermolysis bullosa
OTRs	organ transplant recipients
AK	actinic keratosis
cSCCIS	cutaneous squamous cell carcinoma *in situ*
FDA	Food and Drug Administration
EMA	European Medicines Agency
TKIs	tyrosine kinase inhibitors
ncRNAs	noncoding RNAs
miRNAs	microRNAs
piRNAs	piwi-interacting RNAs
pri-miRNAs	primary miRNAs
pre-miRNAs	precursor miRNAs
AGO	argonaute
miRISC	miRNA-induced silencing complex
lncRNAs	long non-coding RNAs
XIST	X-inactivate-specific transcript
lincRNAs	long intergenic RNAs
NATs	natural antisense transcripts
eRNAs	enhancer RNAs
RBPs	RNA binding proteins
ceRNAs	competing endogenous RNAs
EMT	epithelial–mesenchymal transition
ASOs	antisense oligonucleotides
siRNAs	small interfering RNAs

AMOs	anti-miRNA oligonucleotides
PT	phosphothiorate
LNAs	locked nucleic acids
PNAs	peptide nucleic acids
PMOs	phosphoramidate morpholino oligomers
LNPs	lipid nanoparticles
TREX	TRanscription and EXport
mRNPs	messenger ribonucleoproteins
EVs	extracellular vesicles
MVs	microvesicles
ISEV	International Society for EVs
TGN	trans-Golgi network
MVBs	multivesicular bodies
ILVs	intraluminal vesicles
ESCRT	endosome sorting complexes required for transport
TME	tumor microenvironment
ACVR1	activin receptor type I
PVT1	plasmacytoma variant translocation 1
SK	seborrheic keratosis
ChIRP-ms	comprehensive identification of RNA-binding proteins by mass spectrometry
NTA	nanoparticle tracking analysis
MS	mass-spectrometry

1 Introduction

1.1 The Structure and Functions of Skin

Skin is the largest organ of the body, with a surface area of around $2m^2$, and accounting around 15% of the total adult body weight (Kanitakis 2002). The primary function of skin is to serve as the outermost protective barrier against ultraviolet (UV) light, microorganisms, chemicals and mechanical injury, to produce vitamin D, regulate body temperature, prevent water loss and to permit the sensations of touch, heat, and cold (Cartlidge 2000).

The epidermis, the dermis, and the hypodermis are the three layers made up of the skin:

1.2 The epidermis

The epidermis is the uppermost layer of the skin which consists of keratinized, stratified squamous epitheliumv. It is mainly composed of four layers from the basal lamina towards the skin surface: the stratum basale (basal layer), the stratum spinosum (spinous cell layer), the stratum granulosum (granular layer), and the stratum corneum (cornified layer) (Figure 1). From the stratum basale to the stratum corneum, keratinocytes gradually get differentiated and form the four functionally different layers of the epidermis (McGrath and Uitto 2016). The fifth layer, stratum lucidum, located between the stratum corneum and the stratum granulosum, only exist at certain body locations.

The stratum basale, the bottommost layer of the epidermis, is a single layer anchoring the epidermis to the basement membrane. This basal cell layer contains the keratinocyte progenitor cells which maintain the epidermis throughout the lifetime of an individual. Keratinocytes in this cell layer are undifferentiated and constantly divide to produce new cells, pushing older cells to move up to the surface of the skin where they undergo terminal differentiation (Fuchs and Green 1980; Fuchs 2008; Krieg, Bickers, and Miyachi 2010; Yousef, Alhajj, and Sharma 2021). **The stratum spinosum** is the thickest layer of the epidermis. It contains newly produced keratinocytes from the stratum basale which have permanently withdrawn from the cell cycle. **The stratum granulosum** is made of keratinocytes with flatter, more irregular morphology and thicker cell membranes. The grainy appearance of this layer is due to its keratohyalin granules and lamellar bodies, which create a

permeability barrier to water (Ishida-Yamamoto et al. 2004; Yousef, Alhajj, and Sharma 2021; Krieg, Bickers, and Miyachi 2010). Keratohyalin granules contain the precursor form of filaggrin, called profilaggrin. Filaggrin is a crucial protein that crosslinks with keratin to form a tight barrier in the epidermis (Freeman and Sonthalia 2021). Lamellar bodies are secretory structures which produce components of the lipid layer essential for stratum corneum homeostasis (Raymond et al. 2008). As keratinocytes mature and move upward, their nuclei and other organelles disintegrate, leaving behind keratin, keratohyalin, and cell membranes to form the stratum lucidum and stratum corneum. **The stratum lucidum** is a layer existing only on thick skin, such as the palms of hands and the soles of the feet (Yousef, Alhajj, and Sharma 2021; Krieg, Bickers, and Miyachi 2010). **The stratum corneum** is the uppermost layer of the skin. Keratinocytes in this layer lose cell organelles, become flattened corneocytes and die. These corneocytes make a barrier for the body and shed approximately every 14 to 28 days (Blank 1953; Tobin 2006; Krieg, Bickers, and Miyachi 2010; Yousef, Alhajj, and Sharma 2021).

Keratinocytes account more than 90% of the cells in the epidermis. Keratinocytes provide structural integrity and physical barrier for the skin through producing keratins (intermediate filament proteins) and attaching to the basement membrane and other keratinocytes by hemidesmosomes and desmosomes (Barbieri, Wanat, and Seykora 2014). Keratinocytes also participate in immune response and play an important role in the formation of the skin immune barrier through secreting cytokines and antimicrobial peptides (Barbieri, Wanat, and Seykora 2014). Besides keratinocytes, the other two types of cells in the stratum basale are melanocytes (2-4 % of the cells) and Merkel cells (Figure 1). The role of melanocytes is to produce the pigment melanin and protect body from ultraviolet radiation (UVR) damage; the role of Merkel cells is to serve as a sensation receptor of touch and stimulate sensory nerves. Another type of cell in the stratum spinosum is Langerhans cells (Figure 1), which function as macrophages to prevent infection (Yousef, Alhajj, and Sharma 2021).

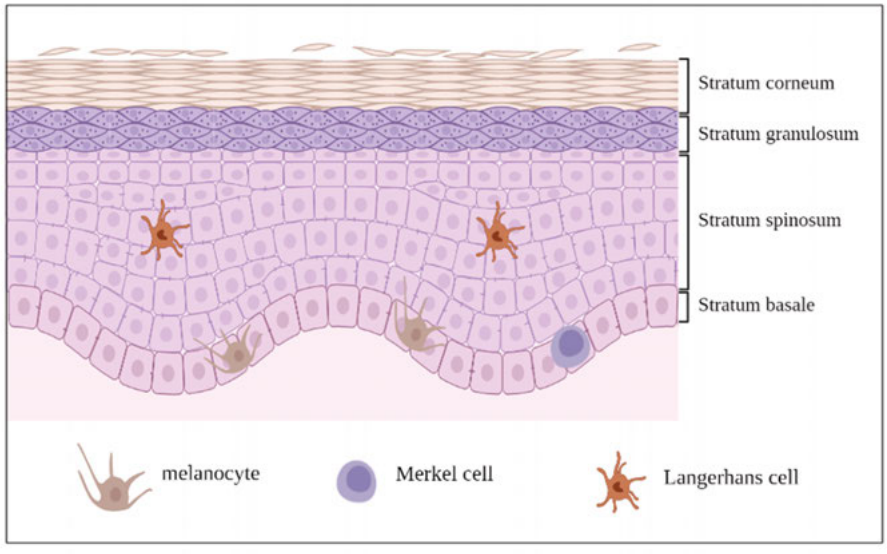


Figure 1. Schematic representation of human skin epidermis (created using BioRender). From bottom to the surface of the skin, the epidermis is composed of four layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The major cell types present in the epidermis including keratinocytes (more than 90% of the cells), melanocytes, Merkel cells and Langerhans cells. The stratum lucidum, hair follicles, and other appendages are not shown.

1.3 The dermis

The dermis, a connective tissue layer lying between the epidermis and hypodermis, is the thickest layer of the skin. It plays key roles in thermoregulation, sensation, skin structure support and protection. The dermis is an integrated structure consisting collagen, elastic tissue, hair follicles, blood and lymphatic vessels, nerve endings and sweat glands (Krieg, Bickers, and Miyachi 2010).

The dermis is made of two layers with an indistinct border: the papillary dermis and the reticular dermis. The papillary dermis is the upper dermis which is composed of loose connective tissue and forms a finger-like border with the stratum basale of the epidermis. Reticular layer is the lower dermis which is much thicker and composed of dense connective tissue. The dermis, rich in extracellular matrix (ECM), contains relatively fewer cells compared to the epidermis.

The predominant cells in the dermis are fibroblasts. They are responsible for the production and organization of ECM in dermis to maintain the structural integrity of connective tissues. Other resident cells in the dermis includes endothelial cells, various immune cells (e.g. macrophages, mast cells and Schwann cells) and stem cells which interact with fibroblasts and form an integrated system.

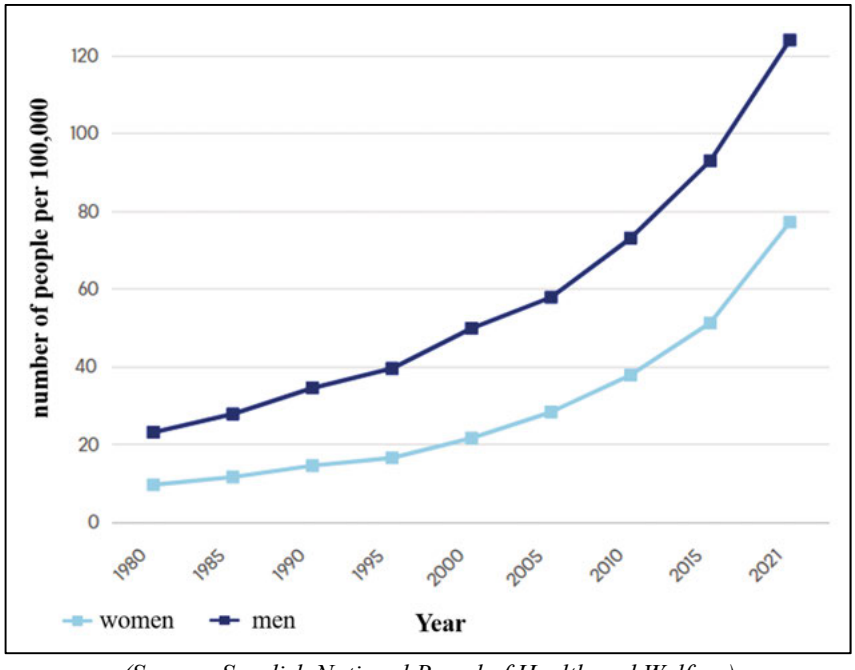
1.4 The hypodermis

Below the dermis is the hypodermis which mainly composed of adipose tissue, connective tissue with larger nerves and blood vessels throughout this layer. The hypodermis stores fat and regulates temperature, also provides mechanical support to upper layer of the skin (Yousef, Alhajj, and Sharma 2021).

2 Cutaneous Squamous Cell Carcinoma

2.1 Epidemiology

Cutaneous squamous cell carcinoma (cSCC) is the second most prevalent type of human cancer which is marked by abnormal, accelerated proliferation of epidermal keratinocytes. The incidence of cSCC continues to rise especially in Caucasian populations (Nehal and Bichakjian 2018; Rogers et al. 2015). In the United States, there is an estimated incidence of 1 million cases of cSCC each year in the Medicare population (Rogers et al. 2015; Muzic et al. 2017). The long-term increasing incidence of cSCC is seen in European countries as well (Rudolph et al. 2015; Brewster et al. 2007; Birch-Johansen et al. 2010). For example, Germany reported a 187% increase in the age-standardized incidence rate for cSCC from 1998 to 2010, and a 141% increase for basal cell carcinoma (BCC), another keratinocyte carcinoma (Rudolph et al. 2015). In Sweden, cSCC is the fastest increasing type of skin cancer. During the last ten years, the incidence of skin cancer increased 4.6% per year among men and increased 5.7% per year among women (Statistik Om Cancer, https://www.cancerfonden.se/om-cancer/statistik/hudcancer). Over the last twenty years, the increase for men was 4.8% per year and for women was 6.2% per year (Statistik Om Cancer). Here, the incidence of skin cancer was analyzed excluding melanoma and BCC, which essentially revealed an increasing trend in cSCC. Until 2021, the growth trend of cSCC in Sweden showed no sign of slowing down, instead, has become more intense (Figure 2). Most cSCC can be surgically excised and thus show a favorable prognosis, however, unlike extremely rare metastasis in BCC, cSCC is one of the common cancers with the potential to metastasize (Que, Zwald, and Schmults 2018a). Around 4% of cSCC patients have lymph node metastases which will lead to a dramatic drop on their ten-year survival rate (Corchado-Cobos et al. 2020). Even though metastasis is not that frequent, due to its high incidence, cSCC is the second leading cause of skin cancer-related death after melanoma and cause a major public health concern (Corchado-Cobos et al. 2020).



(Source: Swedish National Board of Health and Welfare)

Figure 2. From 1980 to 2021, the age-standardized incidence of cSCC in Sweden, excluding malignant melanoma and basal cell carcinoma, was recorded per 100,000 inhabitants, with population standardization based on the year 2000, for both women and men. cSCC, cutaneous squamous cell carcinoma.

2.2 Risk factors

Risk factors for cSCC include chronic UVR exposure, fair skin, older age, male sex and immunosuppression (Nagarajan et al. 2019; Que, Zwald, and Schmults 2018a; Corchado-Cobos et al. 2020; Gloster and Neal 2006; Oberyszyn 2008; Xiang et al. 2014).

Chronic exposure to UVR (primarily UVA- and UVB radiation) is the most dominant risk factor for cSCC. UVR is capable of triggering DNA damage in cells. In response to this damage, cells activate a complex network of pathways known as the DNA damage response (DDR). However, a portion of this damage may remain unrepaired, eventually accumulating over time and triggering tumorigenesis. The UV signature mutation refers to the C to T substitution at a dipyrimidine site (Douglas E. Brash 2015). Interestingly, this UVR-induced C to T mutation has been observed in the tumor suppressor gene *TP53* not only in cSCCs, but also in the premalignant cutaneous lesions - actinic keratosis, and even in UVR-exposed normal skin (Ren et al. 1996; Jonason et al. 1996; Nelson et al. 1994; Pierceall et al. 1991; Campbell et al. 1993; Martincorena et al. 2015).

Xeroderma pigmentosum (XP), first described by Moritz Kaposi in 1870, is a genetic disease with hypersensitivity to UVR and defective repair of UV-induced DNA damage (Kraemer, Lee, and Scotto 1987). Patients with XP carry a higher proportion of C to T mutations. It has been reported that the incidence of cSCC or BCC was 4800 times greater in patients with XP than general population in the United States (Kraemer, Lee, and Scotto 1987; Stary and Sarasin 2002; Kraemer, Lee, and Scotto 1984). The median age of XP patients with skin cancer is 8 years old which is nearly 50 years younger than that in general population (Kraemer, Lee, and Scotto 1987). Epidermolysis bullosa (EB) is a group of rare genetic disorders characterized by mucocutaneous fragility and blister formation. Patients with EB, especially the severe subtype recessive dystrophic epidermolysis bullosa (RDEB), are susceptible to development lethal, metastatic cSCC and have a low age of onset (Dayal et al. 2021; Montaudié et al. 2016; Duong et al. 2021). The mechanisms underlying the high incidence of cSCC in patients with RDEB are not fully understood, but lack of DDR is thought to be one of the contributing factors. Although XP and EB are rare diseases, they are good models for confirming the link between UV-induced DNA damage and skin cancer proneness.

Immune systems of organ transplant recipients (OTRs) are permanently suppressed by using immunosuppressive medications to prevent graft rejection. As a result, immunodeficiency makes OTRs at a high risk for developing malignant tumors with the most prominent risks for cSCC (Jensen et al. 1999; Lindelöf et al. 2000; Euvrard, Kanitakis, and Claudy 2003; Krynitz et al. 2013). A Swedish population-based study revealed that the risk of post-transplant cSCC shot up 100-fold overall, with 198-fold increase in heart and/or lung recipients, 121-fold in kidney recipients and 32-fold in liver recipients and the risk substantially and dramatically increased along with follow-up time (Krynitz et al. 2013).

2.3 Molecular pathogenesis

The development of cSCC involves a multistep process marked by a progression from preliminary stages, including premalignant cutaneous lesions such as actinic keratosis (AK) or cutaneous squamous cell carcinoma *in situ* (cSCCIS), to invasive cSCC, and ultimately metastatic SCC (Que, Zwald, and Schmults 2018a; Ratushny et al. 2012). AK, also known as solar keratosis, is a benign epidermal lesion due to proliferation of atypical keratinocytes. Long-term exposure to UVR is the common predominant risk factor shared by AK and cSCC (Reinehr and Bakos 2019). An estimated 65% of cSCC arise from AK, making AK the most common premalignant lesion of cSCC (Criscione et al. 2009). cSCCIS, also referred to as Bowen's disease, is non-invasive cutaneous malignancy commonly found on sun-exposed skin. cSCCIS is

generally restricted to the epidermis, while approximately 5% of untreated cSCCIS will progress into invasive cSCC (Kao 1986).

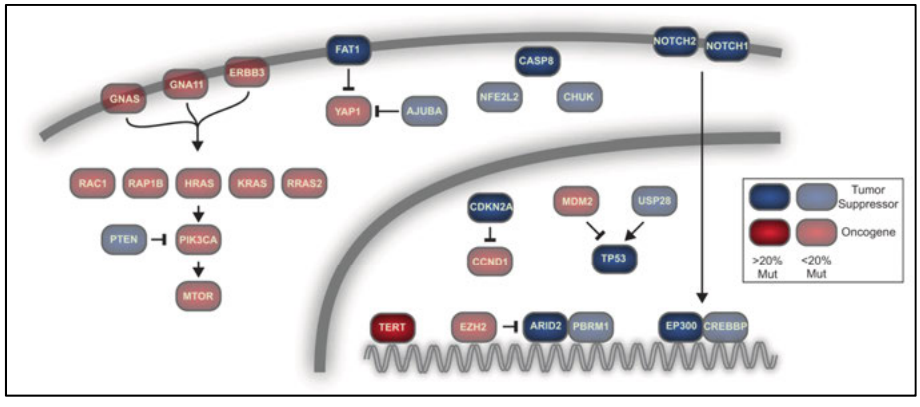
Identifying the driver mutations of cSCC will broaden our understanding about the disease and contribute to the development of targeted therapies and personalized cancer management. However, as a consequence of UVR-induced DNA damage, cSCC is one of the most highly mutated cancers, bearing a mean somatic mutation rate of 50 mutations per megabase pair DNA (Pickering et al. 2014; Y. Y. Li et al. 2015; South et al. 2014; Inman et al. 2018). The high level of background mutations presents challenges in determining the specific genomic changes driving the progression of cSCC. Among the 30 distinct cancer mutational signatures described by the Catalogue of Somatic Mutations in Cancer (COSMIC) project (http://cancer.sanger.ac.uk/cosmic/signatures), signature 7, also known as "UV Signature", exists in most cases of cSCCs (Inman et al. 2018). Signature 7 is characterized by C to T mutations at dipyrimidine sites. Signature 32 is a novel mutational signature discovered in immunosuppressed patients who have received immunosuppressant drug including azathioprine (Inman et al. 2018). Signature 32 is marked by a predominant occurrence of C to T mutations (75%), along with C to A, T to A, and T to C mutations.

Despite the presence of this immunosuppression-associated mutational signature, both immunosuppressed and immunocompetent patients with cSCC share common driver gene mutations (Inman et al. 2018). Well accepted cSCC driver genes include *TP53*, *NOTCH1/2* and *CDKN2A* (Y. Y. Li et al. 2015; Pickering et al. 2014; Inman et al. 2018; Chang and Shain 2021). *TP53* mutation was reported in 54%-90% patients with cSCC (D. E. Brash et al. 1991; Ziegler et al. 1994; D. E. Brash 2006). Mutated *TP53* fails to maintain genomic stability and results in the accumulation of a serial of gene mutations (South et al. 2014; Cho et al. 2018; Chitsazzadeh et al. 2016; Inman et al. 2018; Cammareri et al. 2016). Loss-of-function mutation of tumor suppressor genes *NOTCH1* or *NOTCH2* occurs in 75%-82% of cSCCs (South et al. 2014; N. J. Wang et al. 2011). $p16^{INK4a}$ and $p14^{ARF}$, encoded by *CDKN2A* gene, are two crucial cell cycle regulators in the p53 and RB pathways. Inactivation of either $p16^{INK4a}$ or $p14^{ARF}$ may results in endless cell cycling and cell dividing (V. L. Brown et al. 2004). *HRAS* is mutated in about 3%-20% in cSCC (Pickering et al. 2014; Y. Y. Li et al. 2015; South et al. 2014). Aberrant activation of *RAS* leads to overactive PI3K/AKT/mTOR and RAS/RAF/ERK signaling pathways subsequently. A recent meta-analysis performed on exome-sequencing data nominated 30 driver mutations for cSCC (Chang and Shain 2021) (Figure 3). In addition to those well-known mutations, multiple novel driver mutations for cSCC were identified, including *EP300*, *PBRM1*, *USP28* and *CHUK* (Chang and Shain 2021). Following the mutations of individual genes, a succession of signaling pathways are disturbed in cSCC (Figure 3). The NOTCH pathway and the p53 pathway are perturbed in most cases of cSCC: loss-of-function mutations within the NOTCH pathway occur in 80%

of the cases, and loss-of-function mutations within the p53 pathway are present in 71% of the cases (Chang and Shain 2021). Furthermore, mutations (primarily affecting *CDKN2A*) involved in cell cycle regulatory pathways exist in around 39% of cSCCs (Chang and Shain 2021). Additionally, mutations that activate the MAPK and/or PI3K pathways are found in approximately 31% of cSCCs (Chang and Shain 2021). Collectively, exome-sequencing studies revealed a surprising complexity of the cSCC genome, with a striking heterogeneity in the driver genes.

Surprisingly, AK and cSCC show strikingly similarity in terms of average tumor mutational burden and patterns of driver gene mutations (Thomson et al. 2021). UV-radiation-induced *TP53* mutation is an important early event observed from AK to cSCC (Nelson et al. 1994; Kubo et al. 1994; Thomson et al. 2021; Zheng et al. 2021). Transcriptome analysis was conducted to further elucidate key molecular events associated with progression from premalignant AK to invasive cSCC, which identified the MAPK pathway as a critical determinant of AK to cSCC progression (Lambert et al. 2014). In cSCC, the MAPK pathway can be activated due to *RAS* mutations, and another common cause is overexpression of EGFR (Shimizu et al. 2001). EGFR is a transmembrane tyrosine kinase growth factor receptor that transmits growth-stimulatory signals to cells upon binding with peptide growth factors of the EGF family of proteins (Normanno et al. 2006). Eventually, activated MAPK pathway contributes to cell transformation through allowing evasion of apoptosis and unrestricted cell proliferation.

Overall, the significant genetic heterogeneity of cSCCs is in line with the wide-ranging phenotypic diversity observed in patients. While *TP53* and *NOTCH* stand out as principal driver mutations, present in the majority of cSCCs, the existence of numerous other mutations across patients underscores the intricate and varied genetic landscape underlying cSCC development and highlights the need for a comprehensive understanding of the underlying genetic factors contributing to this disease. Such knowledge is crucial for the development of targeted therapies and personalized treatment approaches tailored to the unique genetic makeup of individual cSCC cases.



(Adapted Chang, Darwin, et al. NPJ genomic medicine 6.1 (2021): 61.)

Figure 3. The landscape of genomic mutations in cSCC. A total of 30 cancer genes were identified through the meta-analysis of exome-sequencing data in cSCC, revealing disrupted signaling pathways. cSCC, cutaneous squamous cell carcinoma.

2.4 Treatment

Surgical excision remains the first-line treatment option for the majority of cSCC patients (Work Group et al. 2018; E Maubec 2020). In addition, destruction (cryosurgery, electrodessication and curettage, and chemical peels), light-based therapies (photodynamic therapy and lasers), radiation, topical treatment (e.g., imiquimod and tazarotene) are options for primary superficial, low-risk cSCCs (Nagarajan et al. 2019). For advanced and unresectable cSCCs, the first line therapy is PD-1 inhibitor-cemiplimab mediated immunotherapy (E Maubec 2020). Cemiplimab is the first drug approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of advanced cSCC, which functions through blocking PD-L1/PD-1 axis mediated cancer immune evasion and allow the immune system to recognize and attack cancer cells more effectively (Migden et al. 2018). Cemiplimab induces response in approximately half the advanced cSCCs accompanied with side effects such as diarrhea, fatigue, nausea, constipation and rash (Migden et al. 2018). However, for immunocompromised individuals whose immune system is not functioning optimally, such as OTRs who are receiving medications to prevent organ transplant rejection, PD-1 inhibitor increases the risk of severe side effects and have potentially compromised treatment efficacy. Second line therapy is cisplatin- or carboplatin-based chemotherapy, which shows clinical response rate lower than 30% and severe adverse events (Que, Zwald, and Schmults 2018b; Cranmer, Engelhardt, and Morgan 2010). Newer alternative for second line therapy is EGFR inhibitor mediated targeted therapy (E Maubec 2020). Considering

the prevalence of EGFR overexpression in cSCC, EGFR inhibitors are tested to treat cSCC. Two types of EGFR inhibitors are designed to inactivate downstream RAS/RAF/ERK and PI3K/AKT/mTOR signaling pathways: monoclonal antibodies (e.g., cetuximab, panitumumab) blocking the binding of ligands and receptor; tyrosine kinase inhibitors (TKIs) (e.g., gefitinib, erlotinib) abolishing the tyrosine kinase activity (Corchado-Cobos et al. 2020). EGFR inhibitors induce a moderate response in the treatment of cSCC but the usage is limited by severe side effects (Que, Zwald, and Schmults 2018b; Eve Maubec et al. 2011).

3 Non-Coding RNA

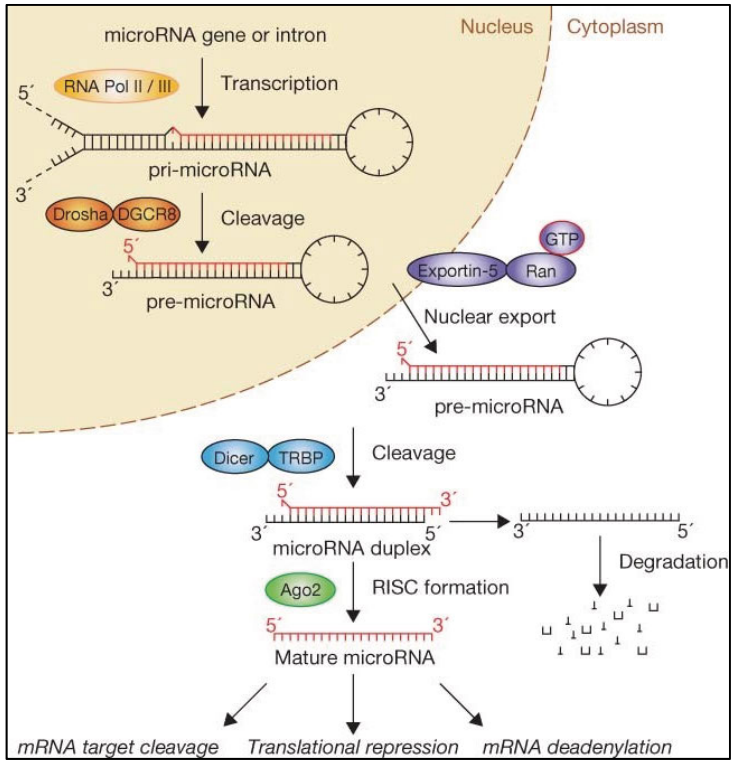
In the human genome, more than two-third of the DNA is transcribed, however the vast majority of them do not encode protein and only 2% genomic DNA is translated into protein (Iyer et al. 2015; ENCODE Project Consortium et al. 2020). The function of transcripts that are not used for encoding for proteins is still unclear, but increasing evidence suggests that they may be involved in both physiological and pathological processes. Collectively, these transcripts are called non-coding RNAs. Based on the length and function, noncoding RNAs (ncRNAs) are classified into different categories: small noncoding RNAs, shorter than 200 nucleotides (such as microRNAs and piwi-interacting RNAs (piRNAs)) and long noncoding RNAs that are longer than 200 nucleotides.

3.1 MicroRNAs

MicroRNAs (miRNAs) is a dominating class of endogenous small noncoding RNAs with the length of ~22 nucleotides, which function as critical regulators of gene expression through mediating RNA silencing (Ha and Kim 2014). The first miRNA lin-4 miRNA was discovered in *Caenorhabditis elegans* in 1993 (R. C. Lee, Feinbaum, and Ambros 1993). Lin-4 was first identified as an important development timing regulator of *Caenorhabditis elegans* in the mid of 1970s (R. Lee, Feinbaum, and Ambros 2004). However, it took almost two decades to further characterize it as a short noncoding RNA with 22 nucleotides which could regulate the expression of lin-14 mRNA at the post-transcriptional level through binding to its 3'UTR region (R. C. Lee, Feinbaum, and Ambros 1993; R. Lee, Feinbaum, and Ambros 2004; Wightman, Ha, and Ruvkun 1993). The second miRNA, let-7, also an important regulator of developmental timing in *Caenorhabditis elegans*, was identified in 2000, seven years after the discovery of lin-4 (Reinhart et al. 2000). Let-7 miRNA is a 21 nucleotide-long miRNA, which regulates lin-41 mRNA expression post-transcriptionally through binding to its 3'UTR (Reinhart et al. 2000). Since then, the studies on miRNAs showed a booming development trend.

3.1.1 MiRNA biogenesis and mode of action

In the canonical miRNA biogenesis pathway (Figure 4), genes encoding miRNAs are transcribed by RNA polymerase II or (more rarely) by polymerase III in the cell nucleus as long primary miRNAs (pri-miRNAs, typically over 1 kb) which are 5' capped and 3' polyadenylated and contain one or more hairpin loop structures (Ha and Kim 2014; Winter et al. 2009). Pri-miRNAs are further processed by nuclear ribonuclease III–like endonuclease Drosha and its essential cofactor DGCR8 into a shorter hairpin loop structure (~65 nt), named precursor miRNAs (pre-miRNAs) (Ha and Kim 2014; Y. Lee et al. 2003). Pre-miRNAs are subsequently exported by exportin 5 to cytoplasm where they are cleaved by cytoplasmic ribonuclease III–like endonuclease Dicer to mature miRNAs duplex (Ha and Kim 2014; Lund et al. 2004). Mature miRNAs arising from the 5′ arm and 3' arm of the hairpin are labelled with -5p and -3p suffixes, respectively (Griffiths-Jones 2004). One strand (guide strand) of the mature miRNAs is anchored to Argonaute (AGO) family proteins (AGO1-4) and form a miRNA-induced silencing complex (miRISC), while the other strand (sometimes called "passenger" strand) will be released and degraded (Ha and Kim 2014). However, it has been reported that passenger strand miRNAs can also be functional (J.-S. Yang et al. 2011). MiRNAs guide AGO proteins to target transcripts through Watson–Crick pairing between miRNA "seed" region (nucleotides 2–7 of the 5' end of the miRNA) and miRNA recognition elements that are usually within the 3'UTR of mRNAs (Bartel 2018). The length of base pairing between miRNA and target mRNA can be as little as 6 nucleotides (6mer site), and extended to the 8th nucleotide of miRNAs (7mer-m8) or start from the first miRNAs nucleotide-A (7mer-A1), or both (8mer) (Bartel 2018). Eventually, miRISCs lead to deadenylation-induced mRNA decay or translational repression (Bartel 2018).



(Adapted Winter, Julia, et al. Nature cell biology 11.3 (2009): 228-234.)

Figure 4. Schematic model of canonical miRNA biogenesis. Pri-miRNA is transcribed by RNA polymerase II or III and processed to pre-miRNA by the microprocessor complex Drosha–DGCR8 in the nucleus. Pre-miRNA is exported to cytoplasm by exportin 5 and cleaved to mature length by RNase Dicer. The guide strand of the mature microRNA binding with Ago proteins forms miRISC, whereas the passenger strand is degraded. miRISCs regulate target transcripts expression through inducing mRNA decay or translational repression. miRNA: microRNA, pri-miRNA: primary miRNA, pre-miRNA: precursor miRNA, Ago: Argonaute, miRICS, miRNA-induced silencing complex.

3.1.2 MiRNAs in cancers

Many miRNAs are conserved among species and highly conserved miRNAs have very many conserved targets (exceeds 400 conserved targets per miRNA family) (Bartel 2009). As more than half of human protein-coding genes are conserved targets of miRNAs, miRNAs are involved in almost all cellular processes, highlighting their significant biological importance (Friedman et al. 2009). The regulation of miRNAs in biological pathways relies on precise temporal and spatial control of miRNA biogenesis pathways in normal physiology. However, the dysregulation of miRNAs expression (usually downregulation) driven by genetic, epigenetic or transcriptional mechanisms is closely

linked to diseases, especially tumorigenesis (Jansson and Lund 2012; Lu et al. 2005). In 2002, Calin, George Adrian, et al. (George Adrian Calin et al. 2002) reported that the deletion of two miRNA genes (miR-15 and miR-16) at 13q14 widespread in the majority of chronic lymphocytic leukemia. This is the first time that miRNAs were associated with cancer. Two years later, they further proposed that miRNA genes are nonrandomly distributed in the human genome but frequently located at cancer-associated genomic regions or in fragile sites (G. A. Calin et al. 2004). These studies suggest the extensive involvement of miRNA in cancer rather than a random event. As of 2023, there have been over 76,000 scientific publications on miRNAs in cancer (PubMed).

The expression and functions of diverse miRNAs have been investigated and reviewed in different kinds of cancers where they function as either oncogenes or tumor suppressors (Jansson and Lund 2012; George A. Calin and Croce 2006; Garzon et al. 2006). Oncogenic miRNAs usually target and decrease the expression of genes suppressing tumorigenesis, for example, the tumor suppressor gene *PTEN* is one of the validated targets of miR-17-92 cluster (Takakura et al. 2008; Mu et al. 2009; Olive et al. 2009). The miR-17-92 cluster, encoded within an 800 bp region on human chromosome 13, is a group of sequence highly conserved miRNAs with cooperative functions, including miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a (Ota et al. 2004). The miR-17-92 cluster is one of the most extensively studied oncogenic miRNAs, which participates in the formation and development of multiple cancers through promoting cell proliferation, inhibiting cell apoptosis and inducing angiogenesis (Takakura et al. 2008; Chen et al. 2011; Mu et al. 2009).

Target genes of tumor suppressor miRNAs usually exert oncogenic roles. MiR-34a is a well-characterized tumor suppressor miRNA that is ubiquitously expressed in normal cells and downregulated in cancers (Tazawa et al. 2007; Qiao et al. 2015; Xi Wang et al. 2015). Similar to other conserved miRNAs, many target genes have been validated for miR-34a. MiR-34a regulates the migration, invasion, and metastasis of prostate cancer stem cells by directly targeting and regulating CD44 expression (Liu et al. 2011). In breast cancer, MiR-34a regulates therapy resistance by directly targeting and regulating the expression levels of HDAC1 and HDAC7 (Wu et al. 2014). Additionally, MiR-34a plays a role in immune response regulation in acute myeloid leukemia by directly targeting and regulating the expression level of the immune evasion mediator PD-L1 (Xi Wang et al. 2015). Therefore, it is not uncommon for a single miRNA to target multiple genes, and miRNAs regulate biological processes by influencing a network of target genes.

3.1.3 MiRNAs in cutaneous squamous cell carcinoma

Analysis of miRNA expression in cSCC by our group and other groups demonstrated that miRNA-expression is altered in cSCC (N. Xu et al. 2012;

Lohcharoenkal et al. 2021; 2016; A. Wang et al. 2014; Fleming et al. 2013; Lefort et al. 2013; Yamane et al. 2013; Dziunycz et al. 2010; Darido et al. 2011; X. Li, Huang, and Yu 2014). Upregulated oncogenic miRNAs promote cSCC through inhibiting the expression of their target genes. For instance, our group identified miR-31 as an upregulated miRNA in cSCC, which promotes cell motility and colony formation ability of cSCC cells (A. Wang et al. 2014). MiR-21, a well-known oncogenic miRNA overexpressed in several types of carcinomas, also contributes to cSCC via regulating GRHL3-PTEN axis (Darido et al. 2011) and TIMP3/PI3K/AKT axis (Yin and Lin 2021). Elevated level of miR-365 promotes cSCC through targeting and suppressing the expression of the tumor suppressor gene HOXA9 (L. Zhou et al. 2018) and NFIB (M. Zhou et al. 2014). Down-regulated tumor suppressor miRNAs (more common in cSCC according to our and other groups' data) increase the expression of their target genes and promote cSCC. Our group found that decreased miR-125b results in enhanced expression of MMP13, MMP7 and MAP2K7 (N. Xu et al. 2012). Another study from our group reported that the expression of miR-203 is downregulated in cSCC and inversely correlates with differentiation grade (Lohcharoenkal et al. 2016). MiR-203 functions as a tumor suppressor in cSCC through directly targeting c-MYC (Lohcharoenkal et al. 2016). Moreover, decreased miR-34a results in enhanced expression of HMGB1 (S. Li et al. 2021) and SIRT6 (Lefort et al. 2013). Decreased miR-199a results in enhanced expression of CD44 (S.-H. Wang et al. 2014), BCAM, FZD6 and DDR1 (B.-K. Kim, Kim, and Yoon 2015). The important roles of miRNAs in cSCC have been revealed by these intensive studies, however, there are still quite a lot of miRNAs whose function remain to be characterized in cSCC, and only a fraction of miRNAs whose expression is altered in cSCC has been investigated in mechanistic studies.

3.2 Long non-coding RNAs

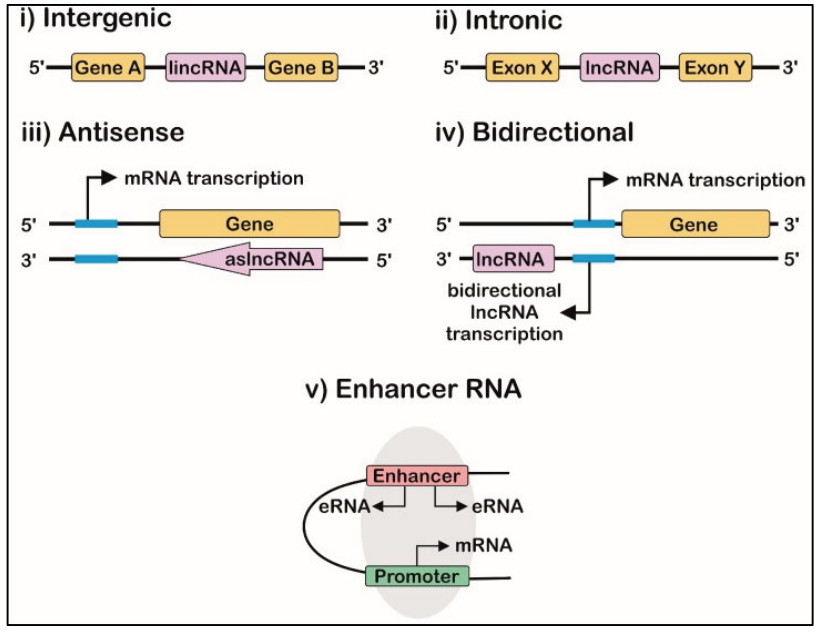
Long non-coding RNAs (lncRNAs) are defined as a group of RNA molecules with a length greater than 200 nucleotides and an apparent lack of protein-coding function (Guttman et al. 2009; Mattick and Rinn 2015). H19 is the first lncRNA discovered in 1990 (Brannan et al. 1990), followed by X-inactivate-specific transcript (XIST) as the second discovered lncRNA in 1991 (C. J. Brown et al. 1991). LncRNAs represent the major fraction of noncoding transcripts in humans. The current GENCODE Release (version 39) comprises 18,811 lncRNA genes encoding 53,009 lncRNA transcripts (GENCODE - Human Release Statistics, https://www.gencodegenes.org/human/stats.html). While lncRNAs were discovered around the same time as miRNAs and constitute a larger proportion of the human genome, our understanding of lncRNAs remains limited due to their structural and functional heterogeneity,

as well as the fact that most investigations have primarily focused on protein-coding genes.

3.2.1 Characteristics and genomic location of lncRNAs

LncRNAs are transcribed by RNA polymerase II so they share some similarity with mRNAs on post-transcriptional modification, such as 5' capping, splicing and 3' polyadenylation (Djebali et al. 2012; Derrien et al. 2012; Melé et al. 2017). However, lncRNAs are more preferentially localized in nucleus while mRNAs are transported to the cytoplasm for translation (Zuckerman and Ulitsky 2019). LncRNAs residing in the cell nucleus are largely inefficiently polyadenylated and spliced (Melé et al. 2017; Cabili et al. 2011; C.-J. Guo et al. 2020; Schlackow et al. 2017). For instance, MALAT1 and MEN β are two lncRNAs localized on nuclear speckles and paraspeckles (Hutchinson et al. 2007; Sunwoo et al. 2009). They lack poly (A)-tails, instead, they are cleaved by RNase P and form triple helical structures which protect them from 3'–5' exonucleases and enhance their stabilization (Wilusz et al. 2012; J. A. Brown et al. 2012; Quinn and Chang 2016). In contrast to protein-coding RNAs, whose sequence is evolutionarily conserved and closely associated with the function of the encoded protein, the low sequence conservation and the uncertain relationship between the lncRNA sequence and its function largely slow down the annotation of the functions of lncRNAs. Furthermore, lncRNAs, in general, have lower level of expression, and show cell type–specific expression patterns (Djebali et al. 2012; Derrien et al. 2012; Sarropoulos et al. 2019). These characteristics make the research for lncRNA-functions more challenging than that for protein-coding genes and make the functional importance of some of the lncRNAs debatable.

LncRNAs are a class of heterogeneous transcripts. The widely used classification for lncRNAs is based on their genomic localization relative to annotated protein-coding genes or DNA elements (Figure 5), and they are defined as: (i) long intergenic RNAs (lincRNAs): transcribed from regions between two coding genes; (ii) intronic lncRNAs: transcribed from the introns of coding genes; (iii) natural antisense transcripts (NATs): overlapped with mRNAs originating from their complementary strands; (iv) bidirectional RNAs: transcribed in the opposite direction with respect to the protein coding gene, but are located within 1 kb from its promoter region; and (v) enhancer RNAs (eRNAs): transcribed from enhancer regions and have enhancer like-function (Fernandes et al. 2019; Kopp and Mendell 2018).



(Adapted Fernandes, Juliane CR, et al. Non-coding RNA 5.1 (2019): 17.)

Figure 5. Schematic model of long noncoding RNAs (lncRNAs) classification according to genomic localization: i) long intergenic RNAs (lincRNAs), ii) intronic lncRNAs, iii) natural antisense transcripts (NATs), iv) bidirectional lncRNAs and (v) enhancer RNAs (eRNAs).

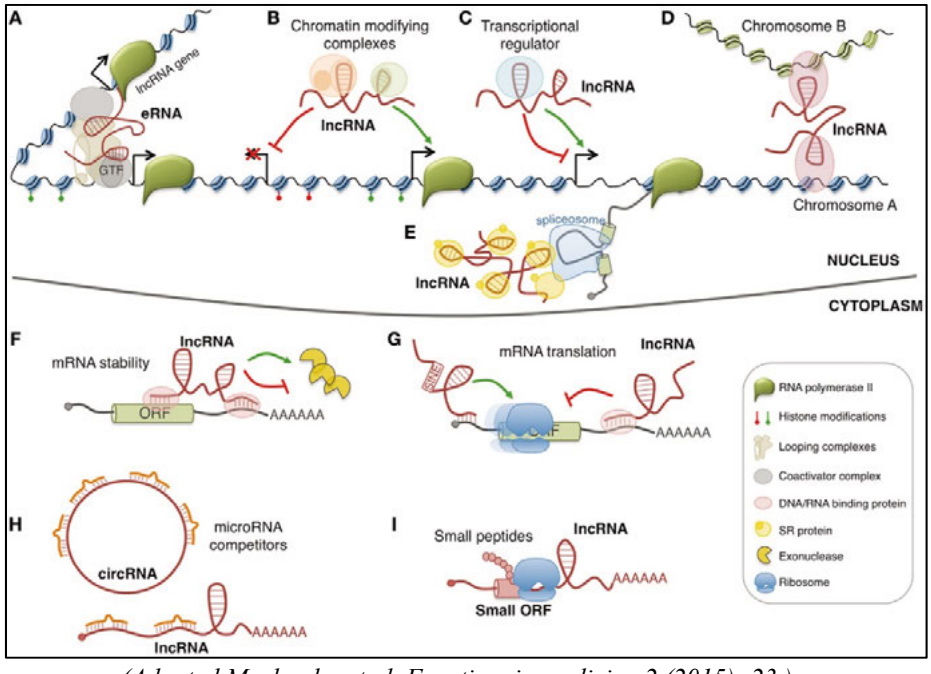
3.2.2 Subcellular localization and modes of action of lncRNAs

LncRNAs were historically ignored or considered as transcriptional noise. However, with the development of high-throughput genomic platforms, a growing number of lncRNAs have been identified as gene expression regulators and play significant regulatory roles in almost every biological process, as well as pathological processes (Ponjavic, Ponting, and Lunter 2007; Ulitsky and Bartel 2013; Perry and Ulitsky 2016; Jandura and Krause 2017). Today, we know that lncRNAs can regulate gene expression at epigenetic, transcriptional, or post-transcriptional levels through interacting with proteins, DNA, and RNA (K. C. Wang and Chang 2011; Mattick et al. 2023). The molecular mechanisms of function largely depend on their subcellular localization (Figure 6):

In nucleus: LncRNAs resident in nucleus play a crucial role in the regulation of gene transcription *in cis* or *in trans* (Long et al. 2017). (A) Enhancer RNAs are transcribed from active enhancer regions and they exert functional impact on transcriptional regulation through contributing to enhancer activity. (B) LncRNAs can function as scaffolds and recruit protein complex to the gene locus or the entire chromosome. Xist, one of the most intensively investigated lncRNAs, is a critical regulator of X-chromosome inactivation in

female embryonic development (Furlan and Rougeulle 2016; Jégu, Aeby, and Lee 2017; da Rocha and Heard 2017; Sahakyan, Yang, and Plath 2018; Brockdorff, Bowness, and Wei 2020; Markaki et al. 2021). Strikingly, a total 50 pairs of Xist lncRNAs (100 Xist molecules per cell), located at 50 spots along the X chromosome, silence all genes across the entire X-chromosome through recruiting a wide spectrum of chromatin architecture regulators, silencing and accessory proteins (Markaki et al. 2021). (C) LncRNAs can block the binding of transcription factors by acting as "decoys". For example, nuclear lncRNA GAS5 functions as decoy of transcription factor CEBPB and abolishes the transcription-promoting effect of CEBPB on growth differentiation factor 15 (GDF15) (L. Guo and Wang 2019). (D) LncRNAs may also regulate gene transcription through modifying the spatial conformation of chromosomes. (E) LncRNAs could modulate pre-mRNA alternative splicing through interacting with splicing factors.

In cytoplasm: (F) LncRNAs localized in cytoplasm can regulate mRNA stability through recruiting RNA binding proteins (RBPs) that promote mRNA decay. For example, 1/2-sbsRNAs are a group of lncRNAs who bind with Alu elements in the 3′UTR region of targeted mRNAs by imperfect base-pairing and recruits RBP STAU1 to induce STAU1-mediated mRNA decay (Gong and Maquat 2011). (G) LncRNAs can also recruit translation suppressive or promoting RBPs to regulate mRNA translation. Cytoplasmic lncRNA GAS5 directly binds with eukaryotic translation initiation factor-4E (eIF4E) and inhibits the initiation of translation on c-MYC mRNA (Hu, Lou, and Gupta 2014). (H) LncRNAs, especially circular RNAs, may work as competing endogenous RNAs (ceRNAs) to completely bind with miRNAs and stabilize mRNA. Circular RNA_LARP4 suppresses malignant behaviors of gastric cancer cells by sponging miR-434 and stabilize LATS1 mRNA (J. Zhang et al. 2017). In breast cancer, lncRNA-PNUTS localized in cytoplasm acts as a competitive sponge for miR-205 to stabilize ZEB mRNA and further promote epithelial–mesenchymal transition (EMT) (Grelet et al. 2017). (I) Although not common, some of lncRNAs have the ability to encode functional polypeptides, challenging the clear separation of coding and non-coding RNAs.



(Adapted Morlando, et al. Frontiers in medicine 2 (2015): 23.)

Figure 6. The modes of lncRNA function are associated with their subcellular localization. LncRNAs accumulated in nucleus execute transcription regulatory functions through acting as enhancer RNAs (eRNAs) (A); recruiting chromatin modifiers (B) or transcription factors (C) and modifying the spatial conformation of chromosomes (D) or influencing pre-mRNA splicing (E). LncRNAs localized in cytoplasm regulate mRNA expression through influencing mRNA stability (F) or mRNA translation (G); completely binding with miRNAs and stabilize mRNA (H). Moreover, there is a small proportion of lncRNAs encode polypeptides (I). lncRNAs: long noncoding RNAs

3.2.3 LncRNA in cutaneous squamous cell carcinoma

RNA-sequencing of cSCC and healthy skin samples performed by our group identified 908 annotated lncRNAs differentially expressed in cSCC (Das Mahapatra et al. 2020). Among differentially expressed lncRNAs in cSCC, 319 were upregulated including oncogenic lncRNAs SNHG12, CASC9, LUCAT1 and PVT1, while 589 were downregulated including tumor suppressor lncRNA TINCR (Das Mahapatra et al. 2020). LncRNA TINCR-terminal differentiation-induced ncRNA, is a key regulator for human epidermal differentiation which post-transcriptionally regulates the abundance of a bunch of differentiation genes such as FLG, LOR, KRT1 and KRT10 (Kretz et al. 2013). Deficiency of lncRNA TINCR results in abnormal epidermal differentiation and lack of intact keratohyalin granules and lamellar bodies (Kretz et al. 2013). Consistently, the expression level of lncRNA TINCR decreases in cSCC (Kretz et al. 2013) and lncRNA TINCR promotes apoptosis and

autophagy via the ERK1/2-SP3 axis in cSCC cells (W. Zhou et al. 2019). Another tumor suppressor lncRNA show decreased expression in cSCC is GAS5 (T.-H. Wang et al. 2017). Overexpression of lncRNA GAS5 inhibits cell proliferation and promotes apoptosis in cSCC cells (T.-H. Wang et al. 2017). Conversely, oncogenic lncRNAs show upregulated expression in cSCC. LncRNA MALAT1 (Gutschner et al. 2013; J. Kim et al. 2018) and HOTAIR (K. Kim et al. 2013; Gupta et al. 2010) are two well-known oncogenic lncRNAs involved in multiple cancers. Enhanced expression of lncRNA MALAT1 (Y. Zhang et al. 2019) and HOTAIR (Yu et al. 2019) is also observed in cSCC and contributes to activated cell proliferation, migration, invasion and EMT process. Despite this, our knowledge about the role of most lncRNAs with altered expression in SCC is only rudimentary and more investigate is needed to understand their roles.

3.3 RNA-targeting therapeutics

RNA-targeting therapeutics, compared to traditional protein targeting drug, is an emerging class of technologies which target to specific RNA molecules and modulate the activity of endogenous RNAs. RNA-targeting therapeutics can work through different strategies. The two main categories of RNA-based therapeutics are antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) (Winkle et al. 2021). Additional therapeutics include miRNA mimics, anti-miRNA oligonucleotides (AMOs), miRNA sponges and CRISPR–Cas9-mediated genome editing (Roberts and Wood 2013; Winkle et al. 2021). Both protein-coding RNA and non-coding RNA can serve as target molecules. With the advent of preclinical research on non-coding RNAs, especially miRNAs and lncRNAs, they are emerging as key players in both physiological and pathological processes. Abnormal expression of miRNAs and lncRNAs has been implicated in a variety of cancer pathogenesis, as well as numerous other diseases, making therapeutic targeting of non-coding RNAs a promising approach for the treatment of these diseases. Over the past decade, a great deal of effort has been devoted to translation of RNA therapeutics and multiple RNA-based therapies have entered clinical trials (Table 1). However, so far, this is still a strategy that presents both opportunities and challenges.

3.3.1 MiRNA-based therapeutics

The fact that different targets of one miRNA can function in a single or multiple cancer-related signaling pathways makes miRNAs particularly attractive cancer therapeutic targets. There are two strategies proposed for miRNA-based therapy: increase of too low miRNA-activity using miRNA mimics and suppression of unwanted miRNA-activity by AMOs or miRNA sponges

(Roberts and Wood 2013; Rupaimoole and Slack 2017). MiRNA mimics are exogenous double-stranded RNA molecules designed to mimic the function of endogenous tumor suppressors miRNAs. In contrast, the function of endogenous oncogenic miRNAs can be neutralized by AMOs or miRNA sponges. The former function through sequestering or degrading mature miRNAs, while the later stabilize target transcripts by competitively binding miRNAs. There are several miRNA-based drugs on clinical trials. For instance, INT-1B3, a lipid nanoparticle formulated miR-193a-3p mimic, is under phase I/Ib study for the treatment of advanced solid tumors (Kotecki et al. 2021). TargomiRs are EnGeneIC Dream Vectors (EDVs, nanoparticles) which are loaded with miR-16-based miRNA mimic and target to EGFR-expressing cancer cells (Van Zandwijk et al. 2017). Phase I clinical trial of TargomiRs for the treatment of malignant pleural mesothelioma has completed with acceptable safety and early indications of activity (Van Zandwijk et al. 2017).

3.3.2 LncRNA-based therapeutics

There are several ongoing observational clinical trials which evaluate the potential of lncRNA as cancer biomarkers, such as the diagnosis and prognostic value of plasma lncRNA MFI2-AS1 in localized clear cell kidney cancers (NCT04946266); the diagnosis value of lncRNA WRAP53 and UCA-1 in hepatocellular carcinoma (NCT05088811); the diagnosis and prognosis value urine lncRNA in prostate cancer (NCT05141383); the diagnosis and prognostic value of plasma circRNA in pancreaticobiliary cancers (NCT04584996). However, currently, there have been no interventional clinical trials conducted related to lncRNA-based therapeutics. Considering lncRNAs have been proved to get involved in various kinds of cancers, they are promising targets for cancer therapy.

There is another lncRNA-related but no lncRNA-targeting cancer therapeutics which utilized lncRNA H19 promoter to express diphtheria toxin gene for the treatment of solid cancers. LncRNA H19 functions as oncogenic lncRNA and show increased expression level in several types of cancer (H. Li et al. 2014; F. Yang et al. 2012; M. Luo et al. 2013; Lottin 2002). H19-DTA (BC-819) is a plasmid that constructed to express diphtheria toxin gene under the regulation of the H19 promoter (Ohana et al. 2002). Clinical phase I/II trials were conducted with BC-819 in different lncRNA H19 highly expressed solid tumors. In the phase I/II clinical trials upon bladder cancer, BC-819 treatment achieved complete ablation of the tumor in 22%-33% of patients (Sidi et al. 2008; Gofrit et al. 2014). In a phase I/IIa clinical study on advanced unresectable pancreatic cancer, BC819 resulted in tumor shrinkage and may improve survival (Hanna et al. 2012). In a phase I/IIa study on ovarian cancer, 4 patients (31%) showed stable disease at 6 weeks after the treatment. There were no complete or partial responses (Lavie et al. 2017).

3.3.3 Challenges for RNA-targeting therapeutics

The clinical use of RNA-based therapeutics is challenged by several issues, including (1) specificity: how to improve the on-target effects of therapeutic RNA molecules in specific organ and cell types, as well as how to reduce the unwanted on-target effects and off-target effects; (2) delivery efficiency: how to deliver the RNA molecules specifically to the cell type of interest without degradation; (3) tolerability: how to avoid the adverse immune response induced by therapeutic RNA molecules. So far, RNA synthesis technology has undergone three generations of improvement through appropriate chemical modifications (Winkle et al. 2021). In the first-generation of chemical modifications, phosphodiester is replaced by phosphothiorate (PT) backbone linkages to enhance stability. In the second-generation, 2'-ribose modifications are used to enhance stability, target specificity, as well as reduced immune stimulation and toxicity. In the third-generation, chemical modifications on furanose ring are used to create locked nucleic acids (LNAs), peptide nucleic acids (PNAs) and phosphoramidate morpholino oligomers (PMOs) to further increase specificity and reduce immunogenicity. Several delivery approaches have been established to improve the efficacy RNA therapeutics, such as lipid nanoparticles (LNPs), polymers, RNA conjugations, metal-based nanoparticles and virus-based delivery systems (Winkle et al. 2021). Although gratifying progress has been made in the field of RNA-targeting therapeutics, unresolved challenges still exist, and numerous clinical trials have been halted due to low efficacy or unexpected side effects. For instance, the clinical trial of MRX34, a liposomal mimic of miRNA-34a designed for the treatment of advanced solid tumors, was halted because of immune related serious adverse events (Hong et al. 2020). To promote the clinical application of RNA-targeting therapeutics, there is still much to be explored. Some promising ideas have been proposed, such as apply extracellular vesicles or bacteriophage or bacterial minicell as vehicles to increase delivery efficacy; use CRISPR–Cas9-mediated genome editing for *ex vivo* manipulations to improve target specificity and utilize metronomic miRNA therapy to reduce immunogenicity (Winkle et al. 2021).

Table 1. RNA therapeutics in phase II or III clinical development (Winkle et al. 2021)

Type	Disease	Target gene and pathway	Phase	Identifier
Pri-miR-451 backbone	Huntington disease	Huntingtin (HTT) mRNA	I/II	NCT04120493
Anti-miR-103/107	Type II diabetes, nonalcoholic fatty liver disease	miR-103/107	I/II	NCT02612662, NCT02826525

miR-29 mimic	Keloid (pathological fibrosis)	miR-29 targetome	II	NCT02603224, NCT03601052
Anti-miR-122	Hepatitis C virus infection	miR-122	II	NCT01646489, NCT01727934, NCT01872936, NCT01200420
ASO	Acute/ chronic myeloid leukaemia	GRB2 mRNA	II	NCT01159028; NCT04196257; NCT02781883
ASO	Dyslipidaemias, hyperlipidaemias, hyperlipoproteinaemias	Angiopoietinlike 3 (ANGPTL3) mRNA	II	NCT04459767, NCT03371355, NCT04516291
ASO	Hereditary angio-oedema, COVID-19	Prekallikrein (PKK) mRNA	II	NCT03263507, NCT04030598, NCT04307381, NCT04549922
ASO	Metastatic NSCLC, resectable early-stage NSCLC, pancreatic cancer, mismatch repair-deficient colorectal cancer	STAT3 mRNA	II	NCT03819465, NCT03794544, NCT02983578
ASO	Squamous cell lung cancer, non-squamous NSCLC, urological neoplasms, metastatic bladder cancer, urinary tract neoplasms, castration-resistant prostate cancer	HSP27 mRNA	II	NCT01120470, NCT01454089, NCT01829113, NCT02423590
ASO	Leber congenital amaurosis type 10 (LCA10), blindness, LCA, vision disorders, sensation disorders, neurological manifestations, eye diseases, hereditary or congenital eye diseases	c.2991+1655A> G-mutated CEP290, pre-mRNA splicing	II/III	NCT03140969, NCT03913143, NCT03913130
ASO	Crohn's disease	ICAM1 mRNA	III	NCT03473626, NCT00063830, NCT00063414, NCT00048113, NCT02525523

ASO	Hereditary transthyretinmediated amyloid polyneuropathy	Transthyretin (TTR) mRNA	III	NCT04302064; NCT03728634; NCT04136184; NCT04136171
ASO (allelenonselective)	Huntington disease	HTT mRNA	III	NCT02519036, NCT04000594, NCT03342053, NCT03761849, NCT03842969
siRNA	Advanced pancreatic cancer	G12D-mutated KRAS mRNA	II	NCT01188785; NCT01676259
siRNA	Cardiovascular disease	Apolipoprotein A (LPA) mRNA	II	NCT03626662, NCT04270760
siRNA	Ocular hypertension, glaucoma	β-Adrenergic receptor 2 (ADRB2) mRNA	II	NCT00990743, NCT01227291, NCT01739244, NCT02250612
	Paroxysmal nocturnal haemoglobinuria, IgA nephropathy, Berger disease, glomerulonephritis	Complement 5 mRNA	II	NCT04601844, NCT02352493, NCT03841448, NCT03999840
siRNA	Dry eye disease	Transient receptor potential cation channel subfamily V member 1 (TRPV1)	III	NCT01438281, NCT01776658, NCT02455999, NCT03108664
siRNA	Hyperlipoproteinaemia	Apolipoprotein A mRNA	III	NCT03070782, NCT03070782, NCT04023552
siRNA	Primary hyperoxaluria type 1 and primary hyperoxaluria type 2, kidney diseases, urological diseases	Lactate dehydrogenase A enzyme (LDHA) mRNA	III	NCT03392896, NCT04555486, NCT04580420, NCT03847909, NCT04042402

ASO, antisense oligonucleotide; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA.

4 mRNA export and the TREX complex

In eukaryotes, protein-coding gene expression begins in the cell nucleus, where DNA is transcribed into RNA. Then RNA undergoes a series of processing steps before being exported to the cytoplasm, where it is translated into protein. The sequential events involved in the process of gene expression are tightly integrated from transcription, 5'-capping, splicing, 3'-polyadenylation to nuclear exportation (Cole 2001; Lei, Krebber, and Silver 2001). Any defect of these interdependent events impairs the progression of the other steps and results in the failure of gene expression. The TRanscription and EXport (TREX) complex plays a central role in coupling the multiple steps during gene expression both physically and functionally (Katja Strässer et al. 2002; Heath, Viphakone, and Wilson 2016).

4.1 Conservation and construction of the TREX complex

The TREX complex is evolutionarily conserved among yeast, plants, insects to mammalian (Katja Strässer et al. 2002; Reed and Hurt 2002; Khan et al. 2020; Rehwinkel et al. 2004). The importance of the TREX complex in coupling mRNA biogenesis from transcription to export was first discovered in *Saccharomyces cerevisiae* (Katja Strässer et al. 2002; Piruat 1998; Chávez and Aguilera 1997). In yeast, the TREX is composed by the THO-subunits (Tho2, Hpr1, Mft1, Thp2 and Tex1) and mRNA export factors Sub2 and Yra1 (Chavez 2000; Fischer 2002; Xie et al. 2021). In metazoans, the TREX complex is comprised of a hexameric THO core complex containing THOC1 (Hpr1 in yeast), THOC2 (Tho2 in yeast), THOC3 (Tex1 in yeast), fSAP79/THOC5, fSAP35/THOC6 and fSAP24/THOC7 and DECD-box RNA helicase UAP56 (Sub2 in yeast), the mRNA export adaptor protein Aly/THOC4 (Yra1 in yeast) (Masuda et al. 2005). The conservation of the TREX complex in a wide range of organisms indicates it crucial physiological importance, for example, THOC1 and THOC5 are essential for murine early embryonic development and loss of THOC1 or THOC5 cause embryonic lethality (Xiaoling Wang et al. 2006; Mancini et al. 2010).

4.2 The role of TREX complex in gene expression

After initiation of transcription, newly synthesized mRNAs bind with a diverse array of proteins and form messenger ribonucleoproteins (mRNPs). These mRNPs are essential for mRNA maturation and export. The function of the TREX complex is to recruit and coordinate the assembly of mRNPs.

TREX complex is recruited co-transcriptionally. In yeast, the TREX complex plays critical roles during gene expression including contributing to transcription elongation, genome stability, mRNA processing and export (Piruat 1998; Chavez 2000; Chávez and Aguilera 1997). Mutations on any subunit of the THO complex result in DNA repeat recombination, impaired transcriptional elongation and mRNA export (Katja Strässer et al. 2002; Chávez and Aguilera 1997; Chavez 2000). Unlike the yeast TREX complex which functions in transcription, the human TREX complex is recruited independently of transcription but during splicing and colocalized with splicing factors in nuclear speckle domains (Masuda et al. 2005).

During splicing, DEAD-box helicase UAP56 is recruited to the pre-mRNA through interacting with splicing factor U2AF65, in turn, UAP56 promotes the assembly of spliceosome (Fleckner et al. 1997; M. Zhang and Green 2001; Libri et al. 2001; Kistler 2001). Additionally, UAP56 binds with mRNA export adaptor protein Aly and recruits it to the spliced mRNP (M.-J. Luo et al. 2001). Therefore, UAP56 coupled the splicing machinery to export machinery. Notably, excess UAP56 exert an inhibitory effect on regulating mRNA export through blocking the recruitment of Aly to spliced mRNP (M.-J. Luo et al. 2001). Once THO subunits and Aly bind to the mRNA export receptor NXF1, RNA-binding domain of NXF1 is exposed (K. Strässer and Hurt 2000; Hautbergue et al. 2008; Viphakone et al. 2012; Hung et al. 2010; Katahira et al. 2009). This allows NXF1 to take over the binding RNAs of Aly and transport them into the cytoplasm through the nuclear pore complex.

5 Extracellular Vesicles

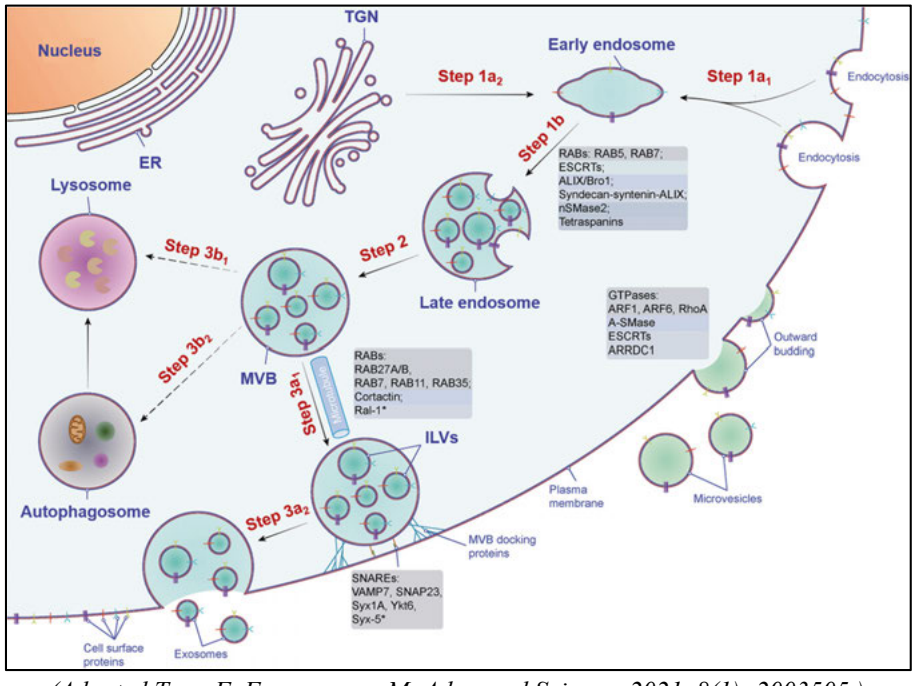
5.1 Classification and biogenesis of extracellular vesicles

Extracellular vesicles (EVs) are a group of highly heterogeneous membrane-bound vesicles of different cellular origin, biogenesis, content and function (Théry 2011; Mathieu et al. 2019). Initially, EVs were considered as “waste carrier” for eliminating redundant cell-components. With the technological advancements, such as improved EV isolation and characterization methods, innovative imaging techniques and advanced high-throughput omics techniques, the significant roles of EVs have been well accepted especially for their functions in mediating intercellular communication in various physiological and pathological processes. EVs are secreted by virtually all cell types and are distributed in various bodily fluids and tissues. The extensive distribution of EVs in the human body allow them to transmit signals between cells with their enclosing biomolecular cargoes (proteins, lipids and nucleic acids) (van Niel, D’Angelo, and Raposo 2018).

Based on their biogenesis, EVs are classified into two major subpopulations: exosomes and microvesicles (MVs) (Mathieu et al. 2019; EL Andaloussi et al. 2013; Wiklander et al. 2019). In addition, apoptotic bodies are vesicles released by apoptotic cells through extensive plasma membrane blebbing (Mathieu et al. 2019). A new member of EVs family is exomeres, a population of non-membranous nanoparticles with a size ≤50 nm (Anand, Samuel, and Mathivanan 2021). Despite the classification and terminology of EVs, it is difficult to assign EVs isolated with distinct methods to a unique biogenesis pathway due to their similar morphology, overlapping size range as well as the absence of truly specific markers for EV-subtypes. Therefore, the International Society for EVs (ISEV) recommends to use the collective term "EVs" instead of exosome and MVs (Théry et al. 2018).

Exosomes (~30–150 nm) are generated through the endocytic pathway in a stepwise manner (Figure 7) (Mathieu et al. 2019; van Niel, D’Angelo, and Raposo 2018; H. Zhang et al. 2018; Teng and Fussenegger 2021): firstly, early endosomes are formed by internalization of the plasma membrane (Step $1a_1$), or in some instances from the trans-Golgi network (TGN) (Step $1a_2$) and mature into late endosomes (Step 1b). Then late endosomes develop into multivesicular bodies (MVBs) and generate intraluminal vesicles (ILVs) by inward invagination of the endosome membrane (Step 2). Exosomes are eventually released into the extracellular environment when MVBs fuse with the plasma membrane (Step $3a_{1,\ 2}$). Alternatively, MVBs fuse with lysosomes or

autophagosomes for degradation (Step $3b_1$, $_2$). MVs (~100–1000 nm) are formed through outward budding of the plasma membrane (Figure 7) (Mathieu et al. 2019; Teng and Fussenegger 2021).



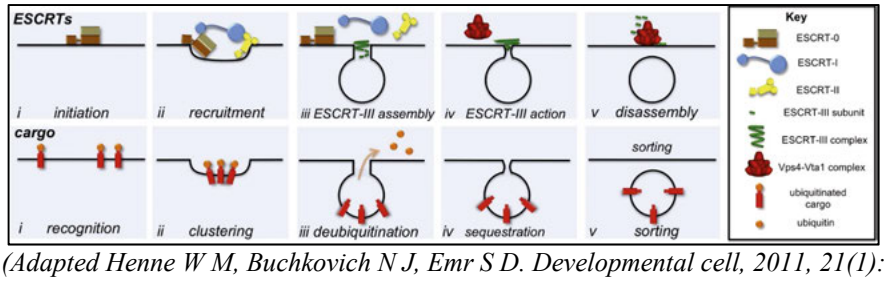
(Adapted Teng F, Fussenegger M. Advanced Science, 2021, 8(1): 2003505.)

Figure 7. Schematic model of EV biogenesis and release. EVs are categorized into exosomes and MVs based on biogenesis. Exosomes are formed in MVB through endocytic pathway by inward budding of endosomal membranes. MVs are formed through inward budding of plasma membrane. EVs: Extracellular Vesicles; MVs: microvesicles; MVB: multivesicular bodies.

5.2 EV cargo sorting

EV cargo sorting is finely tuned by particular sorting machineries through the endosome sorting complexes required for transport (ESCRT) pathway or the ESCRT-independent pathway (van Niel, D'Angelo, and Raposo 2018; Teng and Fussenegger 2021). ESCRT, consisting four cytosolic protein complexes ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, is crucial for the formation of MVBs and EVs cargo sorting, particularly for ubiquitinated cargoes (Henne, Buchkovich, and Emr 2011; Vietri, Radulovic, and Stenmark 2020). All ESCRT-0, ESCRT-I and ESCRT-II have ubiquitin binding ability which is essential for cargo sorting. In the canonical ESCRT dependent pathway (Figure 8), ESCRT-0 is responsible for recognizing and binding with ubiquitinated cargoes and then be recruited to the endosomal membrane (Henne, Buchkovich, and Emr 2011;

Raiborg and Stenmark 2009). Subsequently, ESCRT-I and ESCRT-II are recruited by ESCRT-0 and mediate endosomal membrane invagination (Wollert and Hurley 2010). ESCRT-III components are recruited by ESCRT-II and responsible for recruiting deubiquitinating machinery and cleaving IVLs into the MVBs lumen (Henne, Buchkovich, and Emr 2011; Wollert and Hurley 2010). In the non-canonical ESCRT dependent pathway, scission of IVLs is mediated by ESCRT-III, while membrane budding and cargo clustering can occur independent of ESCRT components, for example, ALIX can function as ubiquitin receptor instead of ESCRT-0 (Pashkova et al. 2013). In the ESCRT-independent pathway, ceramide and CD63 play an important role for ILV biogenesis. Besides ubiquitination, other post-translational modifications of protein can also control protein cargo sorting, such as phosphorylation, SUMOylation, NEDDylation and ISGylation (Anand et al. 2019). The mechanism of the biogenesis of MVs is yet to be revealed.



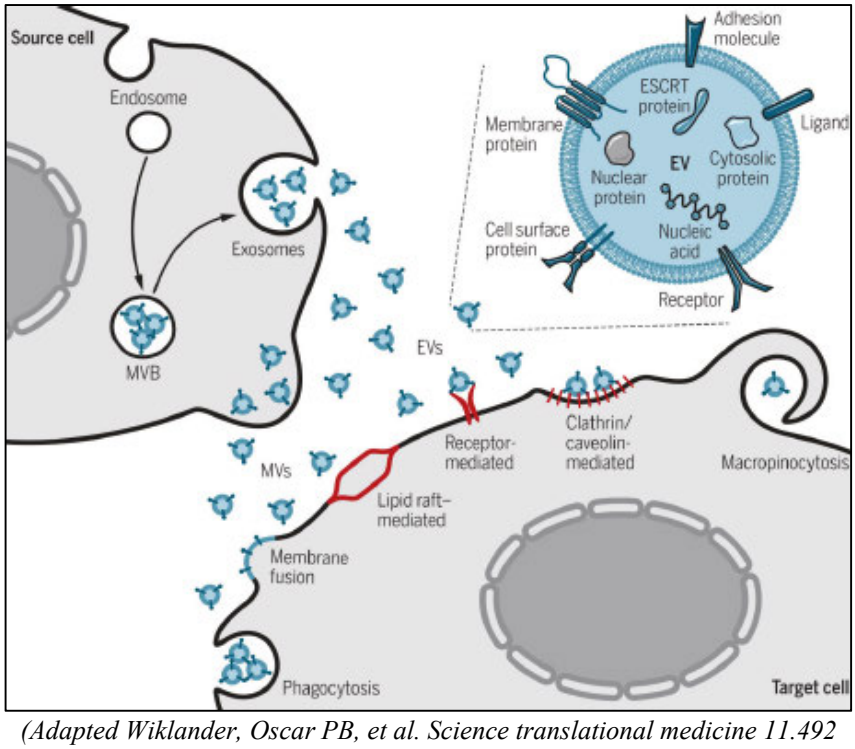
(Adapted Henne W M, Buchkovich N J, Emr S D. Developmental cell, 2011, 21(1): 77-91.)

Figure 8. The canonical ESCRT dependent pathway. ESCRT-0 initiates the pathway by recognizing ubiquitinated cargoes (i). ESCRT-0 hands over ubiquitinated cargoes to ESCRT-I and ESCRT-II which mediate membrane budding and cargo clustering (ii). ESCRT-III is recruited by ESCRT-II and assembles deubiquitinating machinery (iii). ESCRT-III constricts and cleave endosomal membrane and finally packages sorted cargoes into IVLs (iv and v). ESCRT: endosome sorting complexes required for transport; IVLs: intraluminal vesicles.

5.3 Uptake of EVs

Once released by parental cells and entering into the extracellular space, EVs can be recognized and taken up by recipient cells (Figure 9) (Christianson et al. 2013; Feng et al. 2010; Franzen et al. 2014; Tian et al. 2013; Mulcahy, Pink, and Carter 2014). Due to the diversity of EVs, the cellular and molecular mechanisms of EVs binding and uptake by recipient cells are not fully unraveled (H. Zhang et al. 2018). The general modes for EV-uptake could be categorized into two major types: (i) membrane fusion and (ii) endocytosis (Parolini et al. 2009). EV-uptake through the fusion of EVs and plasma membrane allows EVs releasing contents to recipient cells without entry (Figure 9) (Mathieu

et al. 2019; H. Zhang et al. 2018; van Dongen et al. 2016). EV-uptake via endocytosis can be both specific and unspecific (Parolini et al. 2009). Specific endocytosis is mediated by the interaction between membrane-exposed ligands (proteins, sugars or lipids) of EVs and receptors on recipient cells plasma membrane, which could further initiate intracellular signaling pathways (Figure 9) (Svensson et al. 2013; Tian et al. 2014; Escrevente et al. 2011). Impairment of EV-uptake caused by proteinase K-treatments emphasized the necessity of protein-protein interaction between EVs and recipient cells (R. Xu et al. 2018). Moreover, unspecific EV-internalization can be achieved through endocytosis such as phagocytosis or micropinocytosis/ micropinocytosis (Figure 9) (Franzen et al. 2014). Depending on the composition of EVs and recipient cell type, internalized EVs can be recycled and secreted into extracellular space, or degraded by the lysosome and EVs contents will be recycled and metabolized by the recipient cells (Mathieu et al. 2019; H. Zhang et al. 2018).



(Adapted Wiklander, Oscar PB, et al. Science translational medicine 11.492 (2019).)

Figure 9. Schematic model of EV uptake. Uptake of EVs by recipient cells are through membrane fusion or endocytosis. Endocytosis includes EVs surface exposed ligands-cell receptors interaction, phagocytosis and micropinocytosis. EVs: extracellular vesicles; MVs: microvesicles; MVB: multivesicular bodies.

5.4 EVs in cancers

Substantial evidence suggests that EVs can participate tumorigenesis through carrying and transmitting oncogenic proteins and nucleic acids (Le et al. 2014; Fuentes et al. 2020; Takasugi et al. 2017). The tumor microenvironment (TME) is made up of stromal cells (fibroblasts, immune cells, neuroendocrine cells and adipose cells) and extracellular matrix (ECM) surrounding cancers cells which has profound effect on cancer cells behaviors (Spill et al. 2016; Balkwill, Capasso, and Hagemann 2012). EV-mediated bidirectional communication between cancer cells and stromal cells in TME plays a promoting role in cancer initiation, progression, and metastasis (R. Xu et al. 2018; Martins, Dias, and Hainaut 2013). EVs released by cancer cells can drive the transformation of surrounding non-malignant cells (Pang et al. 2015; Webber et al. 2009; Vu et al. 2019), induce endothelial cells angiogenesis (Al-Nedawi et al. 2009; Hsu et al. 2017), promote pre-metastatic niche formation (Peinado et al. 2012; Fong et al. 2015) and suppress immune response (Clayton et al. 2007; Haderk et al. 2017). The other way around, stromal EVs are able to target cancer cells and enhance cancer cell malignant phenotypes. Fibroblast- (Nabet et al. 2017; Boelens et al. 2014), macrophage- (M. Yang et al. 2011) derived EVs are uptake by cancer cells and promote therapy resistance, invasion and metastasis. It has been reported that SCC-derived EVs can regulate TME formation and result in aggressive cancer. Highly expressed C-terminal fragment of desmoglein 2 (Dsg2) in SCC cells enhances EVs secretion which is able to modulate TME and promote tumor growth through carrying cytokines and miRNAs (Overmiller et al. 2017; Flemming et al. 2020). However, further investigation is needed to identify the cargo of EVs and their role in tumorigenesis and development of cSCC.

6 Present investigations

6.1 Paper I. MiR-130a acts as a tumor suppressor microRNA in cutaneous squamous cell carcinoma and regulates the activity of the BMP/SMAD pathway by suppressing ACVR1.

In **Paper I**, our aim was to investigate the function of miR-130a-3p, generally referred to as miR-130a hereafter, in cSCC. We reported that miR-130a expression was decreased in invasive cSCCs compared to healthy skin and precancerous skin lesion - actinic keratosis (AK). This suggests that the decreased expression level of miR-130a is associated with the malignant phenotypes of cSCC. The expression level of miR-130a in cSCC was regulated by the MAPK pathway, where activation of the HRAS-MEK-ERK1/2 pathway resulted in suppressed miR-130a expression at the transcriptional level.

To investigate the function of miR-130a in modulating cSCC tumor growth, we established xenograft models by subcutaneously injecting miR-130a overexpressing cSCC cells and control cSCC cells into immunocompromised mice. By monitoring tumor size and weight, we observed that elevated expression levels of miR-130a exerted a suppressive role on cSCC tumor growth in vivo. Consistently, our in vitro experiments showed that cSCC cells with ectopic overexpression of miR-130a demonstrated diminished abilities in colonization, cell motility, and invasiveness. In contrast, inhibiting endogenous miR-130a enhanced the malignant behaviors of cSCC cells. Therefore, our in vivo and in vitro experiments have demonstrated a cancer-suppressive role of miR-130a in cSCC.

Mechanistically, we identified activin receptor type I (ACVR1/ALK2) as a direct target of miR-130a and showed that miR-130a regulates the BMP/SMAD pathway by directly targeting ACVR1. Taken together, our study recognizes miR-130a as a tumor suppressor in cSCC under the regulation of the HRAS-MEK-ERK1/2 pathway. Our results identify a potential cross-talk between the MAPK pathway and BMP/SMAD1/5 pathway via miR-130a-mediated regulation of ACVR1 in cSCC.

6.2 Paper II. Long non-coding RNA PVT1 is overexpressed in cutaneous squamous cell carcinoma and exon 2 is critical for its oncogenicity.

In **Paper II**, our aim was to investigate the function and the underlying molecular mechanism of lncRNA Plasmacytoma Variant Translocation 1 (PVT1) in cSCC. Our attention was drawn to PVT1 because it was identified as one of the top upregulated lncRNAs in cSCC through our previous RNA-seq analysis conducted on normal skin and cSCCs. To validate the observed difference in PVT1 expression and determine the stage of the cSCC spectrum at which PVT1 expression is altered, we conducted qRT-PCR on a cohort of tissue samples including normal skin, precancerous skin lesion AK and cSCCs. The qRT-PCR data indicated that PVT1 was highly expressed in cSCC compared to healthy skin and AK. To further validate the expression pattern of PVT1 and analyze its clinical relevance, we employed single molecule *in situ* hybridization (RNAScope) on a large cohort samples, encompassing normal skin (n= 59), benign papillomatous proliferation seborrheic keratosis (SK, n=12), AK (n=60), cSCC *in situ* (cSCCIS, n=57), invasive non-metastatic cSCC (cSCC, n=31), metastatic cSCC (n=41) and cSCC metastases (n=7). Quantification of visible PVT1 molecules revealed that PVT1 was expressed at a low level in normal skin, SK and AK; however, its expression was increased in cSCCIS and invasive cSCC. Taken together, our data indicates that PVT1 is associated with keratinocyte transformation and cSCC-development. We identify MYC as an upstream regulator of PVT1 expression and demonstrate that MYC modulates the expression level of PVT1 in both normal keratinocytes and cSCC cells.

The aberrant expression of PVT1 in cSCC indicates that it may be associated with malignant behaviors of cSCC cells. Next, we investigated the function of PVT1 in cSCC. CRISPR-Cas9-mediated deletion of the entire *PVT1* gene locus (around 300 kb) demonstrated that the *PVT1* locus had an oncogenic role in cSCC, as *PVT1* deletion lead to impaired malignant behaviors (proliferation, colonization and tumor spheroid formation) of cSCC cells. LNA GapmeR-mediated knockdown of PVT1-transcripts showed similar tumor suppressive effects as *PVT1* gene locus deletion, suggesting that PVT1 is an oncogenic lncRNA in cSCC independent of the *PVT1* gene locus. As one of the most complicated gene loci, the *PVT1* gene locus encodes more than 35 isoforms which makes the research on PVT1 challenging. We were curious to determine which isoforms of PVT1 are responsible for exerting the oncogenic role in cSCC. We initially focused on PVT1 transcripts containing exon 2 because exon 2 is the most prevalent exon in PVT1 and we found it to be highly expressed in cSCC. Through CRISPR-Cas9-mediated deletion of *PVT1 exon 2*, we identified exon 2 as a crucial element for PVT1's oncogenic effect in

cSCC. Deletion of PVT1 exon 2 inhibited cSCC tumor growth both *in vivo* and *in vitro*. To determine the effect of PVT1 on the transcriptome and signaling pathways in cSCC cells, we conducted RNA-seq-analysis on *PVT1 exon 2* knockout cSCC cells and wildtype cSCC cells. Bioinformatic analysis on the RNA-seq data suggested that cellular senescence-associated genes were differentially expressed in *PVT1 exon 2* knockout cSCC cells. Therefore, we uncovered the molecular mechanism for the oncogenic role of PVT1 in cSCC that lncRNA PVT1 was localized in the cell nucleus and suppressed cellular senescence by inhibiting CDKN1A/p21 expression and preventing cell cycle arrest. Taken together, our data identify lncRNA PVT1 as a potential target molecule for the management of cSCC and a novel biomarker for cSCC progression.

6.3 Paper III. PVT1 regulates the nuclear export of polyadenylated RNAs through interacting with TREX complex.

In **Paper III**, we further explored the underlying mechanism behind the oncogenic role of PVT1 in cSCC. The modes of action of lncRNA are largely depend on their cellular localization. Both single molecule *in situ* hybridization (RNAScope) and cellular fractionation conducted on cSCC cells revealed a predominantly nuclear localization for PVT1 which is consistent with the results obtained in human tissue samples of benign, precancerous and cancerous lesions. We hypothesized that the necessity of exon 2 for the oncogenic role of PVT1 arises from exon 2's role in mediating the nuclear localization of PVT1. Interestingly, we observed that the specific deletion of exon 2 resulted in a diffuse cytoplasmic distribution of PVT1 transcripts in cSCC cells.

RNA-seq performed on wildtype cSCC cells and *PVT1 exon 2* knockout cSCC cells identified a set of differentially expression genes associated with RNA processing, such as mRNA-splicing, capped intron-containing pre-mRNA processing, transport of mature mRNA derived from intron less transcripts and mRNA export from nucleus. To further investigate how PVT1 is involved in the process of RNA processing, we conducted comprehensive identification of RNA-binding proteins by mass spectrometry (ChIRP-ms) to identify the interacting proteins of PVT1. Among proteins specifically binding to PVT1, UAP56/DDX39B (DExD-Box Helicase 39B), URH49/DDX39A (DExD-Box Helicase 39A) and THOC4/ALYREF (Aly/REF Export Factor) were identified which are components of the transcription-export (TREX) complex. The TREX complex is a conserved protein complex which integrates RNA biogenesis from transcription to RNA processing and nuclear export. To investigate whether PVT1 is involved in the process of TREX complex-mediated nuclear RNA export, we determined the distribution of poly

$(A)^+$ RNA in *PVT1 exon 2* knockout cSCC cells. Strikingly, we observed the accumulation of poly $(A)^+$ RNAs in the cell nuclei of *PVT1* and *PVT1 exon 2* knockout cSCC cells. This result indicated that PVT1 is an indispensable molecule for poly $(A)^+$ RNA transport from cell nucleus to cytoplasm. Additionally, we found that Lamin B1 mRNA was retained in nucleus in *PVT1* and *PVT1 exon 2* knockout cSCC cells. Lamin B1 has been implicated in cellular senescence whose expression level is decreased during senescence. Therefore, *PVT1* and *PVT1 exon 2* knockout caused nuclear accumulation of Lamin B1 mRNA could at least partially explain why cSCC cells undergone cellular senescence upon the deletion of *PVT1* and *PVT1 exon 2*.

Collectively, we have identified a segment of the PVT1 transcript responsible for its nuclear localization. Moreover, our findings indicate that PVT1 serves as a key regulator for RNA export from the cell nucleus, unveiling a novel mechanism that explains both the oncogenic role of PVT1 and its involvement in modulating RNA export. Further investigations are currently underway to precisely define PVT1's role in the functioning of the TREX complex and mRNA export.

6.4 Paper IV. Cutaneous squamous cell carcinoma-derived extracellular vesicles exert an oncogenic role by activating cancer-associated fibroblasts.

In **Paper IV**, we conducted a comprehensive analysis of extracellular vesicles (EVs) produced by normal skin cells and skin cancer cell lines, including two primary cSCC cell lines (UT-SCC-111 and A431) and one metastatic cSCC cell line (UT-SCC-7). Our investigation focused on understanding the regulatory effects of cSCC cell-derived EVs on stromal cells within the tumor microenvironment (TME).

EVs were isolated from conditioned medium using size-exclusion chromatography. To characterize isolated EVs, transmission electron microscopy and cryogenic electron microscopy were used to visualize these double-layered phospholipid membrane vesicles. EV markers Alix, CD9 and TSG101 were detected by immunoblotting. Nanoparticle tracking analysis (NTA) was used to determine the size and concentration of collected EVs. Through normalizing NTA-results to cell counts, we found that cSCC cell lines secreted more EVs than normal skin cells. A multiplex bead-based flow cytometry assay was conducted to determine the surface composition of EVs. A total of 37 EV surface protein were detected and a substantial heterogeneity in terms of their EV-surface proteins were identified between metastatic cSCC cells and primary cSCC cells.

To investigate the effect of cSCC cell-derived EVs on cSCC tumor growth, xenograft models were established using cSCC cells possessing normal EVs

secretion ability (control cells) and cSCC cells with impaired EV secretion ability (RAB27A knockdown cells). It was revealed that normal EV secretion ability is essential for cSCC tumor growth *in vivo*. To understand how cSCC cell-derived EVs regulate cSCC tumor growth, we conducted RNA-seq on xenograft tissues, which allowed us to identify differentially expressed genes in both human (representing cSCC cells) and mouse (majorly representing tumor-infiltrating stromal cells) transcriptome. Interestingly, differentially expressed genes in stromal cells in the RAB27A-knockdown-group were significantly enriched in pathways associated with extracellular matrix (ECM) organization, which suggested that cSCC cell-derived EVs may have a regulatory effect on fibroblasts in TME who are responsible for ECM organization. To test this hypothesis, we co-cultured human dermal fibroblasts with either normal skin cells or cSCC cells and found that cSCC cell drove the onset of cancer-associated fibroblast phenotype. Through administrating cSCC-derived EVs to fibroblasts, we found that metastatic UT-SCC-7 cell-derived EVs were able to educate fibroblasts to cancer-associated fibroblasts but not primary cSCC cell-derived EVs, which may be explained by the specific protein cargoes carried by metastatic UT-SCC-7 cells as determined by mass-spectrometry (MS) based proteomics analysis. Additionally, we uncovered that metastatic UT-SCC-7 cell-derived EVs were able to activate the TGFβ signaling pathway, an epithelial-mesenchymal transition (EMT) inducer, in fibroblasts. In conclusion, our study reveals that cSCC cell-derived EVs play an essential role in promoting cSCC tumor growth by mediating cancer-stroma communication through specific protein cargoes.

7 Future perspectives

7.1 Paper I. MiR-130a acts as a tumor suppressor microRNA in cutaneous squamous cell carcinoma and regulates the activity of the BMP/SMAD pathway by suppressing ACVR1.

Considering the decreased expression level of miR-130a in cSCC compared to healthy skin and the precancerous skin lesion AK, miR130a is a promising diagnostic biomarker for cSCC. Additionally, our study reported that miR-130a exerts a tumor suppressor role in the context of cSCC by modulating cSCC cell growth, migration and invasion. This suggests that miR-130a is a potential biomarker associated with the prognosis of patients with cSCC. It would be beneficial to assess the potential of miR-130a as a diagnostic or prognostic biomarker for cSCC through recruiting a large cohort of patients with cSCC at various stages, as well as healthy controls and patients with precancerous skin lesions.

The high mutation burdens caused by chronic excess exposure to UVR make it challenging to identify the driver genes for the progression of AK and cSCC. Our results revealed that the expression level of miR-130a was decreased not only compared to healthy skin, but also in comparison to precancerous skin lesion AK. This suggests that miR-130a is involved in modulating the transformation of AK and the progression of cSCC. Therefore, miR-130a may serve as a molecular target to impede the conversion of AK to cSCC and hinder the progression of cSCC. Additionally, we found that the expression of miR-130a was regulated by the MAPK pathway in cSCC. Considering that EGFR inhibitors are being tested for the treatment of cSCC, it is plausible to hypothesize that a combination therapy of miR-130a and EGFR inhibitors might provide a new option for cSCC management.

7.2 Paper II. Long non-coding RNA PVT1 is overexpressed in cutaneous squamous cell carcinoma and exon 2 is critical for its oncogenicity.

As suggested by our qRT-PCR and single molecule *in situ* hybridization (RNAScope) data, the expression level of PVT1 was increased in cSCCIS and invasive cSCC in comparison to normal skin, benign skin lesions SKs and precancerous lesions AKs. Therefore, PVT1 may be used as a biomarker for diagnosis of cSCC. Additionally, the value of PVT1 as a prognostic biomarker for cSCC can be assessed by analyzing the relationship between its expression level and patient prognosis. In our study, we have discovered that PVT1 transcripts exert an oncogenic role in cSCC, and depletion of PVT1 results in cSCC cell senescence. This finding has illuminated the potential of PVT1 as a therapeutic target for cSCC, through which malignant proliferation of cancer cells will be inhibited by inducing cellular senescence.

Our mechanistic study revealed that the exon 2 is a key element for PVT1 to promote the malignant behaviors of cSCC cells which provide a more precise site for the potential PVT1-based targeted therapy. However, the underlying mechanism for exon 2 acting as an oncogenic element remains to be explored. In addition, although we have discovered the oncogenic role of those exon 2-containing PVT1 transcripts, due to the complexity of the *PVT1* gene locus, we have tried but not yet managed to determine the sequence and expression pattern of various PVT1 isoforms. Advanced RNA-seq techniques may help elucidate how many and which PVT1 isoforms are encoded by the *PVT1* gene locus in cSCC and determine their exact sequence and expression levels. In this case, functional studies will be more precise down to each individual PVT1 isoform, many of which may have different functions.

7.3 Paper III. PVT1 regulates the nuclear export of polyadenylated RNAs through interacting with TREX complex.

Our research has discovered a novel mechanism for the oncogenic role of PVT1 in the context of cSCC: PVT1 is crucial for TREX complex-mediated export of poly (A)+ RNA from cell nucleus to cytoplasm. However, some questions still remain to be explored. It has been reported that the two closely related RNA helicases, UAP56 and URH49, regulate nuclear export of distinct mRNAs (Yamazaki et al. 2010). We would like to determine whether PVT1 modulates the export of poly (A)+ RNA in general or specifically regulates the export of a certain group of poly (A)+ RNAs. We have found that exon 2

of PVT1 is essential for its oncogenic role and nuclear localization in cSCC. We are wondering whether it is exon 2 that mediates the interaction between PVT1 and the TREX complex, and whether this interaction is direct or indirect, and how does it affect the function of TREX. This is the first time that a lncRNA has been found to be involved in the regulation of the function of TREX and nuclear RNA export. We are currently investigating whether the regulatory effect of PVT1 on mRNA-export is specific to cSCCs or if it is a general mechanism present in other normal and malignant cells as well.

7.4 Paper IV. Cutaneous squamous cell carcinoma-derived extracellular vesicles exert an oncogenic role by activating cancer-associated fibroblasts.

Our study has revealed the cargoes of various cSCC cell line derived EVs and emphasized the function of cSCC cell-derived EVs on mediating cancer-stroma communication and promoting cSCC tumor growth. Our results suggest the specific effect of metastatic UT-SCC-7 cell-derived EVs, but not primary cSCC cell-derived EVs, on driving the onset of the cancer-associated fibroblast phenotype. However, future research will be needed with an even larger number of cell lines and possible patient-derived samples. It will be interesting to investigate the effect of UT-SCC-7 cell-derived EVs on the invasive and metastatic capabilities of cSCC using experimental models that test metastatic spread. We could hypothesize that metastatic UT-SCC-7 cell-derived EVs could shape a permissive TME for metastasis through educating stroma cells within TME. To test this, metastasis can be analyzed in xenograft models. In addition, the changes on fibroblast phenotypes triggered by metastatic cSCC cell-derived EVs can be evaluated. A collagen gel contraction assay can be used to assess the contractile ability of fibroblasts, which is important for the remodeling of the ECM. ECM components such as collagen and fibronectin can be detected by immunofluorescence staining to evaluate the ability of fibroblasts to organize the ECM. Nevertheless, the results regarding the enormous heterogeneity of cSCC-derived EVs and their cargo is in line with the genetic and phenotypic heterogeneity of cSCC tumors on patients.

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