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Unusual Presentation of COVID-19: Encephalitis and Syndrome of Inappropriate Anti-Diuretic Hormone Secretion

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Abstract

COVID-19 is a new challenge in clinical medicine. Although typical presentations include fever and pneumonia, we describe a case of COVID-19 presenting with neurological symptoms of encephalitis and infectious-related syndrome of inappropriate anti-diuretic hormone secretion. Because of the epidemic health problems, it is crucial to identify these patients as early as possible to follow the isolation procedures. We suggest that unclear neurological clinical presentations of patients should be considered for COVID-19.

Keywords

COVID-19, SARS-Cov-2, Encephalitis, Syndrome of Inappropriate Anti-Diuretic Hormone, Isolation Procedures

1. Introduction

Coronavirus Disease (COVID-19) has spread as a world pandemic in the last few months. Since this epidemic is sustained by a new strain of coronavirus (Severe Acute Respiratory Syndrome Coronavirus-2: SARS-CoV-2), new aspects of this disease are emerging.

The first clinical descriptions of the disease point to lung disease often leading to pulmonary failure [1] [2]. However, neurotropic properties have been shown for coronavirus and SARS-Cov-2 [3] and neurological manifestations such as loss of smell have been described [4].

2. Clinical Case

Among atypical presentations of COVID-19, we describe here for the first time a neurological presentation with encephalitis and syndrome of inappropriate anti-diuretic hormone secretion (SIADH).

The patient, Ms. P.A., female, 79 years old, was admitted on March 7th, 2020 to the University Hospital of Varese, during the epidemic phase of SARS-CoV-2 in Lombardy Region, Italy.

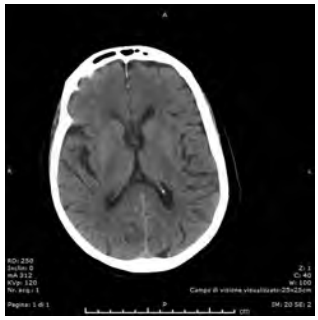
At admission in the emergency department, she presented with neurological symptoms suggesting encephalitis (confusion, somnolence, psychomotor retardation, cephalea). Five to 7 days before admission to the hospital the patient reported gastrointestinal symptoms, with vomit and diarrhea, followed by constipation. In her medical history, the patient referred rheumatoid arthritis treated with hydroxychloroquine sulfate (200 mg/day) and low dose steroid treatment (prednisone 5 mg/day).

At the clinical evaluation, no fever, no dyspnea and no cought were reported. Laboratory tests revealed severe hyponatremia (118 mEq/L; normal range: 136 - 150 mEq/L) and normal values of C reactive Protein (1.6 mg/L; normal range: 0.0 - 5.0 mg/L) and lactate dehydrogenase (235 U/L; normal range: 130 - 240 U/L), and no lymphopenia.

After submitting the patient to brain CT scan (**Figure 1**: non-specific diffuse cerebral atrophy), the neurologist suggested the execution of a lumbar puncture. Cerebrospinal fluid (CSF) analysis showed normal findings: glucose 49 mg/dL, proteins 61 mg/dL; <15 cells /field, negative search for cells and bacteria at microscopy. A search for polymerase chain reaction for Herpes Simplex Virus (HSV) 1 and HSV-2 was conducted on the CSF sample. At electroencephalography triphasic waves were interpreted as non-specific findings and in relation to dysmetabolic changes. At supine chest X Ray there were no signs of lung parenchymal disease (**Figure 2(a)**).

The patient was then transferred on March 10th to the Internal Medicine ward dedicated to non-COVID patients. She progressively continued the correction of hyponatremia, and SIADH was diagnosed [5] based on decreased effective osmolality of the extracellular fluid (P_{osm}: 265 mOsmol/Kg H_2O), inappropriate urinary concentration (U_{osm}: 377 mOsmol/KgH_2O), and elevated urinary Na excretion (189 mmol/24h). A total body CT scan was prescribed for the clinical suspect of a malignant-associated SIADH. The patient was started on acyclovir therapy while waiting for the HSV-DNA result. To further investigate whether a sub-occluding fecaloma was the cause of the mild abdominal pain and constipation reported the days before admission, a rectal exploration was conducted with the finding of malodorous, curry-colored feces.

On March 11th and 12th, hyponatremia was almost completely corrected (130 mEq/L) with a progressive, slow improvement of neurological symptoms, but recovery was incomplete and she was still partially inappropriately confused (to be noted that the patient was fully active and was able of caring the whole family before hospital admission).



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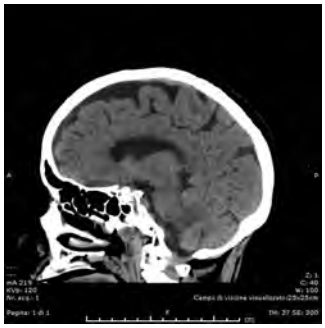
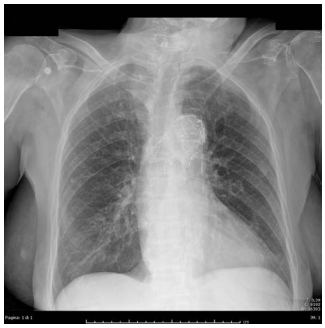
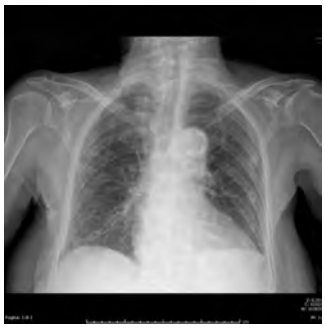


(b)

Figure 1. Brain CT scan. (a) and (b) show non-specific diffuse cortical atrophy at brain CT scan.



(a)



(b)

Figure 2. Chest X Rays at admission and during pneumonia. (a) shows a normal chest X Ray at admission to the hospital (patient in supine position), in the presence of only neurological symptoms and (b) (patient in sitting position) shows mild lung peripheral parenchymal infiltrate, in the presence of mild classical COVID-related symptoms adding to the neurological manifestations.

The total body CT scan was performed on March, 13th and showed normal findings; in particular no malignancies, no pneumonia or lung diseases. Testing for HSV was negative and acyclovir treatment was stopped.

On March 14th she presented fever (temperature 38˚ Celsius) and light cough and, therefore, she performed a nasopharyngeal swab for SARS-CoV-2 which resulted positive (for methods see supplemental material). The CSF obtained 7 days prior to the occurrence of fever in the suspect of neurological disturbances other than COVID-19, was then tested for SARS-CoV-2 with negative results (for methods see supplemental material).

The patient was then moved to a dedicated COVID department where she developed mild signs of COVID-19-related pneumonia (mild dyspnea and O_2 desaturation at pulse oximeter; at chest X Ray (**Figure 2(b)**): mild peripheral parenchymal infiltrate), and was treated with lopinavir/ritonavir, hydroxychloroquine sulfate (200 mg twice/day), antibiotics (piperacillin/tazobactam) and mild non-invasive O_2 therapy (2 L/min). In 15 days she recovered completely from COVID-19 and from the neurological symptoms which caused the admission to hospital. No fluid restriction was needed and no problems about hydro-electrolytic balance persisted during recovery and she was dismissed after performing 2 negative nasopharyngeal swabs.

Informed consent was obtained from the patient to report the case.

3. Discussion

The unusual case of COVID-19 described here showed an initial clinical presentation with exclusive neurological symptoms and an infectious-related SIADH, followed by fever and dyspnea after few days. The oddity of this case is increased by the fact that the patient was already treated (because of rheumatoid arthritis) with drugs used for COVID-19 treatment (hydroxychloroquine and steroids) before admission and before developing more classical symptoms and signs of the SARS-CoV-2-related pneumonia. Possibly, this treatment may have masked for a few days the developing of a COVID-19-related typical lung disease and may have interfered with the finding of SARS-CoV-2 in the cerebrospinal fluid.

Ye *et al.* showed encephalitis as an additional manifestation of COVID-19 coming with the classical symptoms [6] and Poyiadji *et al.* reported images of acute hemorrhagic necrotizing encephalopathy associated with COVID-19 [7]. Our report adds to the one from Moriguchi *et al.* [8] who described a meningitis/encephalitis associated with SARS-CoV-2 in a case of convulsion accompanied by unconsciousness as presenting symptoms.

Preventive measures against pandemics are based on a prompt recognition of cases that can lead to patient's isolation, the only measure to contrast the propagation of infection. The attempt to make an early diagnose of COVID-19 is a crucial step to avoid contaminations of non-COVID medical departments. The unusual occurrence of early neurological symptoms as first manifestation of SARS-CoV-2 infection suggests that health professionals should be advised to

consider COVID-19 in patients showing unclear clinical presentations of complex neurological symptoms.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplemental Material

METHODS: Evaluation of SARS-CoV-2 in biological specimens

Nasopharyngeal swab

After virus inactivation for 1 minute at 90˚C, RNA extraction from the nasopharyngeal swab is performed with Abbott mSample Preparation System (Promega corporation) and an automated extraction system (Extraction m2000SP, Abbott Molecular). Obtained RNA is amplified with GeneFinder COVID-19 plus Real*Amp* PCR kit (ELITechGroup), one step Reverse Transcription Real-Time PCR system targeting RdRp, E, and N genes.

Cerebrospinal fluid

Cerebrospinal fluid was subjected to RNA extraction by QIAmp Viral RNA mini kit (Qiagen) and eluted in 60 µl. One step rRT-PCR was performed using Luna Universal qPCR Master Mix (New England BioLab) with primers targeting the 5'UTR region of SARS-CoV-2. A nested PCR, after retrotranscription with Superscript IV Reverse Transcriptase (Thermo Fisher Scientific) was used to amplify ORF1ab, S, E, and N regions. All tested regions gave a negative result.

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The Influence of Kidney Stones and Salivary Uric Acid on Dental Calculus Formation and Periodontal Status among Some Saudi Patients Aged 25 - 70 Years

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Abstract

Background: The formation of kidney stones is considered a complicated process. Consequently, there are many questions about the link between kidney stones formation and level of salivary uric acid and calculus formation on the teeth surfaces. **Objectives:** To evaluate the correlation between the level of salivary uric acid and kidney stones formation and their influence on dental calculus and periodontal status among Saudi patients aged 25 - 70 years. **Materials and Methods:** 120 Saudi male patients were examined (60 of Kidney stones patients and 60 patients of non-kidney stones patients) for clinical evaluation of plaque index (PLI), gingival index (GI), calculus index of oral hygiene (CI) and clinical attachment loss (CAL). Moreover, lab assessment of uric acid level in the collected salivary samples was done. The findings were analyzed using of ANOVA test and Tukey's test. **Results:** There were statistically significant differences in clinical parameters among kidney stones patients and non-kidney stones patients ($p < 0.05$), but these differences were highly statistically significant in the correlation between calculus index (CI), plaque index (PLI) and gingival index (GI) among kidney stone patients in group II, moreover, PLI and clinical attachment loss (CAL) among kidney stone patients in group III ($p < 0.001$). The statistical analyses revealed statistically significant differences in the level of salivary uric acid (mg/dl) in the comparison between kidney stones patients and non-kidney stones patients in group I and group III, whereas there were highly statistically significant in the comparison between kidney stones patients and non-kidney stones patients in group III. **Conclusion:** At the end of this study, we concluded that

there was a relationship between dental calculus formation, kidney stones formation, and an increase in the level of salivary uric acid.

Keywords

Dental Calculus, Kidney Stones, Uric Acid, Periodontal Status

1. Introduction

The oral cavity is a part of the body that includes various microorganisms, which subsist as a normal community of complex biofilms, structurally organized and multispecies [1] [2]. Therefore, it may be an effective method for the assessment of general and oral health [3].

On the other hand, Saliva is the main liquid in the oral cavity and is essential to keep the oral tissues in healthy status by inhibiting proliferation specific microorganisms, and moreover, inhibiting the build-up of dental plaque and dental calculus [4]. Consequently, saliva may be considered a mirror that can reflect the normal and abnormal states of the body [3].

The anti-oxidative system in saliva includes different enzymes and molecules which are periodontal biomarkers in the evaluation of the periodontal status [5] [6]. Uric acid participates in the antioxidant ability of saliva and blood. Further, it forms nearly 70% - 85% of this ability in the saliva of healthy and periodontitis patients [7] [8] [9]. According to previous studies, there was a possible effect of uric acid and other antioxidants in periodontal disease initiation and progression in addition to uric acid association with some systemic abnormality such as kidney stones formation [10] [11].

A kidney stone is one of the most prevalent systemic diseases of current communities and infects 12% - 15% of the population, and calcium oxalate stones are considered the dominant type of kidney stones worldwide [12] [13] [14]. The possibility of kidney stones recurrence is approximately 10% - 50% through 1 - 5 years from the first infects of kidney stones [15]. In Saudi Arabia, the dehydration and hot weather are reflecting on the estimation of kidney stone risk on the life where the kidney stone incident is at least 50% more than the western countries and, up to 90% of these patients exhibit abnormal oral changes [16] [17].

Dental calculus is the mineralization of dental plaque on the natural tooth surfaces and dental prosthesis in the mouth. Moreover, it has the same biological system of the structure formation process of kidney stones and according to the place of calculus, there are supra and subgingival calculus that are covered by unmineralized dental plaque which is the main etiologic factor of periodontal diseases [18] [19] [20] due to the sharing of kidney stones and dental calculus in the process of calcification and formation factors. Also, there was no prior Saudi study found carrying out among patients from Aseer region to evaluate the influence of kidney stones and salivary uric acid on dental calculus formation and

periodontal status among some Saudi patients aged 25 - 70 years. Therefore, the present study was designed for the assessment of these objectives [21].

2. Materials and Methods

2.1. Ethical Clearance

The current retrospective study carried out at the outpatients' clinics, college of dentistry, King Khalid University between March 2020 G and August 2020 G. The research proposal was approved by the scientific research committee, college of dentistry, King Khalid University. The objectives of the study were fully exhibited to the patients at the beginning of this study, and the patients' informed consent was obtained to fulfill ethical clearance (IRB/KKUCOD/ETH/2019-20/039). The sample size of the study was identified according to the number of patients who come to the outpatients' clinics, the college of dentistry, King Khalid University through the study duration. The G*Power software program was used for performing the sample size calculation [22].

The patients' data related to the patient name, age, gender, and general health were collected from the medical files of the patients. Furthermore, we asked the patients about oral hygiene measures, bad oral habits, and the presence or absence of xerostomia.

2.2. Participant Selection

According to the proposal of this study, a total of 120 Saudi males patients interviewed 60 patients (study group) diagnosed as kidney stones patients based on radiographs and general urine investigations, and 60 patients were diagnosed without kidney stones based on ultrasound in the latter three months (control group). All patients were examined clinically to evaluate the amount of dental calculus and periodontal status; moreover, the salivary uric acid level was measured. The patients were divided according to the ages into three equal groups (n = 40): (Group I) 25 - 40 years, (Group II) 41 - 55 years, and (Group III) 56 - 70 years. All groups included two equal subgroups (n = 20). The subgroups comprised 20 kidney stones patients as study subgroups and 20 healthy patients (non-kidney stones) as the control subgroup.

2.3. Inclusion and Exclusion Criteria

The patients of the study group were without other systemic diseases and, the size of the kidney stone was 20 mm. They were using tooth brushing but did not visit the dental clinics for calculus removal since one year ago. They did not wear orthodontic appliances or fixed or removable dental prostheses. Moreover, they were in a fasting status, whereas the patients of the control group were of good medical status.

The exclusion criteria were the patients who received periodontal therapy within the past 6 months, the patients with bone disease, endocrine disease, blood diseases, medically compromised patients, alcoholic patients, the patients with vitamin D deficiency, and who need calcium supplements. The comprehensive

kidney function evaluation was obtained from patients' medical reports that were within the patients' files to exclude any patients with other kidney diseases.

2.4. Clinical Examination

Periodontal examination was done by the assessment of plaque index (PLI) [23], gingival index (GI) [24], calculus index of oral hygiene index (CI) [25] and, clinical attachment loss (CAL). All clinical parameters were recorded by a Williams probe with a diameter of 0.5 mm (Hu-FriedyIns Co., USA) of each tooth except third molar teeth. PLI, GI, and CI were assessed on mesio-buccal, buccal, disto-buccal, and lingual or palatal, whereas CAL was assessed in interdental areas according to the last classification of periodontal diseases [26] [27] [28]. PLI was recorded before the collection of saliva samples. The clinical findings were recorded in particular case sheets and charts.

2.5. Salivary Collection of Saliva and Biochemical Investigations

The samples of un-stimulated saliva were obtained in the morning after breakfast (9-11 am) to reduce circadian changes. The patients were demanded to swallow of saliva and stay stable 10 min for collection of saliva into a sterilized plastic. The samples were transported on dry ice within less than 30 min for lab investigation after stored at −70˚C. The experiment of saliva collection was conducted by the Modified Navazesh method [29]. A centrifuge was used at 4000 g for 10 minutes at room temperature to remove cell debris, and an Erba kit was applied as an enzymatic method to identify uric acid.

2.6. Statistical Analysis

Statistical analyses of this study were done by ANOVA test for an account and, demonstrate the mean and standard deviation of the clinical results, and biochemical investigations findings. The scale of significance was assessed by using Tukey's test, and it was acceptable at $P < 0.05$, and highly significant when $P < 0.01$.

3. Results

The present study included a total of 120 male Saudi patients (50% kidney stones patients and 50% non-kidney stones patients) into three different age groups. The distribution of patients as kidney stones patients or non-kidney stones patients within the study groups as subgroups, which appears in **Table 1** and **Figure 1**. **Table 1** and **Figure 1** illustrate the age distribution and the mean of age among study groups. The mean age for non-kidney stones patients was 32.01 ys and 31.22 ys for kidney stones patients in group I. Moreover, it was 49.3 ys for non-kidney stones patients and 51.1 ys for kidney stones patients in group II, whereas it was 63.51 ys for non-kidney stones patients and 65.87 ys for kidney stones patients in group III. There were statistically significant differences in the ages between groups I, II, and III ($p < 0.05$) but, there were no statistically significant differences in the age between non-kidney stones patients and kidney stones patients in this study ($p > 0.05$).

Table 1. Age distribution and the mean of age among study groups.

Groups	Range	No	Mean ± SD		ANOVA		Tukey's test		
					F	P-value	I&II	I&III	II&III
GI	25 - 40 ys	40	N-KS	32.01 ± 8.11	4. 322	0.031	0.021	0.013	0.040
			KS	31.22 ± 4					
G II	41 - 55 ys	40	N-KS	49.3 ± 2.46					
			KS	51.1 ± 4.21					
G III	56 - 70 ys	40	N-KS	63.51 ± 6.32					
			KS	65.87 ± 9.71					
N-KS	25 - 70 ys	60	48.27 ± 5.63		1.712	0.186	0.563		
KS	25 - 70 ys	60	49.39 ± 5.97						

G: group, No: Number of participants, SD: Standard deviation, N-KS: non-Kidney stones, KS: Kidney stones.

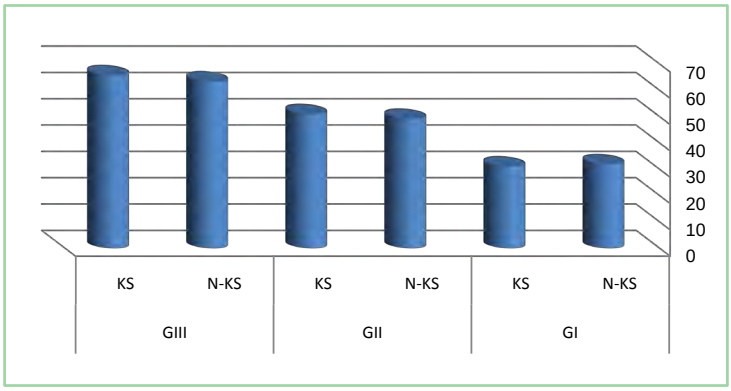

Figure 1. The mean of age among the study groups. G: group, N-KS: non-Kidney stones, KS: Kidney stones.

When we asked the patients about the self-plaque control, the feeling of dry mouth, and bad oral habits, just 45% of kidney stones patients reported that they used a toothbrush in self plaque control compared to 55% of non-kidney stones patients. Moreover, 87% of kidney stones patients were feeling of dry mouth and 13% of non-kidney stones patients clarified that there were no feeling of dry mouth. Opposite we detected in the patients' answers that 78% of patients were smokers (50% kidney stones patients and 50% non-kidney stones patients).

Regarding the comparison of groups I, II and, II in clinical parameter values. There were statistically significant differences in PLI and CI among the kidney stone patients plus GI and CI among non-kidney stones patients ($p < 0.05$), whereas there were no statistically significant differences in GI and CAL among the kidney stones patients moreover PLI and CAL among non-kidney stones patients ($p > 0.05$) (Table 2 and Figure 2).

On the other hand, there were statistically significant differences in PLI, GI, CI and CAL in the comparison between group I, II the kidney stones patients ($p < 0.05$) vice versa in non-kidney stones patients where there were no statistically

significant differences in PLI, GI, CI, and CAL ($p > 0.05$) and also there were statistically significant differences between group I and III in PLI, GI, CI, and CAL among kidney stones patients moreover, PLI and GI among non-kidney stones patients ($p < 0.05$), whereas we did not find statistically significant differences in CI and CAL among non-kidney stones patients ($p > 0.05$). Furthermore, there were no statistically significant differences between group II and III in all clinical parameters except CI among non-kidney stones patients ($p > 0.05$).

In this study, the correlation between clinical parameters with CI of all patients was analyzed by ANOVA test (Table 3). Significant statistically significant differences between clinical parameters and CI were observed among kidney stones patients and non-kidney stones patients ($p < 0.05$), but these differences were highly statistically significant in the correlation between CI and PLI and GI among kidney stones patients in group II moreover, PLI and CAL among kidney stones patients in group III ($p < 0.001$).

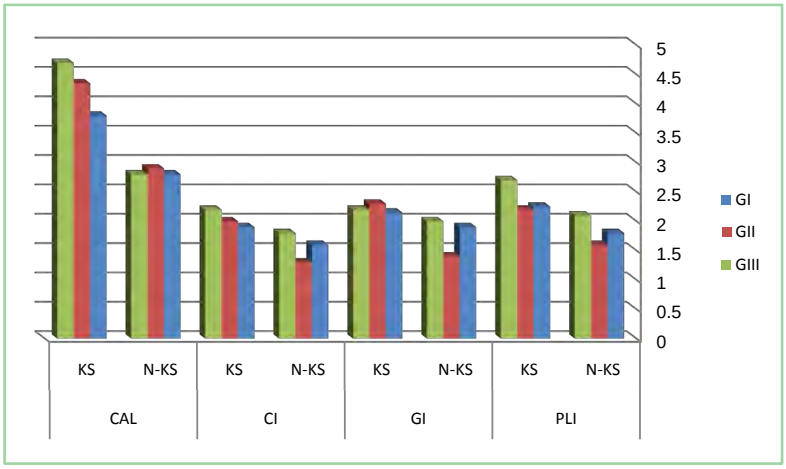

Figure 2. Clinical findings. PLI: Plaque index, GI: Gingival index, CI: Calculus index, CAL: Clinical attachment loss.

Table 2. Clinical findings.

CP		Groups			ANOVA		Tukey's test		
		GI	GII	GIII	F	P-value	I&II	I&III	II&III
PLI	N-KS	1.8 ± 0.2	1.6 ± 0,6	2.1 ± 0.4	2.322	0.115	0.112	0.001	0.279
	KS	2.25 ± 1.2	2.2 ± 0.7	2.7 ± 1.1	1.499	0.044	0.011	0.002	0.232
GI	N-KS	1.9 ± 0.6	1.4 ± 0.5	2 ± 0.3	1.014	0.050	0.498	0.003	0.081
	KS	2.15 ± 0.4	2.3 ± 0.9	2.2 ± 0.4	1.55	0.722	0.001	0.006	0.356
CI	N-KS	1.6 ± 0.2	1.3 ± 0.6	1.8 ± 0.4	1.662	0.000	0.077	0.336	0.014
	KS	1.9 ± 0.4	2.0 ± 05	2.2 ± 0.4	3.618	0.001*	0.004	0.000	0.315
CAL	N-KS	2.8 ± 1.6	2.9 ± 1.5	2.8 ± 1.8	1.148	0.863	0.133	0.622	0.091
	KS	3.8 ± 1.2	4.35 ± 0.7	4.7 ± 0.6	4.47	0.465	0.033	0.000	0.420

PLI: Plaque index, GI: Gingival index, CI: Calculus index, CAL: Clinical attachment loss.

Table 3. Correlation in clinical parameters with calculus index of all patients with ANOVA test.

Groups	CP	PSG	PLI		GI		CAL	
			r	P-value	r	P-value	r	P-value
GI	CI	N-KS	0.573	0.035	0.724	0.040	0.818	0.036
		KS	0.276	0.015	0.229	0.021	0.481	0.041
GII	CI	N-KS	0.602	0.007	0.811	0.011	0.516	0.013
		KS	0.335	0.001	0.298	0.000	0.169	0.022
GIII	CI	N-KS	0.246	0.023	0.447	0.036	0.691	0.024
		KS	0.899	0.001	0.399	0.003	0.188	0.001

G: Group, PLI: Plaque index, GI: Gingival index, CI: Calculus index, CAL: Clinical attachment loss.

Table 4 and Figure 3 demonstrate the mean of salivary uric acid levels among the patients in the different groups (mg/dl) of the present study. The statistical analyses revealed statistically significant differences in the level of salivary uric acid in the comparison between kidney stones patients and non-kidney stones patients in group I and group III, whereas there were highly statistically significant in the comparison between kidney stones patients and non-kidney stones patients in group III.

The correlation between age and all clinical parameters were summarized in Table 5. There were statistically significant differences in the comparison between the age of patients and all clinical parameters except GI.

Regarding the correlation between the level of salivary uric acid and all clinical parameters, it is found to be statistically significant differences in the comparison between the level of salivary uric acid with PLI and GI whereas it was highly statistically significant in the comparison between the level of salivary uric acid with CI and CAL (Table 6).

4. Discussion

Several studies showed the calcifications in the body (stones) with a decrease in the number of studies that tried to demonstrate the connection between these stones. These studies clarified that these stones have the same structure. Therefore, some of the studies considered their formation as metabolic disorders, and their components are identical in different organs [30] [31] [32]. In this retrospective study, the dental calculus formation was detected among most kidney stones patients more than non-kidney stones patients due to the kidneys' work in an important way to organize the levels of uric acid in serum [33]. Similar to the study results of Davidovich E. *et al.* where they demonstrated that the calcium, phosphate, uric acid, and magnesium components in saliva play an important role in the association between kidney stones formation and, dental calculus formation. Furthermore, they found there was a relationship between the distribution of mineral metabolism and calculus formation among kidney disease patients [34].

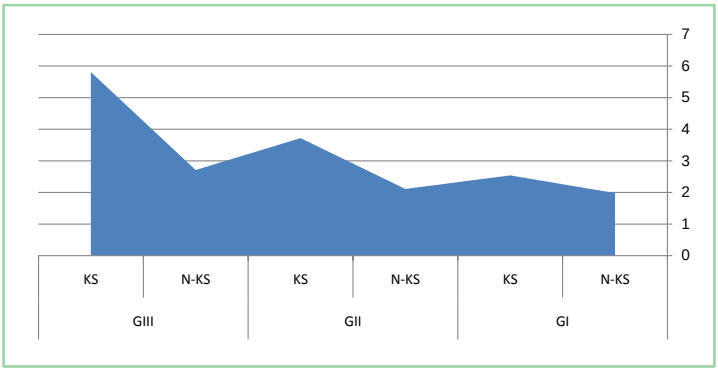

Figure 3. Salivary uric acid level. G: group, N-KS: non-Kidney stones, KS: Kidney stones.

Table 4. The mean of salivary uric acid level among patients in different groups (mg/dl).

Groups		Mean ± SD	ANOVA			
			F	P- value	F	P- value
Group I	N-KS	1.98 ± 0.43	0.615	0.051		
	KS	2.54 ± 0.67				
Group II	N-KS	2.11 ± 0.31	0.798	0.002	0.302	0.001
	KS	3.72 ± 0.70				
Group III	N-KS	2.71 ± 0.52	0.751	0.000		
	KS	5.81 ± 021				

G: group, SD: Standard deviation, N-KS: non-Kidney stones, KS: Kidney stones.

Table 5. The Correlation between Age and all parameters.

Correlations	Age	
	r	P-value
PLI	0.233	0.005*
GI	0.062	0.433
CI	0.03	0.003*
CAL	0.177	0.05*

PLI: Plaque index, GI: Gingival index, CI: Calculus index, CAL: Clinical attachment loss. *statistically significant differences.

Table 6. The Correlation between the level of salivary uric acid and all parameters.

Correlations	**level of** salivary uric acid	
	r	P-value
PLI	0.714	0.004
GI	0.881	0.017
CI	0.136	0.000**
CAL	0.632	0.0001**

PLI: Plaque index, GI: Gingival index, CI: Calculus index, CAL: Clinical attachment loss. **Highly statistically significant.

There was high plaque formation in the present study among kidney stones patients more than non-kidney stones patients, and dental calculus is dental plaque mineralization [18]. The result of the present study has supported this clarification that exhibited there was a correlation between the high value of PLI and an increase of calculus formation among kidney stones patients. Consequently, we evaluated the patients depending on the history of kidney stones, clinical examination of periodontal tissues and, assessment of calculus formation on the teeth surfaces with a uric acid level in saliva.

Results of the former study displayed that the prevalence of kidney stones is more among males (66%) than females and widespread within 31 - 40 years age group [35]. These results are appropriate with the objective of the present study that was done on 120 male Saudi patients within the age group (25 - 70) years, which included the age group (31 - 40) years. We explained to the patients the nature of calculus and, its impact on oral health and periodontal status moreover the possible correlation between calculus formation and the formation of kidney stones. According to previous studies, many researchers found significant links between kidney stones formation and accumulations of dental calculus on the teeth surfaces [34] [36]. Identical to the clinical findings in this study, we detected that there are significant correlations between kidney stones formation and dental calculus accumulations.

The current study and the study conducted by Brito *et al.* showed that GI and CAL were more in kidney stones patients group than non-kidney stones patients [37]. Consistent with the clinical findings of other studies revealed that periodontal destruction was more among kidney stones patients more than non-kidney stones patients [17] [38] [39] [40].

The higher mean values of GI, CI, and CAL among study group in the present study may be due to the higher mean values of PLI scores that recorded among kidney stones patients more than non-kidney stones patients in all study groups with statistical significance difference in all clinical parameters except GI among of kidney stones patients and CAL in kidney stones patients and non-kidney stones patients since dental plaque is the main etiological factor of periodontal diseases [41]. Xerostomia represents an oral complication of kidney stones disease and maybe predispose factors to gingival disease [42]. This is in agreement with the present study where the gingival disease was detected among all the kidney stones patients.

On the other hand, Uric acid is a major salivary antioxidant of healthy and diseased periodontal cases and there were many early studies comparing the salivary antioxidants levels among periodontitis patients and their levels in individuals with healthy periodontal tissues [8] [43]. Our results displayed the presence of a higher amount of salivary uric acid level in kidney stones patients compared to non-kidney stones patients. This result is supported by the results of other studies done by Hadi BA *et al.* (2011) and Xia Y *et al.* (2012) that displayed the levels of salivary uric acid in kidney stones patients were significantly higher than those of non-kidney stones patients [44] [45].

In the present study, there was an association between an increase of periodontal destruction and an increase in the level of salivary uric acid among kidney stones patients compared to non-kidney stones patients. Consequently, the present study demonstrated that there were highly significant differences in the dental calculus mean values among kidney stones patients than the non-kidney stones patients. This may be due to the presence of higher values of salivary uric acid level among kidney stones patients (mg/dl) compared to non-kidney stones patients in all study groups which corresponds to the results of other studies [46] [47]. Finally, many previous studies have tried to explain the relation between renal diseases in general, and the severity and type of periodontal diseases, but their findings were incompatible, that may be due to the massive debate in periodontal disease classification [48]-[53].

5. Strength and Limitations

This study participates in the current efforts to explain the relationship between salivary uric acid levels and periodontal health among Kidney stones patients and non-Kidney stones patients. However, the cross-sectional nature of the study, self-reported medical conditions through medical history in the patient's case sheet, participants' consents and selection of study participants from the patients of a college of dentistry, King Khalid University, absence of female participants, and small sample size considered as limitations of the study.

6. Conclusion

We studied the cases depending on the kidney stones' history, dental calculus and periodontal status evaluation and salivary uric acid measurement where there was a statistically significant difference in dental calculus formation among patients with kidney stones more than non-kidney stones patients. This correlation between an increase of dental calculus formation and kidney stones formation in the current study may be due to the minerals that form the kidney stones that may be accumulate in other areas of the body. This may be evidence that dental calculus can form a diagnostic indicator to help in the detection and diagnosis of kidney stones in case of recurrence of dental calculus formation. Consequently, the detection of a dental calculus during the clinical examination may be considered a predictor of kidney stones present, and this conclusion should be subjected to more studies.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Curaderm, the Long-Awaited Breakthrough for Basal Cell Carcinoma

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Abstract

Background: Basal cells form a continuous cell layer at the bottom of the epidermis, which is the outermost layer of the skin. Basal cell carcinoma occurs when a mutation occurs in the DNA of a basal cell. The mutation inhibits its apoptosis—the programmed cell death mechanism. The cell continues to multiply but does not die, resulting in a change in the skin, such as a growth or sore that will not heal. Basal cell carcinoma is the most common form of skin cancer and the most frequently occurring form of all cancers. Key words searched for the database of this communication were: Curaderm, BEC 5, cancer, skin cancer, basal cell carcinoma, nonmelanoma skin cancer, solamargine, solasonine and solasodine glycosides. **Treatments:** Several types of treatments are available to remove or destroy basal cell carcinoma. All currently used treatments are indiscriminate and also remove or destroy normal skin cells resulting in compromised cosmetic outcomes. **Development of Curaderm Pharmacotherapy:** Curaderm pharmacotherapy discriminates and specifically activates apoptosis at the molecular level in cancer cells but not in normal cells. Accordingly, Curaderm pharmacotherapy for basal cell carcinoma effectively and safely treats virtually all types, sizes and lesion locations. This review describes studies from the inception of Curaderm pharmacotherapy and covers the discovery of the anti-cancer effects, mode of action, preclinical, clinical and field applications with emphasis on efficacy, safety, compliance, tolerance, cost effectiveness and especially cosmetic outcome. In 2018 Curaderm was approved by the European Health Authorities as a Medical Device Class 1 for the indication "Topical Treatment with Keratolytic Action, and Antineoplastic Activity in the Treatment and Healing of Localized Basal Cell Carcinoma of the Skin".

Keywords

Basal Cell Carcinoma, Skin Cancer, BEC, Solamargine, Curaderm, Topical, Pharmacotherapy

1. Introduction

Basal cell carcinoma (BCC) is the most common form of skin cancer and the most frequently occurring form of all cancers. In the U.S. alone, more than 4 million cases are diagnosed each year. BCC is a disease of global health importance that causes substantial psychosocial impacts and requires considerable investment in terms of treatment and technologies. Conventional therapies have several drawbacks, thus, a long-awaited treatment breakthrough for BCC is very welcome.

1.1. Aetiology

BCC begins in the basal cells—a cell layer at the bottom of the epidermis which is the outermost layer of the skin that produces new skin cells as old ones die off. BCC occurs when a mutation occurs in the DNA of one or more basal cells. The process of creating new skin cells is controlled by the basal cell's DNA. The DNA contains the instructions that tell the cell how to behave. A mutation can tell the cell to multiply more rapidly and continue to grow when it would normally die. For every normal cell, there is a time to live and a time to die. Roughly 50 billion cells are born and die each day in humans.

Under normal conditions, a programmed sequence of events, known as apoptosis, leads to the elimination of cells without releasing harmful substances into the surrounding area. Basal cells in the basal cell layer (stratum basale) of the skin are also referred to as basal keratinocytes. Keratinocyte apoptosis plays a critical role in regulating epidermal development and restraining carcinogenesis.

Apoptosis balances proliferation to maintain epidermal thickness and contributes to stratum corneum formation. When basal cells are mutated, apoptosis may not work correctly. Cells that should be eliminated may persist and become immortal, such as the case with BCC.

BCC starts when basal cells grow out of control and crowd out normal cells. In BCC, the process of apoptosis is defunct but cell division is intact resulting in excessive numbers of cancer cells, resulting in a change in the skin, such as a growth or a sore that will not heal.

With BCC, these lesion changes in the skin may express one of the following characteristics:

- A pearly white, skin-coloured or pink bump that is translucent. Tiny blood vessels are often visible. BCC often appears on the face and ears. The lesion may rupture, bleed and scab over.
- A flat, scaly, reddish patch with a raised edge often appears on the back or chest.
- A white, waxy, scar-like lesion without a clearly defined border, called morpheaform.
- A brown, blackish lesion with a slightly raised, translucent border.

DNA damage in basal cells results from natural ultraviolet (UV) radiation from the sun and synthetic UV from tanning lamps and beds. UV radiation is

composed of UVA, UVB and UVC. The most harmful to the human body is UVC. However, UVC is absorbed and reflected by the ozone (O_3) layer. BCC is reported to be associated with intermittent and childhood sun exposure. UVB is a main causative factor in BCC and has been implicated to directly stimulate DNA mutation via covalent bonding between adjacent pyrimidines, whereas UVA stimulates production of reactive oxygen species that contribute to BCC formation. Factors other than exposure to UV (such as exposure to arsenic, pollutants, viral infections, skin type, age, defects in the immune system) may contribute to the risk and development of BCC. Genetic mutations are important factors in the development of BCC. For example, BCC is a well-recognized cancer, with mutations in the components of the Hedgehog signalling pathway. The p53 tumour suppressor gene is implicated in cancer development and mutation in the p53 gene caused by UVB exposure is relevant in the causation of BCC. **Figure 1** displays a general overview of aetiological factors of BCC [1].

1.2. Treatments

Several types of treatments are available to remove or destroy BCCs. Selected treatments are dependent on factors such as tumour size, location, a person's age, general health, and preference.

Excision surgery, Mohs surgery, Curettage and electrodesiccation, Radiation therapy, Photodynamic therapy, Chemotherapy, Cryotherapy, Immune response modifiers and Targeted therapy are currently used to treat BCCs.

All of these treatments for BCC have advantages and disadvantages. Other than targeted therapy, all other treatments are indiscriminate, and do not distinguish cancer cells from normal cells. The lack of specificity and selectivity of these treatments usually limit their applications and translate to poor cosmetic outcomes.

The biggest challenges for treatment of BCC are:

- Education

Understanding BCC causes, risk factors and warning signs may lead to early detection that can be easiest to treat and cure.

- Treatments

The common feature of conventional treatments for BCC is non-specificity without targeting the tumour itself. This leads to unwanted adverse effects in the surrounding tissue, such as scar formation or other cosmetically disfiguring outcomes. Less than favourable recurrence rates are obtained with conventional therapies.

- Depending on the type, location and size as well as preference and ability to do follow-up visits can be daunting for the patient, especially for elderly patients who are also prone to suffer more from this condition.
- An alternative non-invasive, self-treatment modality that is very specific towards the BCC tumour that eliminates small and large BCCs, and is applicable to difficult-to-treat areas with remarkable cosmetic outcomes, resulting in very low recurrence rates, is overdue and welcome.

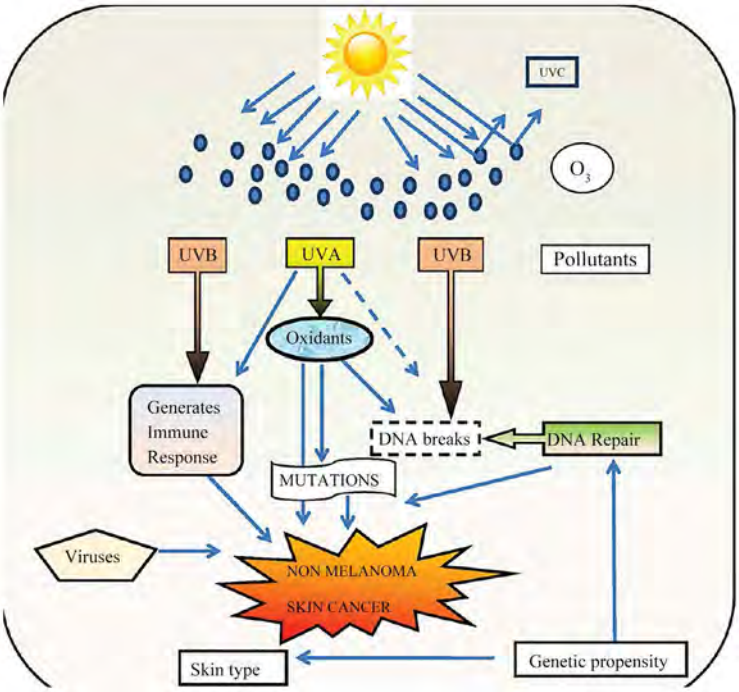


Figure 1. Aetiological factors of basal cell carcinoma (BCC) [1].

1.3. Curaderm

In 2018, the product Curaderm was registered by the European Health Authorities as a Medical Device Class 1 for the indication Topical treatment with keratolytic action, and antineoplastic activity in the treatment and healing of localized basal cell carcinoma of the skin. The prime difference between Curaderm and other treatment modalities for BCC is; Curaderm pharmacotherapy discriminates cancer cells from normal cells, which translates to superior efficacy, safety and cosmetic outcomes.

The incidence of BCC is rising, representing approximately 70% - 80% of all skin cancers. Accordingly, there is a definite need for more acceptable treatments of BCC as verified by the research into the causes, prevention, and treatment of BCC currently ongoing in many medical research centres throughout the world.

Fundamental studies of BCC have been hindered by a lack of a suitable and reproducible tissue-culture model system. Most available treatments for BCC did not require such models because most BCCs are currently, simply cut out or burnt out and no understanding of mechanism of action at the molecular and cellular levels are required for these indiscriminate procedures.

1.4. BEC

In 1987 it was first reported that plant-derived glycoalkaloids solasodine glycosides, including solamargine, solasonine, mono- and di-glycosides of solasodine, known as BEC [2], induced remarkable anticancer effects in cell culture [3], animals [4] and humans [5] [6] [7] [8] [9].

Since then, a plethora of further investigations have resulted in the placement of BEC and its individual components as very promising antineoplastic agents with vast potential to serve as targeted anticancer agents [10].

With BEC, solamargine accounts for 86% antineoplastic activity and solasonine accounts for 9% antineoplastic activity, whereas, the mono- and di-glycosides of solasodine contribute 5% anticancer activity. The anticancer activity of these glycoalkaloids is considered to be concerted and additive [11].

The governing principle that determines the potency of antineoplastic activity is the plant sugar rhamnose that forms part of the glycoalkaloids [12]. BEC targets specific mutant proteins on cancer cell membranes that act as specific receptors. After binding to these characterised specific receptors [13] [14] [15] [16] [17], BEC is internalised by cell receptor-mediated endocytosis followed by the anticancer sequelae of identifiable anticancer properties on a variety of biological pathways, including cell survival pathways [18], tumour suppressor pathways [19], lysosomal pathways [20], mitochondrial pathways [21], caspase activation pathways [22], death receptor pathways [23], protein kinase pathways [24] and signal pathways that impede invasion/migration [13] [24] and multidrug resistance [13] [25].

BEC exhibits much higher cytotoxic effects on cancer cells than currently used antineoplastic agents such as vinblastine, vincristine, camptothecin, cisplatin, 5-fluorouracil, gemcitabine, epirubicin, cyclophosphamide, taxol and doxorubicin [26].

Furthermore, the absolute concentrations of these drugs to obtain comparable efficacy as BEC, are in the order of 6 - 40 times higher [27].

Moreover, the therapeutic index (TI: also referred to as the therapeutic ratio) is much higher for BEC compared with other antineoplastic agents as shown with cell culture studies [28] and animal studies [29]. The high TI of BEC translates to high safety margins.

BEC is active against a wide variety of cancer cells, such as ovarian cancer [30], basal cell carcinoma [31], squamous cell carcinoma [32], melanoma [33], colorectal cancer [34], bladder cancer [35], oral epidermoid carcinoma [36], breast cancer [25], leukemia [37], prostate cancer [38], liver cancer [39], lung cancer [40], pancreatic cancer [41], gastric carcinoma [23], renal cancer [31], uterine cancer [30], mesothelioma [42], glioblastoma [43] and osteosarcoma [19]. In addition, BEC has curative properties in animals with terminal cancer [4] [12].

2. BEC and Basal Cell Carcinoma

The first human clinical studies with BEC were undertaken in the 1980s in Australia, at the University of Queensland, the Royal Brisbane Hospital, and St. Johns Park Hospital, Tasmania. In 1987, it was reported that topical application of a cream formulation containing BEC was effective in the treatment of the malignant tumour BCC. Histological analyses of biopsies taken before, during and after treatment gave compelling evidence of the efficacy of the formulation. The

treated lesions did not recur for at least 3 years after cessation of therapy, and indeed, patients were subsequently followed up for over a decade with no recurrences. Thirteen patients with 24 BCC lesions were treated for 4 to 7 weeks. With the BEC therapy, the BCCs responded rapidly in a characteristic pattern. The pattern comprised initial swelling of the lesion, and erythema of surrounding tissue (approx. 10 mm around the lesion), followed in about 2 days by ulceration until neoplastic cells were destroyed. This was then followed by normal new non-malignant cell growth during the treatment. In the 13 patients treated, 20 of a total number of 24 lesions regressed totally. Partial regression was obtained with the other 4 lesions.

Two normal subjects were treated with the formulation for 8 weeks. Biopsies taken from the treated areas showed no macroscopic or microscopic changes. No clinical reaction was observed with the normal skin. The specificity towards abnormal cells in the skin was the most striking observation.

Biochemical, haematological and urinalytical studies demonstrated that there were no adverse effects on the liver, kidneys or haematopoietic system during or after treatment.

There were no major adverse side effects during BEC therapy except that transient mild itching and burning surrounding the treated lesions occurred in a few cases [5].

Following reports on the anti-cancer effects of BEC in cell culture and in whole animal models, together with the effective treatments for skin cancer, a monograph of BEC was published [44].

In the first human clinical studies, 10% (100,000 mg/L) BEC was used in a cetomacrogol base to treat the BCCs. In the monograph of BEC, it was reported that patients tolerated up to 50% BEC in the cetomacrogol cream without any side effects. It was interesting that in cell culture studies, 8 mg BEC/L cell culture medium was required to kill cancer cells but not harm normal cells. Similarly, 8 mg BEC/kg when injected into mice with terminal tumours resulted in survival. Identical doses of BEC injected into normal animals did not produce adverse effects [4].

The doses of BEC to obtain efficacy for the treatment of skin cancer was compared with the doses of BEC required for the treatment of cancers in cell culture and in animals, there was a difference of a factor of over 10,000. It was subsequently shown that this difference was due to the bioavailability of BEC with the cancer cells. Much further work led to a topical formulation that contained keratolytic components that markedly improved the bioavailability of BEC to interact with skin cancer cells. A concentration of BEC, as low as 50mg/L of cream, was shown to be effective to treat BCC.

2.1. Open Studies

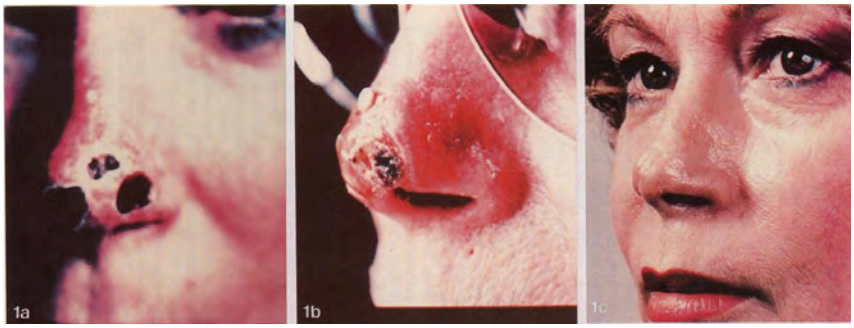
Curaderm and Basal Cell Carcinoma

Consequently, a clinical study was undertaken to treat 28 patients with 39 BCC lesions with very low concentrations of BEC (5 mg%), 10% salicylic acid

and 5% urea in a stabilized cetomacrogol cream formulation, Curaderm. Two patients with BCCs were treated with a placebo formulation. In this open study, clinical and histological observations indicated that all 39 BCCs treated with Curaderm had totally regressed. A placebo formulation had no effect on treated lesions. Curaderm had no adverse effect on the liver, kidneys or haematopoietic system. The treatment period to obtain complete regression of the BCC lesions ranged from 3 to 13 weeks. In the placebo group, treatment period was for 14 weeks, which was 1 week more than the longest treatment period with Curaderm [6].

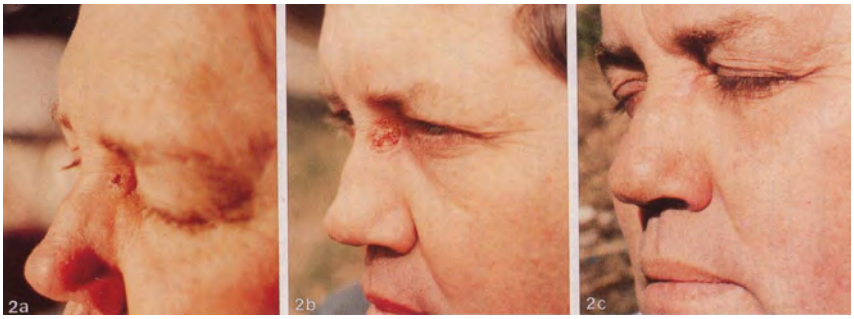
It was remarkable that even at the early developmental stages, Curaderm was able to eliminate BCCs that were almost impossible, due to their locations and sizes, to successfully be treated by other modality treatment procedures.

Photographic documentation of two examples was presented (**Figure 2** and **Figure 3**). **Figure 2** is a BCC of the nose, which was present for at least 12 months before treatment started. Independent prognoses for this patient were plastic surgery of limited effect and/or complete loss of the nose, which was to be replaced with a prosthesis.



(a) (b) (c)

Figure 2. Clinical diagnosis of a BCC on the nose of a patient before treatment with Curaderm (a), during therapy (b), and site of the treated BCC after completion of therapy (c).



(a) (b) (c)

Figure 3. Clinical progress of a periocular BCC on a patient before treatment (a), two weeks after commencement of therapy (b), and site of treated BCC after completion of therapy with Curaderm (c). Treatment period was 5 weeks. Histological analyses before and after therapy showed that the periocular BCC was cleared with Curaderm therapy. There was no recurrence after 5 years

Figure 2(a) illustrates several lesions on the nose before treatment started. Figure 2(b) shows the lesion 3 weeks after the commencement of Curaderm pharmacotherapy. The lesions ulcerated over a large area giving the appearance of one large single lesion. During this period the ulceration progressed to the extent that the soft tissue (cartilage) of the nose was visible. Figure 2(c) illustrates where the BCC was. The patient was treated with Curaderm for 13 weeks. A biopsy taken at the conclusion of the treatment showed histologically that BCC was no longer present. No clinical recurrence was seen 10 years after completion of Curaderm pharmacotherapy. This impressive case showed that during treatment, while the cancer cells were dying, new normal skin cells were replacing the dead cancer cells. This confirms the observations experienced with cell culture and animal studies that BEC preferentially attacks and kills cancer cells and leaves normal cells alone.

Another case was presented that could not be successfully treated by other procedures without losing the eye.

Figure 3(a) shows the lesion that was present for 4 months and was growing rapidly. Figure 3(b) illustrates the lesion 2 weeks after commencement with Curaderm pharmacotherapy. There was a distinct area of ulceration, which was larger in area than the clinically distinguishable BCC prior to the Curaderm treatment. Figure 3(c) shows where the BCC was after treatment with Curaderm. The treatment period in this case was 5 weeks. A biopsy taken at the conclusion of the treatment indicated histologically that BCC was no longer present after treatment with Curaderm. There was no clinical recurrence of the treated BCC for at least 5 years.

The rest is history, many other clinical studies with Curaderm followed, confirming the original observations.

2.2. Double-Blind, Vehicle-Controlled, Randomized, Paralleled Group Studies

In the 1990s a double-blind, vehicle-controlled, randomized, paralleled group study to assess the efficacy and safety of BEC-5 (Curaderm) in the treatment of patients with basal cell carcinoma, was carried out by ten centres in the United Kingdom. The study was conducted in full conformance with the principles of the current Declaration of Helsinki. The study was conducted according to International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) guidelines (1996). In 2008 these Phase III studies were published [45].

In these Phase III studies, the treatment groups consisted of 62 patients who were treated for 8 weeks with Zycure, which is Curaderm, and 32 patients who were treated with placebo for 8 weeks. Curaderm pharmacotherapy proved to be a safe therapy for BCC, with a cure rate of 66% at 8 weeks and 78% at 1-year follow-up.

The mode of action was apoptosis of cancer cells with preferential in its application to transformed cells. Patients experienced regeneration of new epider-

mis at the application site within the first 4 - 5 weeks of treatment, despite twice daily application of Curaderm.

Histological examination showed acanthosis of the regenerated epidermis on an 8-week post treatment biopsy indicating the specificity of Curaderm toward cancer cells and not harming non-cancer cells. Punjabi *et al.* concluded that Curaderm has overall efficacy, patient acceptance, low incidence of local adverse events and no systemic side-effects [45].

Further studies were recommended to investigate the safety and efficacy of Curaderm with BCCs:

- At different sites of the body
- Different histological types
- More invasive forms

These recommended further studies were addressed by a second trial at the Dermatology Department at the Royal London Hospital, comprising 41 patients, which determined that patients with invasive BCC, such as morpheoic BCC lesions, were successfully treated with Curaderm with a success rate of 78% when treated for 8 weeks [46].

It was reported that the cosmetic results offered by treatment with Curaderm were comparable to that resulting from surgical excision, but the risks of surgical intervention were well known.

The investigators at the Royal London Hospital further commented that excision of BCC from the facial area involved reconstructive surgery, of which could be both time consuming and costly.

They stated that an alternative, safe, efficacious and cost-effective method of treatment of BCC that did not require physician or hospital attendance should be encouraged, and that Curaderm was a much-needed alternative to surgery for both primary and secondary skin cancer care [46].

It is noteworthy that the trials in the United Kingdom were designed to treat the patients for 8 weeks, which resulted in a 78% success rate for both superficial and invasive BCCs. In analysing prior and subsequent studies that involved BCC treatments for 14 weeks, it becomes clear that a success rate at 8 weeks treatment was also approximately 80%. Longer treatment periods were required to obtain a higher success rate [27]. These observations complemented the clinical trials.

In order to determine the limitation of topical Curaderm pharmacotherapy, patients with a wider variety of BCCs were studied. In particular, factors indicating poor treatment prognosis in BCC such as size, type, depth, location and previous failure by other treatment modalities, were selected for the study.

2.3. Curaderm Pharmacotherapy and Size of Basal Cell Carcinoma

The recurrence rates of large BCCs (over 2 cm) are very high, regardless of treatment modality and the cosmetic prognosis is very limited. Curaderm topical pharmacotherapy has resulted in remarkable outcomes with very large BCCs with dimensions of 5 cm × 5 cm with protrusions of 1.5 cm from the

skin [27].

Figure 4 shows a large BCC (4 cm × 4 cm × 2 cm) before treatment. Dermatologists and surgeons had recommended surgical excision and radiotherapy followed by surgical reconstruction with skin grafting. The patient who had this BCC for at least 3 years elected to treat the lesion with Curaderm. Histological analysis of a biopsy determined that the lesion was a BCC.

This case, as with many other similar cases, shows that regeneration of new epidermis at the application site during treatment of skin cancers with Curaderm occurs, and supports the preclinical and clinical observations that BEC is preferential in its action towards transformed cells. This also explains the impressive clinical and cosmetic outcomes with topical Curaderm pharmacotherapy as shown in Figure 5 and Figure 6 [47] [49].

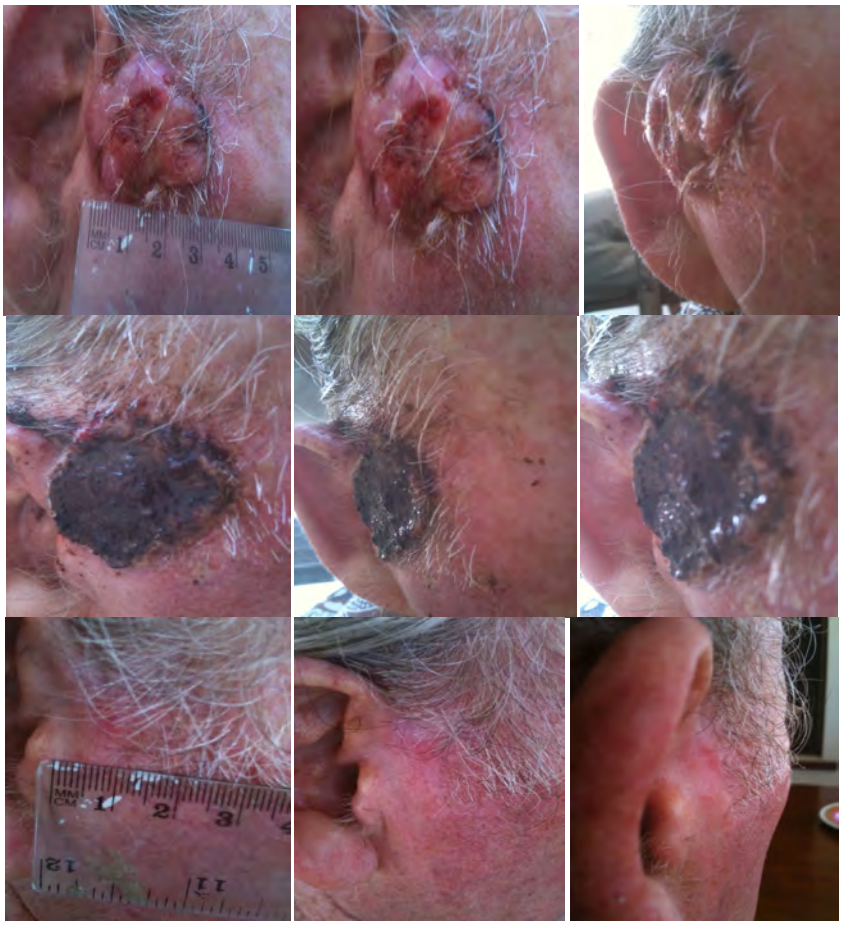
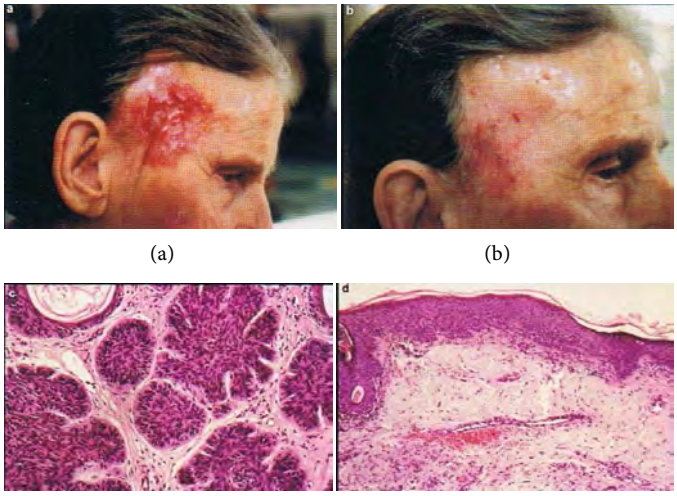


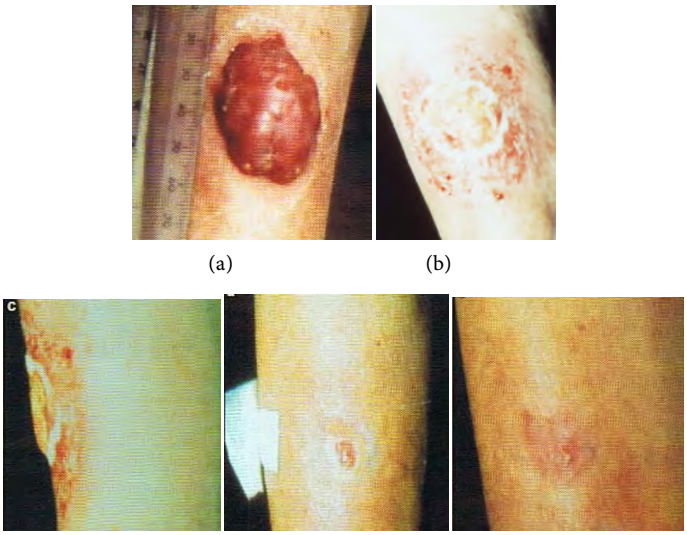
Figure 4. An extensive protruding (4 cm × 4 cm × 2 cm) BCC with central ulceration and raised curly borders on the right side of his face next to his ear is seen in this patient (top row). Treatment with Curaderm resulted in rapid breakdown of the tumour and after 2 weeks of treatment the lesion was reduced to about a half of the original size. Minor bleeding had occurred during this treatment period (middle row). After 14 weeks of treatment the lesion was clinically eliminated. Normal skin cells had replaced the tumour and the cosmetic end result was excellent, with no scar tissue formation. Even the hairs had regrown where the tumour was originally (bottom row).



(a) (b)

(c) (d)

Figure 5. Large BCC on the temple of a woman (a). This BCC had been surgically removed and skin grafts applied on two previous occasions only to return. Four weeks treatment with Curaderm resulting in full regression (b). Note the cosmetic result. The clinical diagnosis was confirmed histologically by punch biopsy (c). After completion of the therapy histopathology determined that no residual cancer was present (d) [49].



(a) (b)

(c) (d) (e)

Figure 6. Large BCC on the leg (a). Note how rapid the cancer was being destroyed by Curaderm during treatment (b-d) and how rapid the wound healed after 5 weeks of Curaderm therapy (e). After completion of the therapy histopathology determined that no residual cancer was present. Clinical assessment 5 years post treatment revealed that there was no recurrence [49].

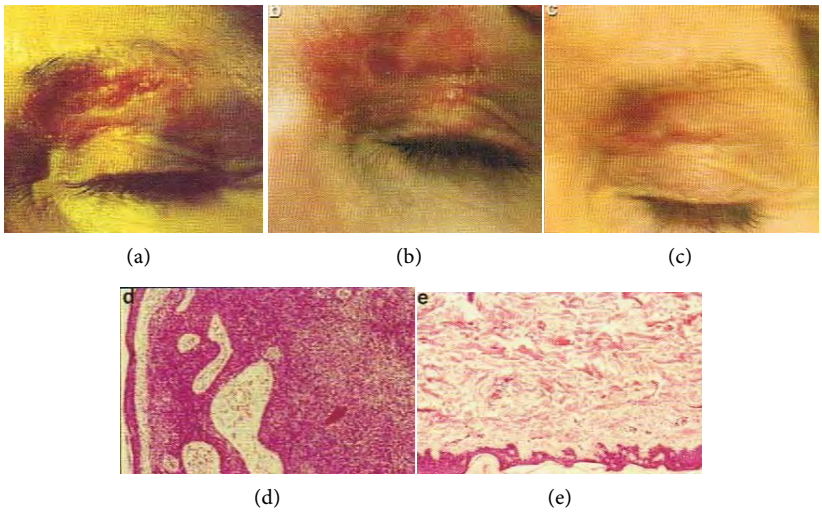
2.4. Curaderm Pharmacotherapy and Type of Basal Cell Carcinoma

Superficial, nodular, infiltrative (morpheaform) with differing depths of BCCs have been successfully treated with Curaderm [5] [6] [8] [13] [15] [17] [26] [27] [28] [31] [36] [47]-[59].

2.5. Curaderm Pharmacotherapy and Difficult to Treat Locations of Basal Cell Carcinoma

Location of BCCs largely determines, and, limits the choice of treatment. Curaderm topical pharmacotherapy was shown to excel in these situations.

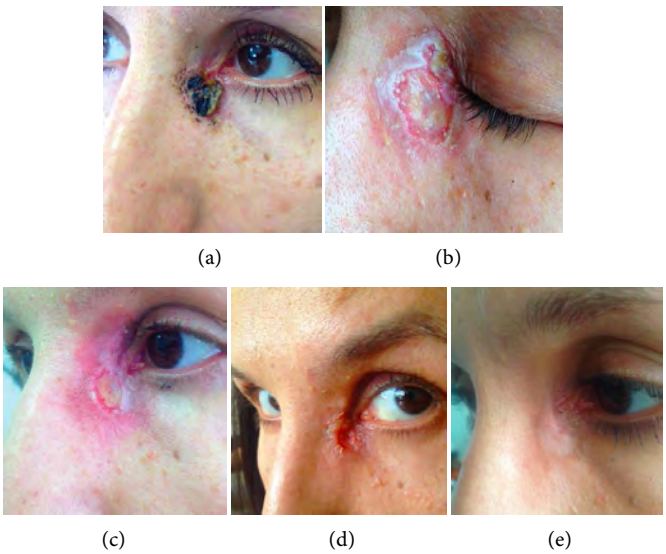
Figures 7-9 illustrate sensitive areas that have been successfully treated with Curaderm, highlighting one of its main attributes, remarkable cosmetic outcomes [5] [6] [8] [15] [26] [49] [51] [54] [63].



(a) (b) (c)

(d) (e)

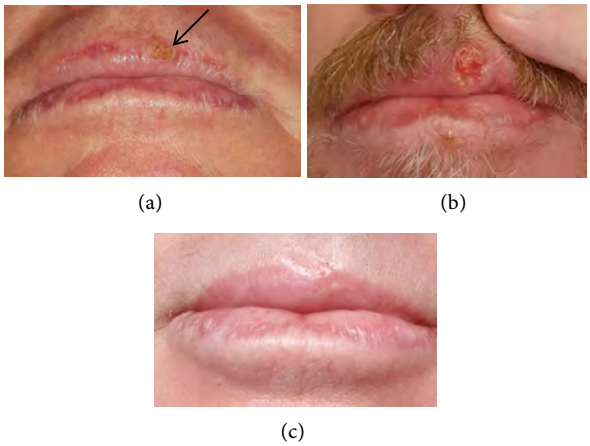
Figure 7. Periocular BCC, superior to the eye including part of the brow, of a patient before (a), during (b) and after (c) Curaderm therapy. During Curaderm treatment the lesion was much smaller and residual tumour can distinctly be seen which was surrounded by some inflammation. After treatment there was no sign of the BCC. Confirmation by histological analysis of the BCC before treatment (d), and after treatment (e) are shown. The total treatment period was 9 weeks. Clinical assessment 5 years post treatment showed that there was no recurrence [49].



(a) (b)

(c) (d) (e)

Figure 8. Patient with a BCC dangerously close to left eye, before Curaderm pharmacotherapy (a), during different stages of therapy (b-d), after 8 weeks treatment (e).



(a) (b)

(c)

Figure 9. BCC on upper lip [see arrow] that had been troubling a 53-year patient for some time, before Curaderm therapy (a), during (b) and after (c) Curaderm therapy.

2.6. Curaderm Pharmacotherapy on Previously Failed Treatment Procedures

A number of treatments are available for topical BCCs with varying success and recurrence rates. When a BCC recurs after treatment with virtually any procedure, scar tissue poses a problem for retreating the lesion. Curaderm pharmacotherapy was evaluated for its application under such circumstances.

Twenty-three patients that had a recurrence by other modality treatments such as radiation therapy, photodynamic therapy, laser therapy, cryotherapy and then subsequently treated with Curaderm, resulted in 52% success rate with no recurrences with 5 years follow up [60].

Figures 10-12 illustrate various lesions that had recurred after treatment with radiation therapy, photodynamic therapy and laser therapy and then treated successfully with Curaderm [56].

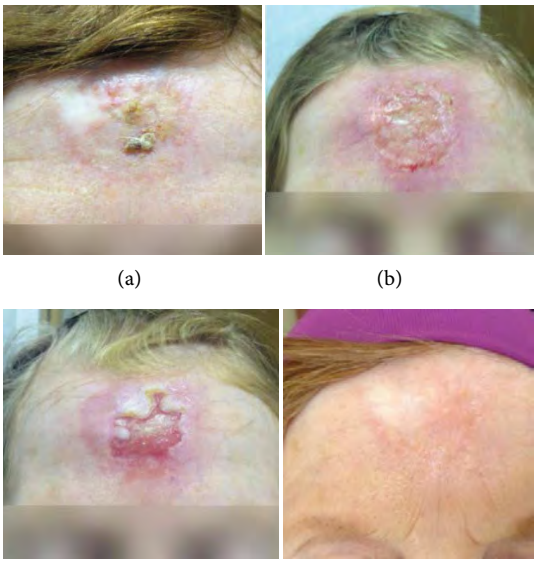
Figure 13 shows a large lesion on the nose of a patient who had previous treatments with surgery, radiation and photodynamic therapy. On all occasions of the treatments, the lesion had recurred. Topical Curaderm pharmacotherapy of the same multiple recurred lesion successfully removed the lesion with no recurrence for over 3 years [61].

2.7. Curaderm and Elderly Patients

There is a correlation between the incidence of BCC and increasing age in humans. Clinicians are usually the frontline to encounter elderly patients with BCCs at their general check-up visits. Once the diagnosis of the BCC is made, both the clinician and the patient are faced with the challenge of treatment. All treatment modalities have special considerations when administered to older adults. The physical condition and ability of the patient and the characteristics of the BCC tumour type and locality may limit the choices of treatment. Dobrokhotova *et al.* [62] have considered the need of an appropriate treatment procedure for elderly patients. These investigators have appreciated that the elderly encounter restrictions when treatment is required for their BCC, and have

evaluated Curaderm as a possible candidate and solution for this large group of patients. In particular, they selected patients with lesions at locations that are considered difficult to treat such as, on the head and neck.

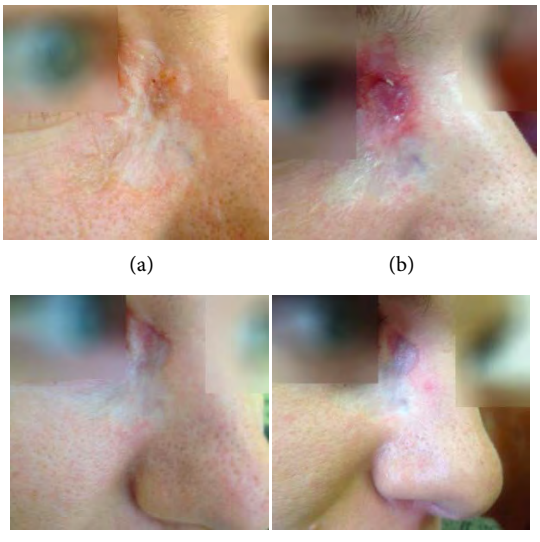
In 2016, they published their findings in the journal "Tumors of the Head and Neck" [62]. They concluded that Curaderm is a successful treatment of BCC of the head and neck. **Figure 14** and **Figure 15** illustrate two elderly patients suffering with BCCs that have successfully been treated with Curaderm and their impressive cosmetic results after treatment. These observations contribute value to the patient and Health Care Providers.



(a) (b)

(c) (d)

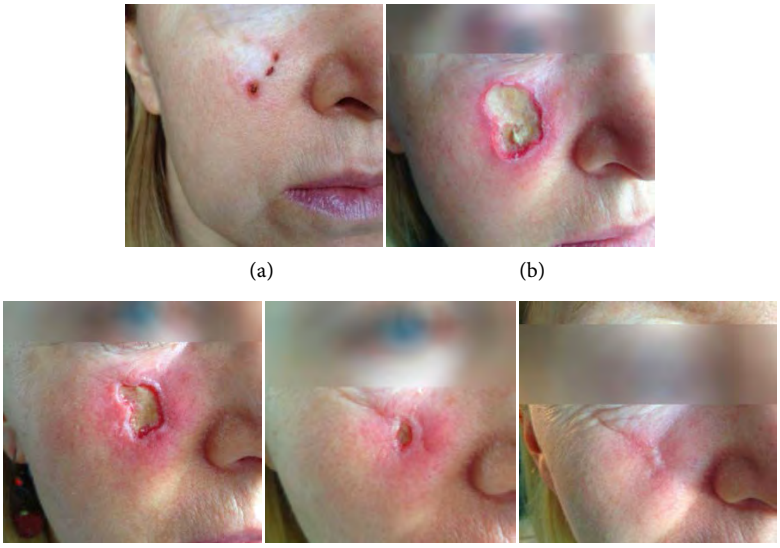
Figure 10. Recurrent BCC after radiation therapy but before Curaderm therapy (a), during treatment with Curaderm (b, c) and after 8 weeks treatment (d).



(a) (b)

(c) (d)

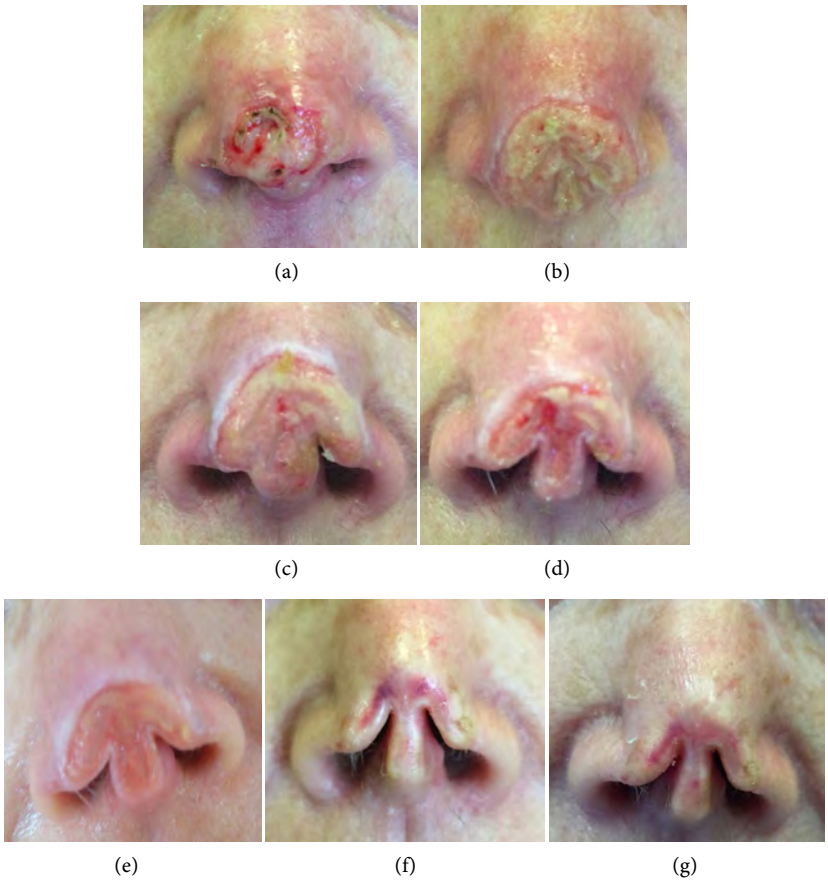
Figure 11. Recurrent BCC close to the eye after laser therapy but before Curaderm therapy (a), during treatment with Curaderm (b, c), and after 8 weeks treatment (d).



(a) (b)

(c) (d) (e)

Figure 12. Relapsed BCC on cheek after PDT but before Curaderm therapy (a), during Curaderm treatment (b, c, d) and after 10 weeks of treatment (e).

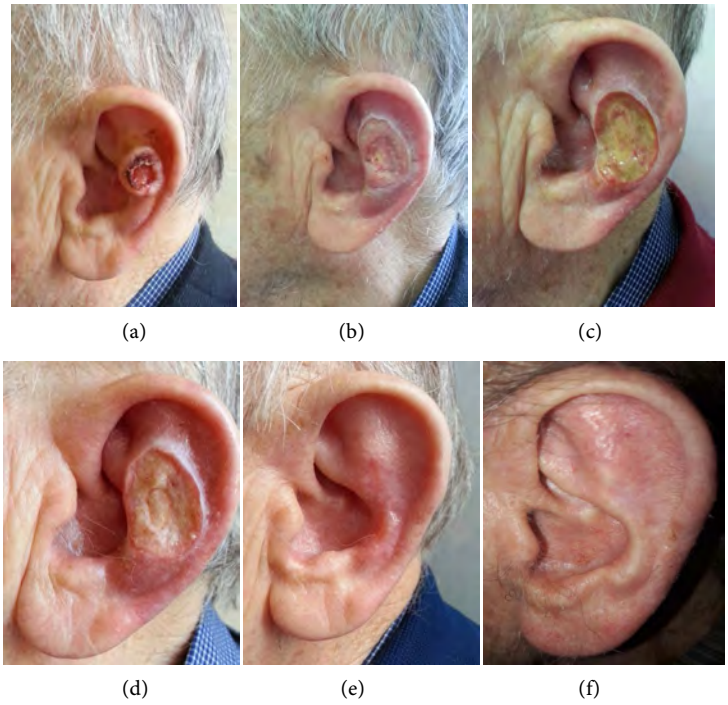


(a) (b)

(c) (d)

(e) (f) (g)

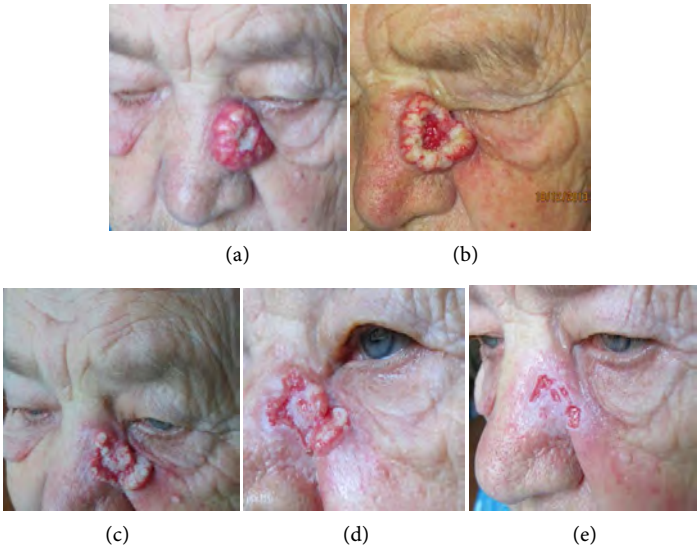
Figure 13. A large infiltrating refractory BCC, which recurred after three failed attempts by widely accepted therapies; (a) immediately before commencement with Curaderm therapy; (b) 2 weeks; (c) 5 weeks; (d) 8 weeks and (e) 14 weeks during Curaderm therapy. Treatment stopped at 14 weeks of therapy. Appearances of treated lesions (f) 7 weeks and (g) 10 weeks after cessation of treatment.



(a) (b) (c)

(d) (e) (f)

Figure 14. Patient, 85 years. BCC on left ear, before Curaderm pharmacotherapy (a), after 17 days treatment (b), after 28 days treatment (c), after 60 days treatment (d), after 74 days treatment (e). No relapse after 5 years treatment (f).



(a) (b)

(c) (d) (e)

Figure 15. Patient, 91 years. BCC close to the left eye, before Curaderm pharmacotherapy (a), during different stages of therapy (b-d), after 14 weeks treatment (e).

2.8. Other Clinically Relevant Studies with Curaderm and Basal Cell Carcinoma

A study showing graphic and pictorial images what occurs clinically during Curaderm pharmacotherapy has been reported in 2015 [55].

The clinical observations with Curaderm pharmacotherapy reveal that initially, the BCC lesion size increases over four-fold due to the interaction of

Curaderm with deeper and more lateral tumour cells, followed by a decrease in size, ultimately resulting in complete elimination of the BCC. Figure 16 shows the changes in areas of the BCC lesion relative to pre-treatment area vs. treatment times, and beyond treatment times. The lesion responded rapidly to the treatment. There was an immediate increase in lesion size after commencement of Curaderm pharmacotherapy. The size of the lesion increased more than four-fold and peaked at approximate 30 days of treatment. On-going treatment with Curaderm then resulted in a decline of lesion size, at day 59 the size had returned to the original pre-treatment size. Continuing treatment caused the lesion to further reduce in size and after 86 days of treatment the lesion was completely eliminated. From approximately day 30 treatment, regeneration of new epidermis at the application site occurred until the end of therapy (day 86) despite continued three times daily application of Curaderm.

Figure 17 illustrates the photographs that correspond to the events described for Figure 16.

The sequence of these observations always occurs when treating BCCs with Curaderm.

The extent of the changes in size of the lesions and the duration of these changes during treatment depend on the original pre-treatment size and characteristic of the BCC.

Very importantly, as was shown in all cases that were treated with Curaderm pharmacotherapy, whilst Curaderm is destroying cancer cells, normal cells are replenishing the dead cancer cells and this exceptional occurrence translates to the observed impressive cosmetic outcomes. There are no other treatments available that render this selectivity.

All other available treatments are indiscriminate with consequential cosmetic limitation outcomes.

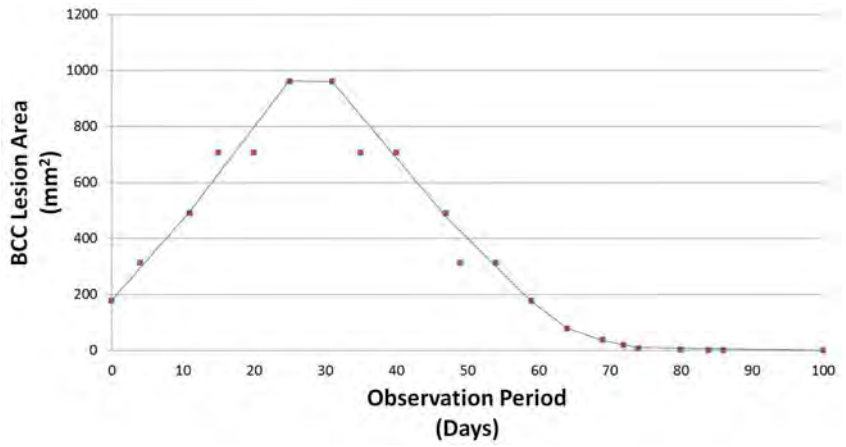


Figure 16. Curaderm therapy caused an immediate change in BCC lesion size and after 30 days treatment, peaked at over a 4-fold increase in size. Continuous treatment after 30 days resulted in a decrease in lesion size and complete removal of the BCC was attained after 86 days of Curaderm therapy. Before treatment started the diameter of the lesion was 15 mm (177 mm^2) [55].

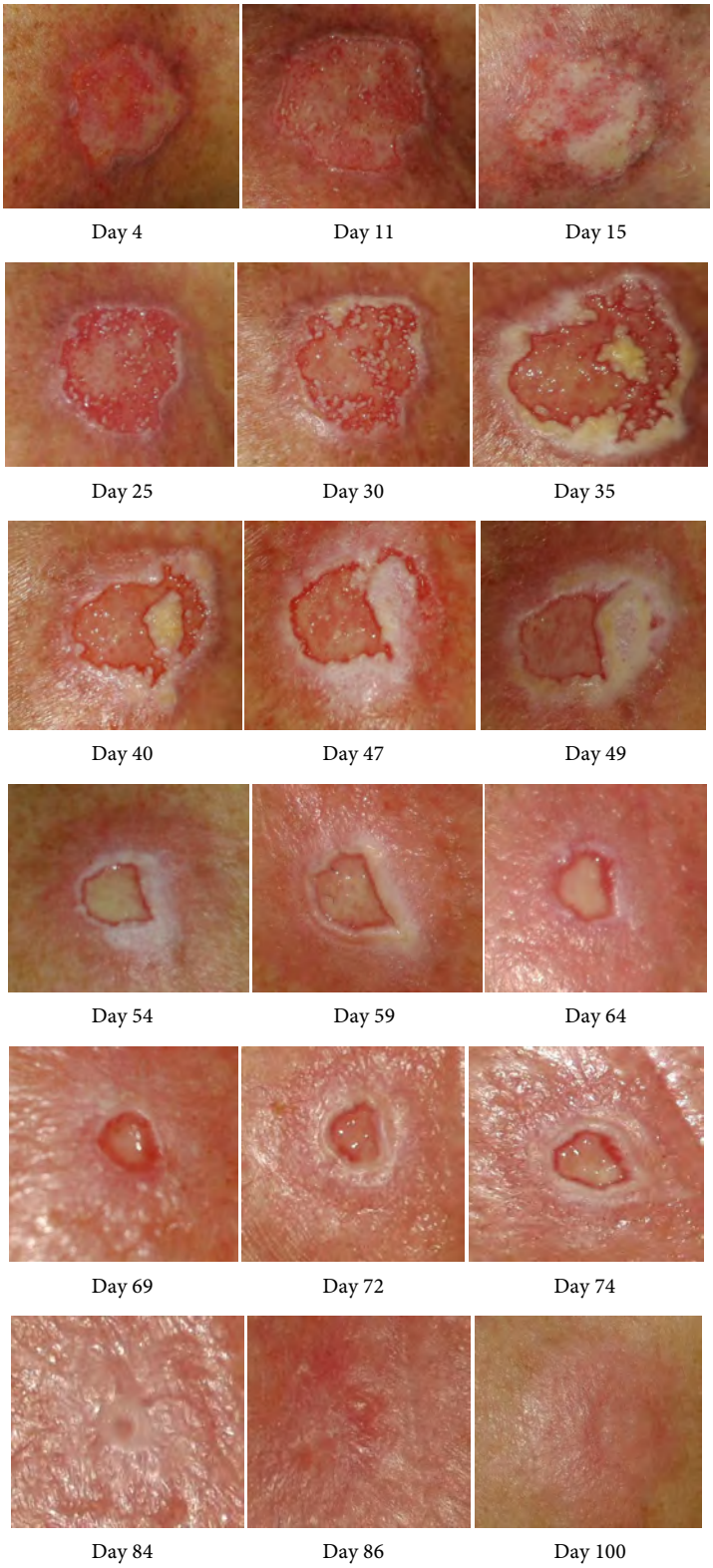


Figure 17. Appearances of BCC lesion during and after Curaderm therapy. Curaderm was applied 3 times daily at a dose of 0.1 g cream and covered with micropore paper tape occlusive dressing. The indicated days refer to the treatment periods. Cancer cells were being eliminated and replaced with normal epidermal skin cells during treatment. Treatment was stopped only after the original BCC lesion had healed (day 86).

2.9. Compliance and Tolerance

Many studies attest to good compliance and tolerance when on Curaderm pharmacotherapy. The only reported side effects were mild erythema with transient burning sensations at the treatment site. These sensations were dependent on the sizes of the lesions. Nevertheless, the treatment was well tolerated by all patients [63], confirming many other studies [5] [6] [11] [26] [27] [31] [48] [49] [51] [52] [53] [55] [56]-[62].

2.10. Population Studies with Curaderm

Curaderm pharmacotherapy has been used in different populations and ethnicity throughout the world with similar outcomes. For example, in the Russian Federation, 95 patients were treated with Curaderm at the Russian Cancer Research Centre of Russian Academy of Medical Sciences. The duration of treatment with Curaderm ranged from 4 weeks to 14 weeks. Seventy-two patients with primary BCC when treated with Curaderm resulted in 82% success rate. The cosmetic outcomes after treatment with Curaderm were remarkable [60].

2.11. Rare and Long-Term Adverse Effects of Curaderm

Phase IV studies with Curaderm were designed to detect any rare or long-term adverse effects over a much larger patient population and timescale than was possible during the initial clinical trials. Curaderm has been available and used for over two decades and only a very small number of patients have experienced minor scar tissue caused by the salicylic acid in the Curaderm cream. The reasons for the scar tissue were applications of too much cream to the site, sensitivity to salicylic acid, and application to non-indicated conditions such as dry sensitive skin. These observations have secured Curaderm pharmacotherapy as having exceptional effective and safety profiles.

2.12. Coping with BCC Treatment, Does Curaderm Pharmacotherapy Assist?

There are different types of side effects, usually not reported, that patients endure when diagnosed and treated for BCC.

Side effects such as emotional, physical and financial cost of BCC can be very high depending on mainly, size and location of the lesion.

Emotional and social effects, as well as physical effects, may include sadness, anxiety, anger or not being able to control stress levels. Not knowing the cosmetic outcome of the selected treatment procedure, places tremendous stress on the patient and if the cosmetic outcome of their treatment is not to their satisfaction, the patient has to live with this trauma from day to day, and the patient may lose confidence in the medical profession.

2.13. The Financial Stress Can Have a Significant Bearing on the Patient

BCC treatment can be expensive. It is often a big source of stress and anxiety for

people with BCC and their families. In addition to treatment costs, there are possibly extra, unplanned expenses related to their care. High cost of medical care may result in the patient stopping or not completing the treatment. This can put the health of the patient at risk and may lead to higher cost in the future.

The many advantages of having available self-treatments under the supervision of a health care professional are significant for both the patient and for the Health Care Professional. The safety and treatment outcomes are the prime considerations for the patient. Curaderm has proven to be very safe and the cosmetic outcome is impressive and is similar, if not better, than surgery, which may include reconstruction and grafting.

The lower cost of the treatment with Curaderm, compared to established therapies, is beneficial for the patient and for the Health Care System. Any modality that can reduce the already financial burden to the Health Care System and to patients should be considered seriously.

2.14. Limitation of Curaderm Pharmacotherapy

The main limitation of topical pharmacotherapy with Curaderm is the duration of treatment, which depends on the type and size of the BCC and may take several weeks up to several months therapy for the complete removal of the BCC. Transient stinging and burning sensations are experienced in some patients for several minutes after application of Curaderm cream to the lesions. However, these limitations are considered minor when compared with the efficacy and impressive cosmetic outcomes, especially at difficult to treat locations.

3. Conclusions

The conventional treatments for BCC are surgical excision, Mohs micrographic surgery, curettage and electrodesiccation, chemotherapy, radiation, cryotherapy or laser therapy. The common feature of all of these treatments is that they are nonspecific without targeting the tumor itself. This leads to unwanted adverse effects in the surrounding tissue such as scar formation or other cosmetically disfiguring outcomes. In addition, treatment successes and morbidity of these treatments are questionable and mostly depended on the skill of the operator of the procedure. Hence, an alternative treatment modality that is very specific towards the BCC tumour that eliminates small BCCs rapidly, and also eliminates large BCCs, and difficult-to-treat areas that contain BCCs with amazing cosmetic outcomes, is overdue and welcome.

This astonishing BCC treatment has been achieved by extensive investigations of the natural compound BEC that has been shown invariably to suppress and reverse the cancer-causing biochemical changes. This has led to Curaderm, a pharmacological intervention for cutaneous BCC.

Self-administered, non-invasive topical Curaderm pharmacotherapy offers innumerable benefits unavailable with conventional therapies. Curaderm pharmacotherapy is preferable to invasive procedures, especially in cases of multifocal lesions, unclear lesion edges, risk of hypertrophic scarring and/or keloids re-

sulting in disfiguring scars, surgical risk factors and localization in some areas such as the face. Molecular targeting therapy with BEC for the treatment of BCC fills the gap of conventional therapies.

Finally, the adage, "prevention is better than cure" is very applicable to BCC.

There are many known risk factors that contribute to the cause of BCC. Many of the known risk factors, if taken into consideration when developing preventative measures, should result in fewer required treatments. Since solar radiation is a major environmental cause of BCC, a combination of sun protection measures should be encouraged. With the advanced understanding of the science of BCC and its aetiology, novel approaches should be motivated to prevent this disease from occurring. To that end, research has shown that BEC in combination with sunscreen agents in a formulation Curasol eliminates very early stages of skin cancer. This preventative manner of approach to influence the fight against BCC should be stimulated.

BCC is the most common form of skin cancer and the most frequently occurring form of all cancers. If allowed to grow, BCC can become disfiguring and dangerous. Neglected BCCs can become locally invasive, grow broad and deep into the skin and destroy skin, tissue and bone. It is therefore important to treat BCC as early as possible to avoid serious repercussions. There are various treatments available. The treatment choice depends on size, location, age of the BCC, age of the patient and the patient's overall health.

The most widely used treatments are all indiscriminate, these treatments do not distinguish cancer cells from normal cells and the ultimate effective and cosmetic success of the treatment relies on the skill of the treating doctor.

On the other hand, Curaderm pharmacotherapy distinguishes cancer cells from normal cells and only eliminates cancer cells at the molecular and cellular levels, resulting in impressive cosmetic outcomes as verified by Phases I to IV clinical studies and open field observations. The recurrence rate of BCC treated with Curaderm is minimal compared with other treatment modalities.

Curaderm pharmacotherapy has been approved in 2018 by the European Health Authorities as a Class 1 Medical Device for the indication "Topical Treatment with Keratolytic Action, and Antineoplastic Activity in the Treatment and Healing of Localized Basal Cell Carcinoma of the Skin".

Curaderm pharmacotherapy offers the long-awaited breakthrough for the treatment of skin cancer, in particular BCC.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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The Pattern of Eosinophil Count among Nigerians with Frequent Use of the Commonly Available Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

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Abstract

Introduction: Non-steroidal anti-inflammatory drugs (NSAIDs) use is very common. NSAIDs use could be associated with elevated eosinophil count which could be a class effect or patient-related. Inflammation could be the link between NSAIDs use and eosinophilia. **Aims:** To compare the pattern of eosinophil count in the peripheral blood of frequent users of NSAIDs and healthy controls. **Methodology:** Two hundred (one hundred frequent users of NSAIDs and 100 healthy controls) participants who had no known risk factor for kidney disease and had given informed consent were recruited. Blood was taken to determine the white cell count and differentials, serum electrolyte and creatinine, and random blood sugar. **Results:** The mean age of NSAIDs users was not significantly different from controls, P = 0.3. The mean eosinophil count was higher in males than females. The incidence of eosinophilia in NSAIDs users was 4%. The mean Eosinophil count of NSAIDs users was insignificantly higher than controls, 164.3 ± 51 6 vs 135. 6 ± 53.4, P = 0.4. The mean platelet count of NSAIDs users was significantly higher compared to controls, P = 0.04. The mean hematocrit of NSAIDs users was significantly lower than the controls, P = 0.02. Propionic acid derivatives were

associated with the highest eosinophil count. Eosinophil count was positively related to age and serum creatinine and inversely related to blood glucose, hematocrit and glomerular filtration rate. **Conclusion:** The incidence of eosinophilia was 4%. The eosinophil count was higher in frequent NSAIDs users than occasional and non-users, in males than females and with use propionic acid derivatives compared to other NSAIDs. The Eosinophil count was positively related to age and platelet count. Being commoner in inflammatory states, the tissue destruction associated with elevated EC can be avoided by the prevention and prompt treatment of inflammatory conditions.

Keywords

Eosinophilia, Kidney Function, Non-Steroidal Anti-Inflammatory Drugs, Hematocrit, Platelet Count, Propionic Acid

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are cheap and readily available agents used in treating pain [1]. NSAIDs use is very common in low-income nations like Nigeria, where there are significantly larger population of manual laborers and artisans due to the very low level of industrial mechanization compared to the developed countries [2]. NSAIDs use in treating rheumatic conditions is quite common in the elderly [3].

At the community level, Agaba *et al.* reported a 13% prevalence rate of NSAIDs use [2]. Twenty nine million Americans (12.1%) were reported to be regular users of NSAIDs in 2010 [4]. Zeinali *et al.* also reported a high prevalence of NSAIDs use among Iranian with 19.3% of all prescriptions having at least, an NSAID and 7% of these being combination NSAIDs [5].

NSAIDs use has been reported to be associated with eosinophilia and tissue eosinophilic infiltration [6]. These drugs inhibit cyclooxygenase (COX) pathway thereby inhibiting the release and actions of prostaglandins (PGs) which are made up of the following subunits: PGD_2, PGI_2, PGE_2 and PGF_2 [7]. Eosinophilia, with its chemo attractant actions, is mediated through NSAIDs effect on its PGD_2 subunit [8]. Eosinophilia mediates airway remodeling and induces disease progression resulting in fibrosis of chronically inflamed cells that involves angiogenesis [9]. It is not known if the actions of NSAIDs on leucocytes are class effects or not as only Indomethacin has been reported to exhibit these features [8] [10] [11]. The relationship between NSAIDs use and inflammatory tissue damage, through the degranulation and release of cytopathic eosinophils and basophils has been reported from studies in the western world [12] [13]. Ironically, in sub-Sahara Africa and other low-income countries where NSAIDs use is commoner, the relationship between NSAIDs use and the pattern of eosinophil distribution is rarely reported. In this study, we determined the pattern of eosinophil distribution among Nigerians with frequent NSAIDs use, defined as daily use for up to a month [14].

2. Methods

A prospective, comparative study carried out at the Federal Medical Centre, Abeokuta, Nigeria, from January 2016 to December 2016, in which, two hundred (one hundred frequent NSAIDs users and 100 age and sex-matched healthy controls), eighteen years and above who gave consent were consecutively recruited. Ninety-two NSAIDs users were recruited from the orthopedic clinics and eight from among manual laborers/artisans working within and around the hospital construction sites. The controls were recruited from healthy hospital staffs and the surrounding community. Participants less than 18 years, with hypertension, diabetes, sickle cell anemia, diseases of the kidneys, heart or liver or risk factors for these diseases were excluded. Also excluded were participants who sneeze a lot, or had recurrent stuffy or running nose, watery eye, tight chest or itching on exposure to strong smell (perfumes, boiling oil or fumes), sandy air or to any food or drink and any other form of allergy. Participants with infection, hypertension, diabetes and proteinuria were also excluded.

Socio-demographics and drug history were obtained through an interviewer-administered questionnaire and from participants' case files. The NSAIDs users were shown packets, sachets and containers of the commonly used NSAIDs in the locality to ascertain those used by them, alone or in combination. Participants were described as frequent NSAIDs users when they take at least a unit (tablet, capsule, patch, ointment or suppository) daily for at least 1 month [14]. All participants had stool microscopy, culture and sensitivity for ova and parasite, prior to sample collection.

Participants' height and weight were measured without shoes and on very light clothing using a SECA standiometer and weighing scale respectively, and the body mass index (BMI) was calculated. Participants' pulse rate and blood pressure were taken after 5 minutes rest. Five milliliters of blood was taken from each participant into an ethlenediamine tetraacetic acid (EDTA) containing bottle, blood was mixed gently and immediately taken to the laboratory to determine the full blood count (FBC) including the total white cell count (WBC) and differentials including the eosinophil using the counting chamber. Another 3 ml was taken for determination of serum electrolytes, urea and creatinine and the estimated glomerular filtration rate (eGFR) was calculated.

Definitions

Frequent NSAIDs use-daily use of at least a unit for ≥1 month [14].

Eosinophilia-peripheral blood eosinophil count of $\geq 450 \times 10^6$/l [15].

Hypereosinophilia->1500×10^6/l [16].

Kidney dysfunction-eGFR < 60 ml/min [17].

Anemia-hematocrit < 39% [18].

Sample size was calculated from the formula on comparative study using a previous study's prevalence [19].

Statistical analysis

Continuous variables were presented as mean with standard deviation and

compared using student's t-test while categorical variables were presented as proportions and compared using chi-square or Fisher's exact test. Pearson correlation test was performed to determine the degree of correlation between eosinophil count and participants' characteristics. The level of $P < 0.05$ was considered statistically significant.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of the Federal Medical Centre, Abeokuta approved the study. The institutional ethical committee of the Federal Medical Centre approved all study protocols ((FMCA/238/HREC/09/2015). Accordingly, written informed consent was taken from all participants before any intervention

3. Results

Two hundred participants (100 frequent NSAIDs users and 100 age and sex-matched healthy controls) were recruited for the study. Forty-nine males and fifty-one females in each group participated. The mean age of the NSAIDs users and controls were 46.5 ± 14.2 and 46.2 ± 14.3 respectively, P = 0.3. There was no significant difference between the age, sex and diastolic BP of the NSAIDs users and the controls, P = 0.5, P = 0.3, P = 0.6 respectively. The demographic and clinical characteristics of the participants are shown in Table 1. The mean BMI and systolic BP of the NSAIDs users were significantly higher than those of the controls, P = 0.03 and P < 0.001 respectively.

Table 1. Socio-demographic and clinical characteristics of participants.

	Variables	NSAIDs users N = 100 (%) Mean ± SD	NSAIDs users N = 100 (%) Mean ± SD	X^2 t-test	P-value
Gender	Males	49 (49)	49 (49)	0.55	0.5
	Females	51 (51)	51 (51)		
Age, *years*	18 - 39	28 (28)	34 (34)	0.70	0.3
	40 - 59	53 (53)	49 (49)		
	>60	19 (19)	17 (17)		
WHR		1.0 ± 0.1	1.0 ± 0.04	0.01	0.8
Mean Age, *years*		46.5 ± 14.2	46.2 ± 14.3	0.3	0.3.
Mean BMI, kg/m^2		28.1 ± 13.1	26.4 ± 13.2	3.04	0.03
Mean SBP, *mmHg*		123.5 ± 10.4	114.0 ± 1.2	5.92	<0.001
Mean DBP, *mmHg*		75.7 ± 8.2	74.5 ± 7.2	0.03	0.6

NSAIDs = non-steroidal anti-inflammatory drugs, SD = standard deviation, WHR-waist hip ratio, BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, S = serum, eGFR = estimated glomerular filtration rate, CKD-EPI = chronic kidney disease epidemiology collaboration.

Four (4) NSAIDs users had eosinophilia as against none among the controls. None of the participants had hypereosinophilia nor leukocytosis. Table 2 shows the laboratory results of the participants. There was no significant difference between the mean white cell count and eosinophil count of the NSAIDs users and the controls, P = 0.1 and P = 0.4 respectively. There was a significant difference between the platelet count and the hematocrit of the NSAIDs users and the controls, P = 0.04 and P = 0.02 respectively. There was a significant difference between the serum creatinine and glomerular filtration rate of NSAIDs users and the controls, P < 0.001 and P < 0.001 respectively.

The mean eosinophil count was higher in the males than females in both NSAIDs users and the controls. Table 3 compared the eosinophil count of NSAIDs users and the healthy controls. The eosinophil count was positively associated with the age and BMI but had an inverse relationship with the GFR in both the NSAIDs users and controls.

Among the NSAIDs users, there was a positive relationship between the eosinophil count and the doses of each drug. Table 4 shows the relationship between the eosinophil count and the various doses of single NSAIDs used by participants. The mean eosinophil count was highest in Ketoprofen and Ibuprofen and it was least with Aceclofenac. The difference between the smaller and the larger doses of NSAIDs, in terms of mean eosinophil count, was statistically lower in Ketoprofen (P = 1.0) and Ibuprofen (P = 0.8) compared to Aceclofenac (P = 0.5) and Meloxicam (P = 0.4). The mean eosinophil of single NSAIDs users was 156.15 ± 23.61 compared to 176.46 ± 28.16 for those that used two or more NSAIDs. The difference was statistically significant, P = 0.04.

As the BMI of NSAIDs users increased, the eosinophil count increased and the difference was statistically significant, P = 0.04. There was a direct relationship between the eosinophil count and the duration of NSAIDs use, P = 0.01. The determinants of eosinophil count amongst the NSAIDs users are shown in Table 5. The eosinophil count increased with the age of participants, and the systolic and diastolic blood pressure but the differences were not statistically significant, P = 1.6, and P = 0.05 and P = 0.9 respectively.

4. Discussion

Our series found a non-statistically significant increase in eosinophil count in frequent NSAIDs users compared to a healthy population. The degree of this increase was directly proportional to the duration of NSAIDs use. The increase was also more in males than females as it was for participants who were overweight or obese compared to those who were underweight. The increase in eosinophil count was more in the older age group than in the young. The increase in eosinophil count in NSAIDs users mirrors findings by Satoh *et al.* [8] and Kataoka *et al.* [20] who reported in separate studies that NSAIDs use is associated with elevated eosinophil count but they noted that the only drug associated with the hypereosinophilic syndrome was Indomethacin, which unlike other NSAIDs,

is a potent agonist of the PGD_2 receptor, chemoattractant receptor-homologous molecule expressed on T helper type 2 cells ($CRTH_2$). The decreased eosinophil response to PGD_2 was associated with reduced priming of the chemotactic actions of eosinophil as a result of downregulation of $CRTH_2$ cell surface expression [10]. We, therefore, infer that the low incidence of eosinophilia was secondary to the non-availability of Indomethacin for use by participants.

Table 2. Laboratory results of participants.

Variables	NSAIDs users	Controls	t-test	P-value
	N = 100 (%)	N = 100 (%)		
	Mean ± SD	Mean ± SD		
Mean Total WBC, $\times 10^6/L$	5.2 ± 2.3	5.1 ± 1.7	0.6	0.4
Mean Eosinophils, $\times 10^6/L$	164.3 ± 51 6	135. 6 ± 53.4	0.8	0.4
Mean Hematocrit, %	36.8 ± 7.3	40.2 ± 11.5	1.7	0.02
Mean Platelet count $\times 10^9/L$	386.5 ± 44.7	349.8 ± 56.2	1.1	0.04
Mean FBS, *mmol*	4.8 ± 1.4	4.9 ± 1.2	0.1	0.4
Mean Creatinine, *umol/l*	99.6 ± 13.3	69.5 ± 9.1	5.7	<0.001
Mean eGFR, *ml/min*	87.8 ± 3.1	115.0 ± 2.7	9.4	<0.001

NSAIDs-non-steroidal anti-inflammatory drugs, SD-standard deviation, WBC-white cell count, FBS-fasting blood sugar, eGFR-estimated glomerular filtration rate.

Table 3. Comparison between the Eosinophil counts of NSAIDs users and controls.

Variables		NSAIDs users	Controls	t-test	P-value
		Eosinophil ($\times 10^6$)	Eosinophil ($\times 10^6$)		
		Mean ± SD	Mean ± SD		
Eosinophil count, $10^6/L$		164.3 ± 51.6	136.9 ± 53.4	0.8	0.4
Sex:	Males	191.1 ± 22.7	146.0 ± 30.2	1.2	0.2
	Females	137.6 ± 20.9	128.2 ± 29.6	0.2	0.8
Age, *years*	20 - 39	138.6 ± 14.8	122.6 ± 33.9	0.1	0.9
	40 - 59	163.6 ± 17.3	133.6 ± 18.6	1.4	0.1
	>60	191.4 ± 29.5	153.4 ± 36.2	0.4	0.6
BMI, kg/m^2	<19.5	150.3 ± 16.3	128.8 ± 42.2	1.7	0.07
	19.5 - 24.9	154.7 ± 21.4	125.7 ± 11.9	1.5	0.06
	>25.0	188.2 ± 24.5	157.6 ± 23.2	1.5	0.05
eGFR, *ml/min*	<60	183.4 ± 19.9	144.0 ± 21.4	0.2	0.8
	>60	146.1 ± 18.6	129.1 ± 16.6	1.1	0.2

NSAIDs-non-steroidal anti-inflammatory drugs, eGFR = estimated glomerular filtration rate, CKD-EPI = chronic kidney disease epidemiology collaboration.

Table 4. Relationship between eosinophil counts and various doses of each NSAID.

Variables	Frequency (%)	Mean Eosinophil ($\times 10^6$) Mean ± SD	t-test	P-value
Aceclofenac, *mg*				
100	1 (1)	123.2 ± 22.8	0.45	0.5
200	2 (2)	131.2 ± 18.2		
Diclofenac, *mg*				
50	10 (10)	137.3 ± 31.5	0.31	0.7
100	14 (14)	142.1 ± 44.3		
Ibuprofen, *mg*				
600	4 (4)	172.4 ± 33.8	0.23	0.8
1200	2 (2)	179.5 ± 40.3		
Ketoprofen, *mg*				
100	4 (4)	178.3 ± 58.7	0.11	1.0
200	6 (6)	179.9 ± 66.5		
Meloxicam, *mg*				
7.5	5 (5)	154.4 ± 25.8	0.52	0.4
15	11 (11)	163.2 ± 31.8		

NSAID = non-steroidal anti-inflammatory drug.

Table 5. Determinants of eosinophil count among frequent NSAIDs users.

Variables		Frequency N = 100 (%)	Mean Eosinophil ($\times 10^6$) Mean ± SD	t-test	P-value
Gender	Males	49 (49)	191.1 ± 22.7	2.16	0.05
	Females	51 (51)	137.6 ± 20.9		
Age, *years*	18 - 39	32 (32)	138.6 ± 14.8	0.42	1.6
	40 - 59	53 (53)	163.6 ± 17.3		
	>60	15 (15)	191.4 ± 29.5		
NSAIDs types	1	59 (59)	163.7 ± 130.8	0.25	1.9
	>2	41 (41)	166.0 ± 25.6		
Duration, *months*	0 - 6	23 (23)	142.6 ± 36.1	3.02	0.01
	7 - 12	36 (36)	149.1 ± 32.6		
	13 - 60	35 (35)	184.5 ± 42.9		
	>60	6 (6)	222.8 ± 32.9		
BMI, kg/m^2	<19.5	17 (17)	150.3 ± 16.3	2.42	0.04
	19.5 - 24.9	49 (49)	154.7 ± 21.4		
	>25.0	34 (34)	188.2 ± 24.5		
Systolic BP, *mmHg*	<120	29 (29)	148.6 ± 77.4	2.32	0.05
	120 - 139	71 (71)	180.8 ± 29.4		
Diastolic BP, *mmHg*	<80	36 (36))	159.7 ± 41.9	0.8	0.9
	80 - 89	64 (64)	169.2 ± 41.9		

NSAIDs-non-steroidal anti-inflammatory drugs, BMI-body mass index, BP-blood pressure.

The mean age of the NSAIDs users was less than what other studies found in Nigeria, Iran and in the United States [3] [5] [6]. This difference could be attributed to the fact that chronic diseases, commonly found in the elderly, like hypertension and diabetes, were excluded in this study. One would have expected more women participation in the study considering that the fact that females are more commonly associated with rheumatologic disorders, coupled with cyclic pains experienced by women during their menstrual circle [1]. The higher mean BMI in NSAIDs users than in healthy controls is similar to findings by Schwartz *et al.* who found an increased incidence of acute kidney dysfunction in NSAIDs users with attendant fluid retention and weight gain [21]. The higher eosinophil count in males mirrors findings by Pardo *et al.* [22] and Tariq *et al.* [23] who found this pattern more common in participants without kidney disease. Ogbogu *et al.* [11] and Loules *et al.* [24] separately reported higher eosinophil count in males and attributed it to the presence of the pre-mRNA 3'-end-processing factor FIP1-platelet derived growth factor receptor A (FIP1L1-PDGFRA) fusion genes in males. The authors reported that when eosinophils are pretreated with Indomethacin, eosinophilic migration towards PGD2 was suppressed [24].

We found higher eosinophil count in the older age group and this agrees with Praga *et al.* [25] who attributed this to the higher incidence of eosinophilia associated with acute interstitial nephritis in advancing age. There was a positive relationship between eosinophil count and body size and this mirrors findings by Amani *et al.* [26]. The findings however disagree with findings by Berair *et al.* [27] who found no relationship between eosinophilia and obesity. Obesity is associated with elevated triglycerides, total cholesterol and glycated hemoglobin as well as reductions in high-density lipoprotein [26].

Even though hypertensives were excluded from our study, we found that within normal ranged blood pressures, eosinophil counts were positively correlated with blood pressure. Hypertension and obesity are chronic conditions associated with elevated cytokine release and oxidative stress which are associated with elevated eosinophil count [28]. Masenger *et al.* [29] on behalf of the American Heart Asssociation (AHA) reported the association between hypertension and eosinophilia. Hypertension is associated with increased infiltration of macrophages into the kidneys, increasing victims' risk for chronic kidney disease (CKD) with increases commonly found in IL-6 and IL-17 with concurrent reductions in the anti-inflammatory IL-10 [30] Madhur *et al.* [31] also reported angiotensin II induced hypertension associated with elevated IL-17, further emphasizing the relationship between elevated eosinophil and IL-17.

Elevated eosinophil count was positively related with the platelet count in our study and this mirrors findings by Shah *et al.* [32] The association between eosinophil and platelets is said to be symbiotic, as eosinophil activates platelets in a dual, indeed bimodal faction as, eosinophil derived inflammatory mediators stimulate platelets while some eosinophil derived mediators are reported to inhibit platelet activities. The findings of eosinophil in mural thrombus associated

with acute coronary events like myocardial infarction, a condition associated with platelet aggregation further confirms the symbiotic association between the two [33]. The negative relationship between eosinophil count and the hematocrit in this study is in agreement with findings by Sweidan *et al.* [18] who reported a case of autoimmune hemolytic anemia with Ibuprofen use. The pro-inflammatory features of eosinophils stimulate hemolysis, from disruption in membrane proteins, leading to altered cell cellular adhesion, increased permeability and osmotic fragility.

The finding of an inverse relationship between eosinophils and the blood glucose level in this study agrees with findings by Zhu *et al.* [34] and Ment *et al.* [35]. The suppressive effect of glucocorticoids on the eosinophil with background leukocytosis could be multifactorial, one mechanism being increased apoptosis induced by reductions in IL-5, an anti-apoptotic agent that stimulates eosinophil maturation and prevent its destruction [36]. The inverse relationship between eosinophil and HbAic, and also with, the severity of Cushing syndrome is further explained by the interaction between glucocorticoids and eosinophils. The higher eosinophil count in NSAIDs users compared to healthy controls could be attributed to the widely reported findings that the eosinophil counts are elevated in kidney disease [37]. Inflammatory mediators are commonly elevated in KD, particularly chronic kidney disease (CKD), and these stimulate eosinophil release. The infiltration of the kidneys and the perivascular spaces by macrophages and other inflammatory cytokines, can cause renal dysfunction or cause acute depression of kidney function in patients with background CKD. It therefore becomes apparent that a "cause and effect" relationship exists between elevated eosinophils count and kidney disease [38].

Even though no significant difference in eosinophil count was found between the different doses of each of the commonly used NSAIDs, it is worth noting that higher doses were associated with higher eosinophil levels. We infer that this finding strongly suggests that the kidney function decline in NSAIDs users is more dependent on the drug type than the drug dosage. This is more so considering the fact that Ibuprofen, probably the most nephrotoxic of the NSAIDs used, showed the least difference between the two dosages that were compared [39]. We found a positive relationship between the length of drug exposure and the eosinophil count. In acute inflammatory conditions, eosinophils stimulate the release of acute inflammatory mediators like IL-1, TNF-α, IL-6, platelet-activating factors (PAF) and various adhesion molecules leading to tissue injury associated with increased extravasation of fluid and the release of pro-apoptotic agents which reduces peripheral platelet count [40]. In chronic inflammatory conditions, eosinophils cause the release of transforming growth factor β (TGFβ), IL-4 and IL-13. These profibrotic mediators cause tissue fibrosis and dysfunction. [41]. This pattern is also supported by findings of progressive decline in kidney function in acute interstitial nephritis progressing to chronic interstitial nephritis (CKD) in NSAIDs users who have used the drugs for more than a month. [21]. The increase in echogenicity, tubular atrophy, tu-

bular wall dilatation with papillary calcification seen in analgesic nephropathy is morphologically represented in the small, indented calcified kidneys seen in this condition [42].

The usefulness of NSAIDs in the control of pain needed to be balanced with various consequences of their use including the attendant risk of kidney dysfunction, induction of the inflammatory cascade, cytokine release and tissue damage, fibrosis and loss of function. These changes would therefore be more in the elderly, obesity and people with background kidney disease. It becomes imperative therefore that these population groups should be given lower doses of these drugs or given other pain suppressing agents as a way of avoiding/minimizing the tissue damage associated with NSAIDs.

We acknowledge some limitations encountered in our study. Some allergic conditions could have been present which participants could have misunderstood as other health conditions. The reliability of the screening process is dependent more on the sensitivity and correctness of the stool examination findings. The determination of the eosinophil count could also be operator-dependent.

5. Conclusion

The use of NSAIDs is very common worldwide, more so, in low-income nations. The incidence of eosinophilia was 4% in NSAIDs users. NSAIDs use was associated with elevations in BMI, blood pressure within normal, eosinophil count, platelet count and serum creatinine as it was associated with reductions in the hematocrit, eGFR and blood glucose. Eosinophilia was common in males, advancing age, kidney dysfunction and in combined and prolonged NSAIDs use. The eosinophil count was also positively related to the dose of an NSAID. The study showed that higher eosinophil count was associated with inflammatory conditions and conditions with increased risk for inflammation hence it was associated with advancing age and kidney dysfunction. There is therefore need to minimize eosinophilia and its attendant consequences by preventing and/or treating inflammatory conditions.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Manifestation of Pathological States of Numerous Diseases in the Largest Organ of the Human Body: (II) From Pancreatitis to Pancreatic Cancer Invasion, Formation of Stroma around the Primary Tumor in the Fascia, to Early Detection of Non-Coding microRNAs in Body Fluids and Development of Drugs to Treat Different Stages of Pancreatic Cancer

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Abstract

Patients suffering from pancreatic ductal adenocarcinoma (PDAC) have an average survival time of 4 - 6 months after confirmed diagnosis. The primary tumor is surrounded by a thick interstitial fluid with high pressure and dense distribution of collagen, forming a huge stroma, rendering the tumor resistant to chemo- and radiotherapy. From the genetic point of view, pancreatic carcinogenesis is driven by mutations, resulting in common activation of the oncogene KRAS, and/or inactivation of one or more of the tumor suppressor genes CDKN2A, TP53, SMAD4 [1]. The pancreas is a mixed exocrine and autocrine organ, with different cell types building up the organ. The pathogenesis involves more than 13 signaling pathways at different stages. Off-balance of the function of the proteins in these pathways due to the stated 4 plus other mutations could readily lead to carcinogenesis. We first present the basic mechanism of these 13 relevant pathways. We then provide a detailed analysis of the progression of this disease, from pancreatitis to tumor formation and metastasis, with special attention on the roles played by the newly dis-

cover calcium channel Piezo, stellate cells, stem-cell-like cells, and the concept invadopodium. Thirty potential drugs, based on *in vitro* and xenograft experiments from different groups, are discussed, including vitamins A, Tocotrienols-E, and D, chemical compounds, non-coding micro RNAs, circular RNA, piwi-interacting RNAs. The recent detection of exosomes enclosing many of these RNAs in body fluids gives us hope of developing early detection methodology because these RNAs carry messages for cell-cell communication at a distance. Delivery of potent drugs by nanoparticles gives us chance to send drugs through the stroma to target the tumor. Since body fluids form a circulating system, together with the connective tissues (where the tumor is associated) form the largest organ—the fascia, we conclude that manifestation of successive pathological states of pancreatic carcinogenesis can be found in compartments of the fascia. We present 17 figures, hoping to ease off the complexity of the pathogenesis of this most lethal cancer disease.

Keywords

Pancreatic Ductal Adenocarcinoma, Signaling Pathways in Carcinogenesis, Piezo 1,2, Exosomes Containing Micro RNA in Body Fluids, Stellate Cell, Cancer Stem Cell, Potential Agents to Treat Pancreatic Cancers, Fascia

1. Introduction

1.1. Interaction of the Fascia and the Organs

During embryonic development, wound healing and tissue homeostasis in adulthood, there is a complicated cellular network linking different biochemical, biophysical cellular pathways that result in 1) proliferation, 2) differentiation, 3) migration, 4) cell cycle progression, 5) apoptosis, and 6) autophagy. In general, groups of different cell types build up an organ (which can be a small blood vessel or a small nerve fiber). All organs are embedded by the largest organ of the body—the fascia, which is an intriguing interwoven structure of collagen fibrils/fibers, proteoglycans, glycoproteins, interstitial fluid, with resident cells and cells in transit. Different parts of the fascia have a special arrangement of their constituents to cater to the functions of organs embedded by these parts of the fascia (for details, see [2]). In the close neighborhood of an organ, the part of the fascia is generally called extracellular matrix (ECM). Fibroblasts apply tract forces to collagen fibers (which contain also elastic fibers) and transmit the force to other cells and connective tissues in the fascia. Such tension can change the phenotyping of the fibroblasts. Mast cells send out biochemical signals for physiological and immunity processes. Mesenchymal stem cells can change phenotypes according to the biophysical and biochemical signals received. The numerous compartments of the fascia, *i.e.* the ECMs (with the resident cells and cells in transit) associated with different organs (which can be a blood vessel, a nerve fiber) are reactive to the associated organs by mechanotransduction.

Inside the fascia, which contains the interstitial fluid as a ground substance, there are growth factors such as the transforming growth factor molecules, that are stored in a dormant state. Mechanical force can release these growth factors that are (naturally) ligands for special receptors at the cell membrane. Once docked at the receptor, one or more cellular processes leading to events stated in 1) - 6) can be initiated. Cells in the fascia, or cells in the bloodstream, can generate cytokines, hormones that can pass from the blood vessel by extravasation to the interstitial fluid and these secretions can also be received by the specific receptors at the cell membrane of an organ(s). Moreover, there are special channels to allow passage of these biochemical molecules and others (such as ions in the extracellular stores) into the cells. In a healthy organ, biophysical and biochemical interactions between the ECMs and the organs would intriguingly trigger off cellular processes 1) - 6) in a balanced manner, so that homeostasis of the physiological processes are maintained with no manifestation of pathological states of the organs, for survival.

However, mutations of genes often happen, leading to the existence of defective proteins/peptides in the cytoplasm. If these defective proteins are not corrected (by rewinding) by heat shock proteins, or sent to apoptosis inside the cell, with lysosomes playing important roles (see details in [3]), these defective proteins exist in the cytosol of the cell. If these defective proteins happen to be members participating in one of the 1) - 6) pathways, cytokine-induced inflammation, abnormal growth, early apoptosis, uncontrolled cell-cycle progression, improper differentiation occur. These pathological states could lead directly or indirectly to carcinogenesis at the end. We have previously explained in detail the manifestation of the pathological states of the tendons diseases in the fascia [2]. We will show in this paper signatures of carcinogenesis of the most lethal cancer can be found in the fascia. In fact, detection of these signatures in the fluids of the fascia and drug delivery via the interstitial fluid gives us hope for early detection and therapeutic treatments.

1.2. Objectives and Setting of This Article

There are different cells in a pancreas and pancreatic cancer of any cell type, often resulting in pancreatic ductal adenocarcinoma (PDAC), which is the 4th leading cause of deaths in the USA and is well known, unfortunately, to have the worst prognosis among all cancers. The mean survival period of PDAC is only 4 - 6 months [4]. It is estimated that PDAC (the disease) would become the second leading cause of cancer-related deaths in the USA before 2030 [5].

Compared to other cancers, PDAC is not a heavily mutated tumor. Recently, only four genes have been identified to be associated with the highest frequency of occurrence involved in the carcinogenesis of PDAC in humans: 1) KRAS (Kirsten rat sarcoma viral oncogene homolog), 2) CDKN2A (cyclin-dependent kinase inhibitor 2A, or p16), 3) TP53 (tumor protein 53) and 4) SMAD4 (mothers against decapentaplegic homlog-4). Other mutations have been found in dif-

ferent pathways during carcinogenesis also in the PDAC specimens [6]. The objectives of this article are as follows: a) Review and extend the signaling pathways involved in different stages of pathogenesis of pancreatic cancers. There are more than 10 such signaling pathways (**Sections (3)-(11)**), the content of which is certainly relevant to the later part of the paper. At the end of each crucial pathway, at least one chemical as potential treatment (mainly compounds) is introduced, though most of them have not undergone clinical trials. b) Since the pancreas is a "mixed organ" with an autocrine and an exocrine function, different types of cells build up this organ. We analyze the progression of pathogenesis in **Sections (12)-(17)**, starting from pancreatitis, through the epidermal to mesenchymal transition (EMT), finally to PDAC [7], leading to the presentation of an overall picture of tumorigenesis in **Section (17)**. During our analysis, we pay special attention to the roles played by the stellate cell, the (pancreatic) stem cell-like cells, the newly discovered calcium ion channel Piezo1 [8], the concept of invadopodium [9], and the formation of the stroma [10]. c) Review the status of prevention of these diseases, plus other plausible treatments targeting proteins/genes in the pathways already discussed, including the functions of vitamins A, D, and tocotrienol-E; such analysis is included in **Section (18)**. **Section (19)** is devoted to treatments with micro RNAs with efficacy based on *in vitro* evidence and *in vivo* animal xenografts investigation. The efficacies of some experimental results cannot be pinned down to specific pathways so far known. d) To look for early detection and therapies of this most lethal cancer, we describe specific examples of the non-coding circular and long non-coding RNAs in **Section (20)**. These non-coding RNAs act as a sponge to "absorb" or interact with other RNAs and therefore have profound effects on the augmentation or attenuation of the pathogenesis. It has been discovered in the recent several years that these non-coding RNAs are messages conveying the status of the sending cell and being carried by vesicles. These vesicles have been found in several body fluids. The concluding remarks in **Section (21)** summarise the analysis of this paper with special attention to the fact that the pathological state of the progression of pancreatic tumorigenesis manifests itself in different compartments of the fascia [2], which includes the interstitial and other body fluids that carry those vesicles just mentioned. We also emphasize the fact that a huge stroma is developed around the primary tumor in the extracellular matrix, with a high density of collagen fibrils and viscous interstitial fluid, rendering the well-known chemotherapy and radiotherapy resistance. We see the hope of early detection of the vesicles in the fascia compartment just mentioned and consider the usage of nano-particles to deliver "drugs" as a workable strategy; these drugs could be pharmaceutical, micro RNA mimics (or their inhibitors). All the crucial statements of deductions are justified with the experimental results of different research groups. 17 figures are presented for the convenience of the reader to follow the complicated signaling pathways and deductions. A list of abbreviations for the relevant biological/biochemical names is provided at the end of the article.

2. Some Basics of the Pancreas—The Pancreas Is a Mixed Gland with Endocrine and Exocrine Functions

2.1. The Islets Containing α, β, δ, and γ Cells That Secrete Different Hormones through the Blood Vessels to Fulfil Endocrine Functions

As an endocrine gland, the pancreatic islets (painted in light green with a thin red boundary in Figure 1) of Langerhans secrete mainly four hormones: 1) insulin, 2) glucagon, 3) somatostatin, 4) pancreatic polypeptide, into the bloodstream. The enlarged diagram of an islet is shown in the lower right corner of Figure 1. Each islet is surrounded by about 20% of (total cells in each islet) α cells (red), which secrete glucagon, a hormone that prevents blood glucose dropping to too low a level. There are blood vessels in each islet, bringing glucagon to the liver where some stored glycogen is converted to glucose. 70% of the cells in each islet are β cells (light green), which secret insulin, that is delivered first by the blood, and then by the interstitial fluid to the muscles. The third type of cells in each islet are the δ cells (yellow, several percent of cells in an islet) which secret somatostatin, a hormone that inhibits the secretion of glucagon and insulin as a regulator of these two hormones. Stimulated by eating, exercising, and fasting, the γ cells secrete pancreatic polypeptide, which can inhibit gallbladder contraction and pancreatic exocrine secretion; the mechanism of secretion and function is still unclear [11].

2.2. The Spleen as a Blood Filter and Blood Cells Storage Provides Clean Blood Supply and Immunity Service to the Pancreas

Blood vessels are connecting the pancreas and the spleen, whose main functions are: 1) As a filter for blood—old red blood cells are recycled in the spleen. 2) Storage of platelets and white blood cells. The spleen is an important organ of the immune system.

The pancreas is thus a main part of the digestive system and source of "fuel" (in the form of hormones stated above) for skeletal movement and organ function in general. The malfunction of the pancreas would lead to defects in the functions 1) and 2) of the spleen too [12].

2.3. As an Exocrine Organ, the Acinar Cells with Their Zymogen Digestive Enzymes Secrete Digestive Juice into an Epithelium (the Duodenum, Rather Than the Bloodstream) through the Pancreatic Duct; the Pancreas Also Provides a Route for the Bile into the Duodenum from the Gallbladder (as Storage) and the Liver

As a digestive organ, it secretes 2) pancreatic juice that contains bicarbonates for the neutralization of stomach acid that enters the duodenum region as well as digestive enzymes (Figure 1) that break down fats, proteins, and carbohydrates of the food that has entered the duodenum region. Moreover, the bile duct of the gallbladder, passes through the duodenal pillar near the "head" of the pancreas,

supplying bile to digest fat too. The main type of pancreatic cells, the acinar cells (purple) form ring-shaped structure attached to the pancreatic duct (light purple) via the square-shaped centroacinar cells (light blue) and (branch) ductal cells. The acinar cells synthesize Zymogen granule digestive enzyme, which in turn secretes the pancreatic digestive juice into the duodenum via a branch of the pancreatic duct. The bile duct (deep green) from the gallbladder has a zig-zag structure, and together with the hepatic duct (also deep green), forms a single duct joining the pancreatic duct at the head of the pancreas. This "mixed duct" pierces through the connective tissues of the pancreas and the duodenum to supply bile juice plus digestive juice to digest food—the entrance to the duodenum is shown by the white arrow. In some people, this joint tube is separated into two (see X-ray photo in Figure 225.1 of [13]).

The adult pancreas is highly plastic to keep its integrity in response to internal stress such as activation of digestive enzymes, obstruction of pancreatic ducts due to the development of gallstones. It can also suffer from external stresses such as trauma and alcohol interaction. Since this organ is rather fragile to mechanical injury, it is anatomically protected by the diaphragm during evolution. Only the major cells are labeled in **Figure 1**, without the immunity cells repairing this organ after injury. During pancreatitis, acinar cells undergo a morphologic and transcriptional change into duct-like cells with embryonic progenitor

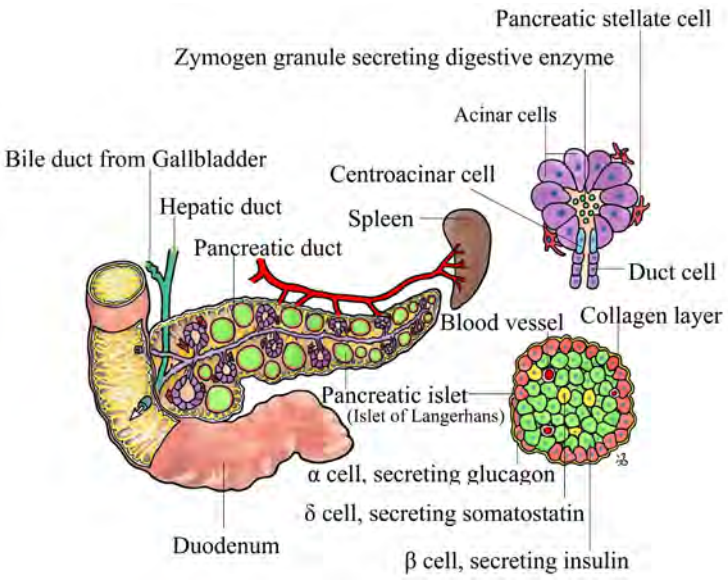

Figure 1. The pancreas, duodenum, pancreatic duct, acinar cells (enlarged in the upper right corner), islets (enlarged in the lower right corner). The deep green ducts deliver bile juice from the gallbladder (as storage) and liver and merge with the pancreatic duct in most people. Digestive juice plus bile juice are secreted into the duodenum, whereas several hormones (glucagon, insulin, somatostatin) are secreted by the islet cells into the blood vessels. An islet is composed mainly of *α* cells (red), β cells (light green), and δ cells (yellow). The pancreatic stellate cells are painted in red—they play active roles in cancer formation and metastasis. There are some stellate cells near the islets too (not shown here) See text for more details. This figure was painted by author PCWF.

cell properties, developing back to adult acinar cells at the end stage of the repairing process; such a wound healing process is now called acinar-to-ductal metaplasia (ADM) [14]. Note that the exocrine part of the pancreas composes at least 95% of the organ's mass.

2.4. The Stellate Cells Plus the Stem Cell-Like Cells Are Found around the Acinar Cells and Quiescent Stellate Cells Are Found in the Islets of Rat Recently

The red cells around the enlarged "acinar ring" in the upper right corner are pancreatic stellate cells, plus some stem-like cells, which will be shown later to play important roles in pancreatic carcinogenesis and metastasis, if activated into the myofibroblast form [15]. Recently quiescent stellate cells have been found in the islets of rat; we infer that such distribution of cells is also similar in human islets [16].

3. The Several Conserved Basic Pathways in Most Cells

3.1. The Akt-mTOR 1,2 and LKP1-AMPK-(TSC1/TSC2) Pathways in Almost All Cell Types—The Imbalance of Any One or More Would Lead to Disease States, Including Those to Be Discussed Later in This Article

Let us first turn to the upper right side of **Figure 2**. Receptor tyrosine kinases (RTKs, painted in red) are cell surface receptors for many (polypeptide) growth factors, cytokines, and hormones, including insulin. Phosphatidylinositol-3 kinase (PI3K, yellow), which is a signaling molecule, is recruited to the cell membrane by the activated growth factor receptors. Phosphatidylinositol 4,5-bisphosphate or PIP_2 (yellow) or called $PI(4,5)P_2$, is a small phospholipid component of the cell membrane. Being activated by PI3K, the lipid unit PIP_2 converts to another lipid unit PIP_3 (yellow, also residing inside the cell membrane), leading to the activation of the phosphoinositide-dependent protein kinase-1 (PDK1, yellow), which is also called the "master kinase PDK1".

Now PDK1, as the "master kinase", activates the following enzymes: 1) Protein kinase A, (PKA) whose activity is dependent on cellular levels of cyclic AMP and is a regulator of glycogen, sugar, and lipid metabolism. 2) Protein kinase C, (PKC), a member of the family of protein kinase enzymes that are involved in controlling the function of other proteins. 3) Ribosomal protein kinase β-1 P70-S6K (or S6K1), the phosphorylation of which induces protein synthesis at the ribosome (already mentioned in the synthesis process of proteins). 4) Protein Kinase B or Akt (yellow). In the figure, mTOR1,2 proteins stand for mammalian target of rapamycin complex 1,2. Now Akt activates the mTOR2 protein (activation is a two-way process [17].

The PI3K/Akt/mTOR1,2 pathways play key roles in numerous normal cellular functions including proliferation, cell adhesion, metabolism, migration, invasion (during growth and wound repair), and survival [18]. During the past three decades, many of the up-stream and down-stream regulations have been discov-

ered. Here we emphasize only the functions of the relevant enzymes involved in proliferation, angiogenesis, apoptosis, migration (including invasion).

In the "growth state", a GTP-binding protein Rheb (also known as Ras homolog enriched in the brain, painted in green) causes mTOR1 to trigger gene expressions in the nucleus, and protein biosynthesis. A ribosome, which is generally free in the cytosol, is needed for such synthesis. A ribosomal protein kinase β-1 P70-S6K (or S6K1, yellow) helps to activate the ribosome, whose small unit is then attached to mTOR1 and the large unit is attached to the rough part of an endoplasmic reticulum (ER). One ribosome (bright green) structure is schematically shown in the figure, being attached to mTOR1. When a gene is triggered off to synthesize the related protein, the RNA "zipped" through between the small and big units of the ribosome; with the help of transmission RNA (tRNA, light yellow), amino acids are picked up and a related native protein is synthesized. This native protein is fed into the ER for quality control: heat shock proteins (such as HSP 70 and HSP 90) check and refold the misfolded native or "first round proteins". Hence the function of HSPs is necessary for a healthy cell to synthesize proper proteins. In the lower-left side of the figure, mTOR1 also activates heat shock factor 1 (HSF1) which leads to the synthesis of HSPs in the nucleus, as indicated by the arrow and the patch marked "HSPs expression".

The growth state must be regulated by the "AMPK pathway" as a negative control. The liver kinase B1 protein (LKB1, green) functions as a tumor suppressor. It activates the 5' adenosine monophosphate-activated protein kinase (AMPK, green) which in turn mobilizes the tuberous sclerosis complex (TSC), that is built of two basic units: TSC1 (also called hamartin) and TSC2 (also called tuberin). This complex stops the action of Rheb, and thus stops protein synthesis.

Note that mTORC2 (orange) is located close to a mitochondrion (purple and white) and an endoplasmic reticulum (deep green). The mitochondrion generates energy (ε). The bioenergy ε in the cytosol inhibits the action of AMPK (back to the left side of the figure), giving the chance of activation of mTOR1 and growth. Moreover, the protein FOXO1,3 leads to apoptosis, as protection of the host, during the condition of insufficient bioenergy or pathological state [19]. Hence, sufficient intracellular energy also rescues the cell from apoptosis. Besides, there is also evidence that Akt can inhibit the action of the (TSC1/TSC2) complex [20], letting the protein Rheb free to mobilize mTOR1 for growth. The regulation of the PDK1-Akt pathway is based on the inhibition of the phosphatase and tensin homolog protein (PTEN) on the conversion of PIP_2 to PIP_3, thus stopping the action of the PDK1-Akt pathway altogether [21]. The processes described above are balanced intriguingly, for most cell types. Now we understand that the duty of PTEN is very important, and mutation of the gene encoding this protein would readily lead to carcinogenesis. The other two pathways on the extreme left and right will be discussed in the following section.

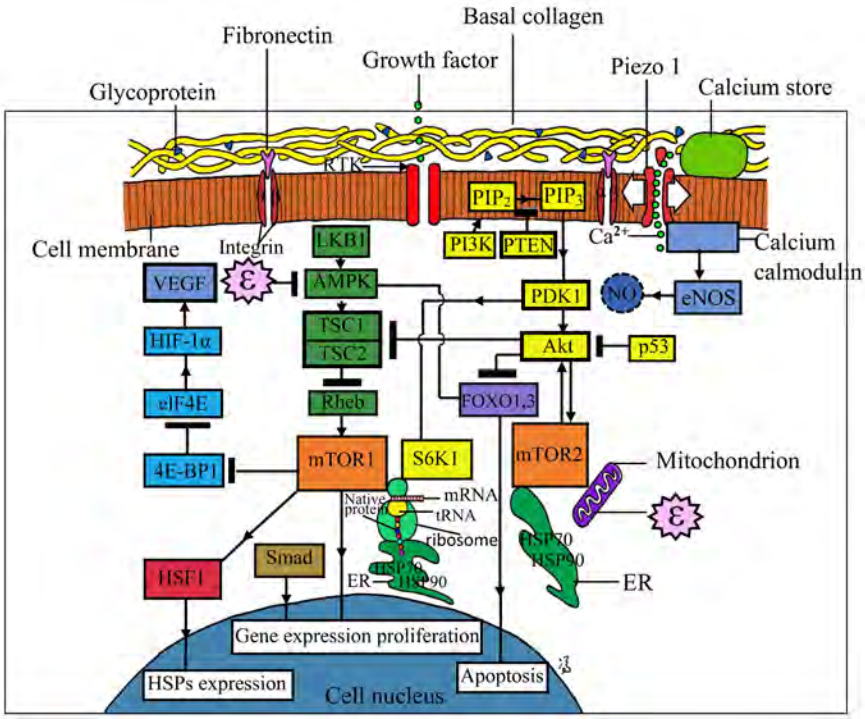

Figure 2. The LKP1-AMPK-(TSC1/TSC2) pathway (dull green) inhibits cell proliferation and protein synthesis. The PI3K-PIP2-PIP3-PDK1-Akt pathway (yellow) stimulates cell growth. Protein Rheb triggers cell proliferation also. Protein PTEN is a key tumor suppressor. HSF1 activation leads to the synthesis of various heat shock proteins (HSPs) for quality control of synthesized proteins. The two blue pathways on the extreme left and right pertain to the endogenous synthesis of vascular endothelial growth factor (VEGF) and nitric oxide (NO) for vessel dilation, the details of which will be amplified in the next section. For other details, see text. This figure was hand-painted by author PCWF.

3.2. Synthesis of Vascular Endothelial Growth Factor (VEGF) and Nitric Oxide—These Two Processes Are Physiological during Embryogenesis and Wound Healing, But Are Involved in Basically All Types of Carcinogenesis

The eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1, encoded by the gene EIF4EBP1), is a member of a family of translation repressor proteins [22]. The protein 4E-BP1 directly interacts with eukaryotic translation initiation factor 4E (eIF4E), whose activity is then inhibited; these two proteins are commonly treated as a complex in specifying pathway activation. 4E-BP1 is a well-established substrate of the rapamycin (mTOR1) signaling pathway [23]. Now, eIF4E activates the hypoxia-inducible factor-1α (HIF-1α), leading to a synthesis of the vascular endothelial growth factor (VEGF), resulting in angiogenesis. Note that HIF-1 α is a master transcriptional regulator of cellular response to hypoxia. In short, angiogenesis occurs when mTOR1 is in action (by growth factor stimulation, such as insulin, as one example) [24]. This pathway is shown on the left side of **Figure 2**. During embryonic development or wound repair, cells would have to synthesize VEGF for blood vessel growth.

Recently the calcium channels Piezo1,2 have been discovered in cell mem-

branes of many cells, including cells of islets in the pancreas [25]. A Piezo1 channel (relevant to pancreatic cells) is schematically painted in **Figure 2**. At the quiescent state, the entrance is blocked. When triggered by mechanotransduction, such as that activated by integrins in the cell membrane, the calcium channel is opened to calcium ions from extracellular stores. Calcium ions mobilize a small protein called calcium calmodulin (blue), which is attached to the inner side of the cell membrane. The protein calmodulin activates the enzyme endothelial nitric oxide synthase (eNOS, one of the three isoforms, painted in blue) which converts L-arginine to citrulline, releasing nitric oxide (NO) that can pass through water and cell membrane, and can be delivered by red blood cells [26]. The NO generation process is schematically represented at the upper right corner of **Figure 2**. Cancer cells need a lot of oxygen and nutrients to be able to survive. To cancer cells, angiogenesis is the first establishment. Moreover, all blood vessels receive vessel dilator and constrictors to adjust the blood flow rate for the obvious reason of survival. NO is a strong vessel dilator (as against angiotensin). Hence cancer cells need to generate a large amount of NO molecules. With the excessive generation of VEGF and NO, the "angiogenic switch" of pancreatic cells could readily occur, as mentioned in [27].

3.3. The Anti-Cancer Effects of Irisin (Hormone) Targeting the AMPK-mTOR1 Pathway

Uncoupling protein 1 (UCP1) is a membrane protein found in the mitochondrial inner membrane of brown adipose cells. Activating UCP1 by cold or diet, energy derived from the oxidation of fat is not available to drive the phosphorylation of ATP and is consequently dissipated as thermal energy, playing a role in regulating energy metabolism. Irisin is an exercise-induced cytokine, produced by the proteolytic hydrolysis of fibronectin type III domain-containing protein 5 (FNDC5) [28]. There is also evidence that Irisin could induce browning of white adipose cells by upregulating the peroxisome proliferator-activated receptor (PPAR)-γ and UCP1.

Recently, apart from the secretion from the muscle and skin, Irisin has been identified also as a myokine in the heart and organs of the digestive system, including the pancreas [29]. Using human pancreatic cancer cell lines MIA PaCa-2 and Panc03.27 (pancreatic adenocarcinoma epithelial cells), it has been shown that 1) Irisin receptors could exist on the surfaces of pancreatic cancer cells. 2) Irisin inhibits pancreatic cancer cell growth via the AMPK-mTOR1 pathway (see **Figure 2**).

In another study [30], employing the human pancreatic cancer cell lines, PANC-1 (isolated from a pancreatic carcinoma of ductal cell origin) and BxPC-3 (adenocarcinoma origin), it has been reported that 1) Irisin decreased the proliferation of pancreatic cancer PANC-1 and BxPC-3 cells. 2) Irisin's action led to apoptosis of the pancreatic cancer BxPC-3 cells. 3) Migration and invasion of pancreatic cancer BxPC-3 cells were attenuated by the application Irisin. 4) Irisin suppressed the protein phosphorylation of the PI3K/Akt signaling pathway. Re-

ferring to **Figure 2**, suppression of this pathway amounts to inhibiting the action Akt to stop apoptosis via FOXO1,3, with the result of letting the cancer proceed to natural cell death. Both the studies of [29] and [30] substantiate each other using four different human pancreatic cancer cell lines.

Developing drugs that can promote the endogenous secretion of Irisin seems to be one plausible therapy to treat pancreatic cancer. We should note, however, this peptide has no and even adverse effects on a few other types of cancer, as remarked in [31]. The first chemical synthesis of irisin using two sequential ketoacid-hydroxylamine (KAHA) ligation giving milligram quantities has been reported four years ago [32].

4. The Most Significant Pathway Responding to Mechanical Stimulation Initiated in the ECM and Its Correlation with Pancreatitis

4.1. The Mitogen-Activated Protein Kinase (MARK) Cascade or the Ras-Raf-Mek-Erk Signal Transduction Cascade Triggered by the Newly Discovered Calcium Ion Channel Piezo1 Plus Internal Calcium Store, Leads to Transcription, Mitosis

The (activation) mutation of the KRAS gene has been found to be at ~95% in late stages of pancreatic cancer [1]. The gene KRAS encodes the protein Ras. We will discuss straight away the signaling pathway initiated by the over-activation of Ras here. Referring to **Figure 3**, there are many receptor tyrosine kinases (RTKs, reddish-orange) at the cell membrane; activation by growth factors (like epithelial growth factor, EGF) when docking on the receptor caused activation of the enzyme phospholipase enzyme (PLC-epsilon1, bluish-green) by these growth factors. PLC-epsilon1 is attached to the cell membrane and catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$), or $PI(4,5)P_2$ or PIP_2 (see **Figure 2**) bluish-green) to give two second messengers: 1) phosphatidylinositol 1,4,5-triphosphate (IP_3, also bluish-green) and 2) diacylglycerol (DAG, light purple, not shown in **Figure 2**) [33]. There is evidence this protein Ins (1,4,5) P_3 docks the receptor (represented by the symbol R, dull green) in the surface of endoplasmic reticulum (deep green). The internal calcium ion store (medium green) is then opened, releasing Ca^{2+}(bright green small circles) [34] to a concentration of even up to micro-Mole level, much greater than the value of 100 nano-Mole, which is the value during the rest state. So that is the source intracellular calcium ions.

Note that DAG activates the protein called Ras (light purple, encoded by the gene KRAS) which is a member of a class of proteins called small GTPase, and these proteins are expressed in all animal cell lineages and organs, involving mainly in transmitting signals within cells. DAG can activate Ras only if calcium ions are present, and Ras is then "switched on". Ras can also be switched off in the absence of Ca^{2+}. The active Ras subsequently activates the Rapidly Accelerated Fibrosarcoma, or Raf protein (light purple). Raf causes the phosphorylation of Mitogen-activated protein kinase (Mek, light purple), extracellular sig-

nal-regulated kinases (Erk, light purple) in a series. Activated Erk phosphorylates Ribosomal s6 family kinases (RSK, light purple). The Ras-Raf-Mek-Erk pathway is then said to be formed. Both RSK and Erk (after activation) translocate to the cell nucleus (deep blue) where they activate multiple transcription factors resulting in effector protein synthesis; there are consequential causal changes in cell growth, differentiation, and survival—we use only the terms transcriptions and mitosis to represent such consequences in **Figure 3** [35]. The above Ras-Raf-Mek-Erk pathway is also called mitogen-activated protein kinase (MARK) cascade. Apart from the fat cell, this cascade functions in basically all other cells in the body.

By 2017, more than 90% of pancreatic cancers have been found to have mutations in the form of amplifications, deletions, translocations, inversions, frameshifts, and substitutions [1]. Aberrant KRAS mutation, resulting in aberrant Ras-Raf-Mek-Erk signaling, activates inflammatory signaling pathways that play crucial roles in promoting pancreatic intraepithelial neoplasia (PanIN) and the progression of PDAC [36].

At the molecular level, genes mutation can first enhance/subside the function of a related protein, making it even constitutively active or loss of function, with complete abrogation in the extreme case. Subsequently, the changes in protein functions can cause uncontrolled proliferation, motility, and adhesion of cells, protection from apoptosis or autophagy, DNA repair problems, the establishment of a cancerous niche, resulting in the development and growth of cancers. The Ras protein is a small GTPase, which binds guanosine triphosphate and diphosphate nucleotides. It is activated or deactivated when bound to GTP or GDP respectively. At the protein level, activated protein Ras binds and activates Raf family kinases, Raf1. Activated Rafs phosphorylate and activate Mek1,2 kinases, which in turn phosphorylate and activate Erk1,2. The Ras-Raf-Mek-Erk pathway eventually leads to transcription, cell proliferation, and mitosis as explained briefly above.

In passing, note that the protein PI3K (orange), which is attached to the cell membrane, is drawn in both **Figure 2** and **Figure 3**. The activation of the PI3K-PDK1-Akt-mTOR1,2 has already been discussed in the last section. The crucial consequence of that pathway is the blockage of the apoptotic process, rendering this cell viable. The same protein PI3K appears in both figures. We show a short symbol "inhibits apoptosis" in **Figure 3** to highlight this fact, referring the readers to **Figure 2** above again.

The two main pathways shown in **Figure 3** are: 1) the Ras-Raf-Mek-Erk pathway and 2) the Rho-Rock pathway. Note that integrin can be activated by mechanical force from ECM, and growth factors. The focal adhesion, composed of several proteins, is formed. Fibronectin has an affinity to integrin. Pathway 1) leads to transcription and mitosis, whereas pathway 2) initiates the formation of the F-actin (α-SMA) chain(s) starting from the focal adhesion. The "glued" α-SMA chains can contract, leading to the morphological change of cell shape and migration. Some of these chains also join the nucleus surface, mediating

transcription. Mechanical stimulation such as acupuncture/acupressure/massage causes grouping of the integrin sets together; the tension forces are extended on the cell membrane to pull the cation channel Piezo, leading to the entry of calcium ions from the external calcium store. The E-cadherin is a transmembrane protein joining adjacent cells (usually of the same type). The indices 1,2 in Piezo1,2 are omitted, as each type is found in specific cells. In a neuron, Piezo2 is found. In an epithelial cell, the channel is Piezo1.

We still have not answered the question: where do the Ca^{2+} ions come from to replenish the metabolized internal calcium ions? The recent discovery of the calcium channel Piezo1,2 provides the source of calcium entry from extracellular calcium store due to mechanical traction of the integrins in the cell membrane. This issue is followed up in the next section.

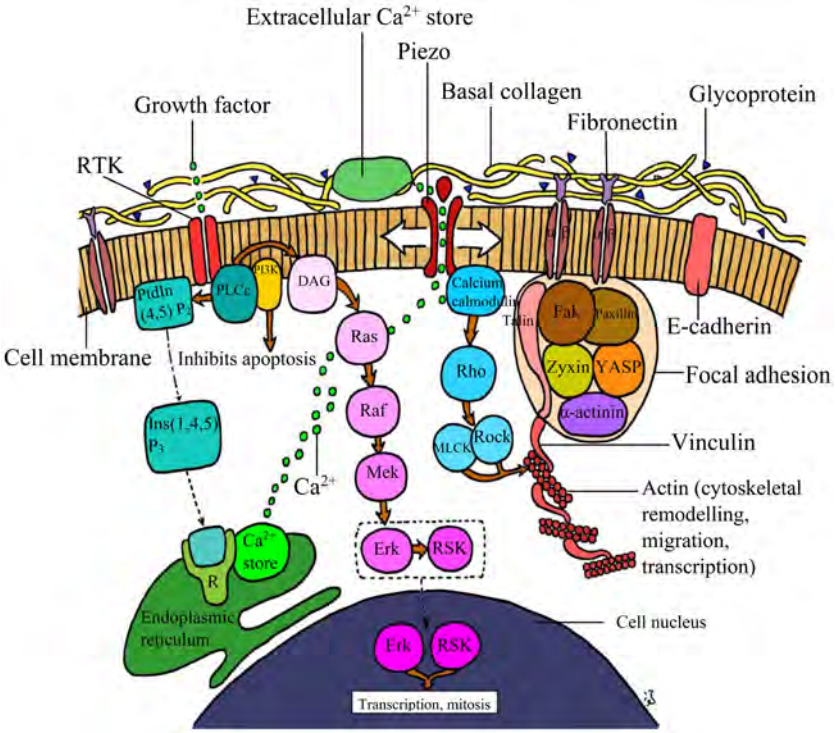

Figure 3. Emphasizing the several pathways in most cells resulting from mechanical stimuli applied at the extra-cellular matrix. The symbols inside the circular/elliptical "bags" are names of the proteins which are defined and specified in the text. The thin yellow fibrils are constituents of the basal membrane (composed of mainly collagen type IV) structure that embeds every organ. The small triangles in the ECM are proteoglycans/glycoproteins, which form part of the non-collagen proteins. RTK is a typical cell membrane channel that allows the docking of growth factors that activate Proteins PLC-epsilon and PI3K. The dash-dot line with an arrow means "becomes after phosphorylation or other chemical reaction". The deep orange arrows indicate the activation of proteins. We observe that on activation, both the internal and external calcium ion stores can release calcium ions which triggers (a) the Ras-Raf-Mek-Erk pathway and (b) the Rho-Rock pathway. Integrin can be activated by mechanical force from ECM, and growth factors. The focal adhesion contains several proteins as specified in Section (4.2), Piezo is a newly discovered calcium ion channel discussed in Sections (4.2) - (4.4). For other details, see text in Section (4.1). This figure was hand-painted by author PCWF.

4.2. The Important Role Played by the Newly Discovered Calcium Channel Piezo 1,2

Referring to **Figure 3**, an integrin set has two units-the alpha (red) and the (slightly longer) Beta (red), the general function has been reviewed previously. When an integrin set is attached to the ligand (fibronectin, purple [37]); it becomes active, and the integrin set can recruit specific proteins forming a group called focal adhesion. According to function, the focal adhesion complex may be roughly divided into three layers. The signaling layer, closest to the integrins cell membrane, contains the highly phosphorylated signaling proteins 1) focal adhesion kinase (Fak, brown in **Figure 3**), which can be considered as a crucial integrator of cell mobility, and 2) Paxillin (lighter brown), whose main function is to adhere the cell to the ECM. Paxillin is expressed at focal adhesions of non-striated cells and at costameres of striated muscle cells. The second layer of the focal adhesion complex may be called the force transduction layer, which has members such as 3) Talin (pinkish) and 4) Vinculin (reddish-orange); they are adapter proteins that connect the integrin complex to the actin chain that can contract. Talin has an elongated structure, spanning basically the whole (force transduction) layer, with its head binding to integrins and the rod connecting actin (which can be 30 nm away from the integrins). Mechanical stretching of Talin promotes Vinculin binding; such binding stabilizes the focal adhesion complex [38]. The third layer may be called the actin regulatory and actin stress fiber layer. This layer contains proteins such as 5) Zyxin (ochre color), a zinc-binding phosphoprotein, serving as a messenger in the signal transduction pathway, by modulating the cytoskeletal organization of actin bundles. 6) Vasodilator-stimulated phosphoprotein (VASP, orange) and 7) Alpha-actinin (red), which is a microfilament protein. The boundary of this layer can be 60 nm away from the integrin layer. We have painted a boundary (peach color) surrounding the key proteins in the focal adhesion complex (there are other proteins too). Now, this focal adhesion complex is attached to one or more integrin sets. The formation of the focal adhesion is an important step in physiological functions and pathological progressions. We will follow up on what focal adhesion leads to, in relation to the action of the calcium ion channel, in the next section. In the meantime, we turn to the pathway involving one of the four most frequent mutated genes in pancreatic cancer.

When a group of integrin sets are activated, they are crowded together, leading to contraction of the cell membrane (light brown). At the quiescent state, there is a triangular structure like a cork of a wine bottle (built of amino acids) blocking the opening in a typical Piezo structure. We shall leave out 1 or 2 in the term Piezo1,2 as they are cell-type-dependent. When the integrin sets are in action, a transduction force (represented by the white arrows) automatically acts on the cation channels Piezo. Upon being pulled on both sides via mechanical stimulation, there is some space effectively opened to the entry of positive ions Ca^{2+}, as governed by the arrangement of the residues (with electric

polarization) as described in [8]. With such understanding, the internal calcium ions which are used in triggering the Ras-Raf-Mek-Erk signal transduction cascade are then replenished, a missing link confirmed only by 2010 [39]. With the rat model, using fluorescence imaging microscopy detection of dendritic spine of neuron in real-time, it has been demonstrated that the structural plasticity of the dendritic spine of neuron of rat first activates the small calcium calmodulin protein (near the cell membrane) and then the protein GTPase Rho A (see **Figure 3**). We propose that the structural plasticity would open the cation channel Piezo also. This proposal has been supported by recent experiments on several types of cells (see e.g. [8]). In turn, GTPase Rho A activates the Rho-associated protein kinase (Rock) plus Myosin light-chain kinase (MLCK). This Rock-MLCK complex activates myosin II protein which lines up the actin molecules, forming the actin chain(s), as shown in **Figure 3** (see also [40]). When the actin chains are formed, cytoskeletal remodeling occurs, leading to migration. This actin structure can be extended to the nucleus, causing certain transcription of proteins depending on the mechanical stimulation sensed by the integrin-Piezo complex. Thus, the tension of the collagen fibrils/fibers outside the basal membrane, which embraces the cell groups of an organ (even a small blood vessel, a small nerve fiber) has profound effects on the proteins synthesized by the associated group of cells.

4.3. Mechanical Pressure Applied to the Pancreas Would Readily Induce Acute Pancreatitis and the Evidence of Abundant Expression of Piezo1 in Acinar Cells of the Rat

Recently, using the *in vivo* rat model, it has been demonstrated that channel Piezo1 is found to be expressed in pancreatic acinar cells based on RT-PCR analysis, plus the following observations: 1) Intraductal pressure would cause acute pancreatitis (using high-pressure transducer, as a starting point of the known phenomenon). 2) The acinar cells were full of calcium ions when stimulated (via calcium indicator Calcium 6-QF analysis). 3) Yoda1, which is a Piezo1 agonist, would induce acute pancreatitis. 4) GsMTx4 (inferred to be working as Piezo1 antagonist) would reduce the severity of acute pancreatitis 5) The degree of severity of pancreatitis in acinar cells with genetic deletion of Piezo1 is much less, using the rat model [8].

4.4. Piezo1 Are Abundant in Pancreatic Stellate Cells of Mice Models and Also in Beta Cell Lines of the Rat's Islet

Pancreatic ductal adenocarcinoma (PDAC) has a fibrotic stroma, known to be acidic. The stroma of the pancreatic ductal adenocarcinoma has a gel-fluid phase full of interstitial fluid, compressing the tumor with a high hydrostatic pressure up to even 100 mm Hg by direct measurement using a transducer in mice model study [41]. Moreover, whereas the normal pancreatic juice is alkaline (to balance the stomach acid), the desmoplastic PDAC microenvironment is found to be acidic with a pH value below 6.5 [42]. High expressions of Piezo1 mRNA have

been found in murine PSCs [43]. Stimulation of the Piezo1 channel led to calcium influx. In the *in vitro* experiment, PSCs were seeded on a matrix resembling the desmoplastic matrix found in the PDAC stroma; application of Piezo1 activator Yoda1 mobilized PSCs to migrate in such matrix (see Figure 3A of [10]).

There is a puzzle on the discovery of low Ph value in the tumor region because PSCs would have proceeded to apoptosis under such acidity. In animal studies, PSCs were found to invade together with the pancreatic cancer cells [10]. Piezo1 channels have been found in the beta cells (in the islets) of the rat's cell lines INS-1 and BRIN-BD11, and the ion channels participate in the insulin secretion mechanism [25].

4.5. On the Efficacy of Gemcitabine Plus Nimotuzumab to Treat PDAC; the Evidence of Phenols and Sulfonamides Having Affinity to Mutated Ras Protein

Aberrant Ras-Raf-Mek signaling plays a role in activating mutations found in pancreatic cancers of different stages/types (70% - 90%) [44]. The Kras (G12D) is a specific variation in the Ras; this variant, with no mutation at amino acid position 12, has a glycine. The mutated Kras G12D protein has an aspartic acid, or D for short in position 12 instead. In order to identify compounds that bind directly to this muted protein, a fragment screen [6] using uniformly ^{15}N labeled GDP-bound Kras (G12D). From the NMR-based screen of 11,000 fragments, the approximately 140 fragments yielded have been identified to bind to GDP-bound Kras (G12D) in the study of [45]. Six small molecules were identified to have an affinity to mutated Ras protein: 1) phenols; 2) sulfonamides; 3) three analogs of 1) and 2) were synthesized (see Figure 1 of [45]).

A randomized study (with 192 patients) was carried out to investigate the efficacy of gemcitabine (gem) plus nimotuzumab (nimo), which is an anti-epidermal growth factor receptor monoclonal antibody, compared with gem plus placebo as first-line therapy in patients with advanced pancreatic cancer. Gemcitabine functions by blocking the creation of new DNA, resulting in cell death, and is a well-known nucleoside analog family of medication. One-year overall survival (OS)/progression-free survival (PFS) was 34%/22% for the combined gem plus nimo compared with 19%/10% for gem plus placebo ($p = 0.02$) [46].

5. CDKN2A, Which Encodes an Essential Cell-Cycle Regulator, Is the Most Frequently Mutated (with Alteration) Tumor Suppressor Gene, with Loss of Function in More Than 90% of Ductal Adenocarcinomas, Particularly in the Early Phases

5.1. The CDKN2A Pathway

The gene CDKN2A has several splicing sites which can generate transcript va-

riants. This gene, which encodes an essential cell-cycle regulator, is the most frequently mutated (with alteration) tumor suppressor gene, with loss of function in more than 90% of ductal adenocarcinomas [47] [48].

CDKN2A encodes the following two tumor suppressor proteins: 1) p16(INK4A) and 2) p14(ARF) proteins. The p16 (INK4A) protein binds to two other proteins CDK4 and CDK6 both of which normally stimulate the cell to continue through the cycle and divide. However, binding of p16 (INK4A) blocks CDK4's or CDK6's ability to stimulate cell cycle progression—p16(INK4A) is thus a tumor suppressor. However, as is noted in [49], long-term expression of p16 (INK4A) pushes cells to enter senescence. The p14(ARF) protein protects the well-known tumor suppressor protein p53 from being broken down; p14(ARF) is also a tumor suppressor. Overexpression of proteins 1) and 2) or the gene CDKN2A have anti-cancer effects.

In the cytosol, p14(ARF) protein inhibits the function of the human double minute 2 (Hdm2) which forms a complex with another protein called Hdmx. This complex inhibits the action of p53, which activates a cyclin-dependent kinase inhibitor 1 (p21), which is a general inhibitor of CDKs, including CDK2, CDK4, and CDK6 [50]. These three CDKs suppress the action of the retinoblastoma protein pRB (encoded by the RB1 gene in mammals), which is the key "check-point" protein for the cell cycle to proceed from the G_1 phase. Now the transcription factor E2F family (such as E2F1) leads to transcription of genes related to the G_1-S phase progression. The pRB protein inhibits the function of E2F. Following through the logic of activation and inhibition, we learn that activation/overexpression of p14(ARF) and p16(INK4A) would result in cell cycle arrest. Thus the two "products" of the gene CDKN2A stops cancer growth. The mutated gene CDKN2A is inactive in its function as the cell cycle check-point [51]. Thus we can summarise the above results and represent a flow chart in **Figure 4** for the action of the anti-cancer gene CDKN2A.

5.2. Methylation of the CDKN2A Gene Is Correlated with a High Risk of Pancreatic Cancer, and Inhibitors of CDK4/6 Are Looked for—We Pay Attention to Abemaciclib

There is another type of mutation that frequently occurs in the pathway discussed. First, we note that DNA methylation is well known by now to be a process by which methyl groups are added to certain sites of the DNA molecule. Methylation can change the activity of that DNA segment without changing the sequence. When methylation occurred in a gene promoter, repression of transcription of that gene occurs. Using meta-analysis methods, based on samples from 45 chronic pancreatic patients, 155 pancreatic intraepithelial neoplasia (PanIN) patients, and 418 pancreatic cancer patients, the result in [52] indicates that methylation of the CDNK2A is correlated with a high risk of pancreatic cancer. The degree of methylation of CDNK2A promoter has been proposed as a prognostic marker of pancreatic carcinogenesis.

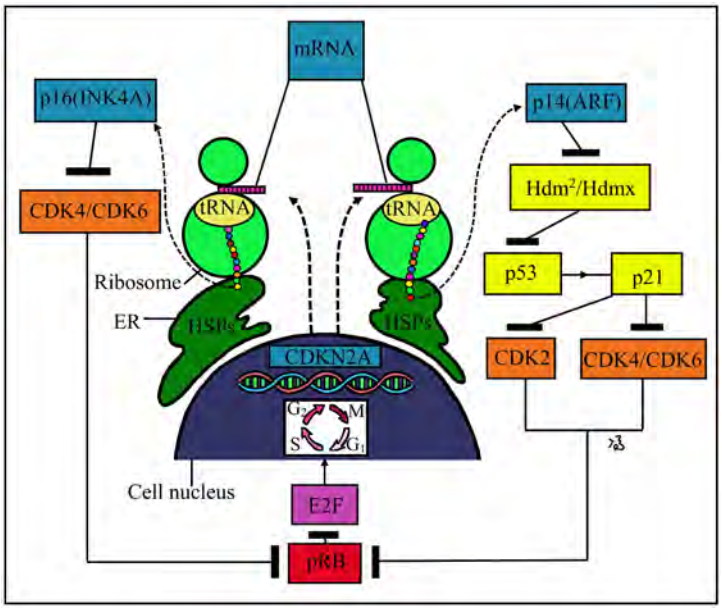

Figure 4. The CDKN2A pathway. The gene CDKN2A can transcript two variants. The messenger RNA of one variant p16(INK4A) synthesizes the protein bearing the same name via the ribosome and the help of transmission RNA (tRNA) as shown on the left side. The product protein inhibits the cyclin-dependent kinases (CDK4 or CDK6) which drives the cell cycle, particularly the G phases. The other variant of CDKN2A leads to the generation of another protein p14(ARF) similarly, as shown on the right side. On the other hand, the Hdm2 forms a complex with Hdmx. This activated complex inhibits the function of p53 (a cancer suppressor) which activates the cyclin-dependent kinase inhibitor 1 (p21). Now p21 inhibits the actions of all CDKs, including CDK2, CDK4, CDK6. These three CDKs suppress the action of protein pRB, the key "check-point" protein for the cell cycle to proceed from the G1 phase. Now the transcription factor E2F is the one related to the G1-S phase progression. Following through the logic of activation and inhibition, we learn that activation/overexpression of p14(ARF) and p16(INK4A) would result in cell cycle arrest. For other details, see text. This figure was painted by author PCWF.

Based on the discussion in the above two subsections, clinicians are looking for suitable CDK4/6 inhibitors to treat a broad band of solid cancers, including pancreatic cancer. Of note, the CDK4/6 inhibitor Abemaciclib has received approval from FDA in 2017 to treat hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative advanced/metastatic breast cancer according to [53]. Last year, the same drug has been applied to treat pancreatic cells with efficacy. Whether this drug can be put on clinical trial, we believe the situation should become clear within one year or two [54].

5.3. Iron Chelators as Potential Anti-Cancer Drugs—The Compound DD44mT, as an Example, Promoted Activity of p21 in the CDKN2A Pathway in Figure 4

We note that more than a decade and a half ago, to look into the mechanism of interaction of Fe chelator with gene expression, gene arrays were used to assess gene expression after incubating cells with two Fe chelators, 1) desferrioxamine

(DFO) and 2) 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (abbreviated as 311) [55]. In that analysis, only the N-myc downstream regulated gene 1 (NDRG1) was specifically up-regulated by Fe chelation. Such up-regulation was mediated by hypoxia-inducible factor-1 (HIF-1)-dependent and -independent mechanisms [55]. Several months ago, the cancer suppressor function mechanism of Ndrg1 has been found to participate in the activation of p21 (effectively upregulation of p53) [56].

On the other hand, over 2 decades ago, detection of the expressions of Ndrg-1 in breast epithelial cells as well as breast cancer cells, it was pointed out in [57] that Ndrg-1 participated in cell cycle progression.

Using two human colorectal cancer cell lines HCT116 p53+/+ & HCT116 p53−/− in the study of [58], a detailed analysis suggested that Ndrg-1 is necessary for p53-dependent apoptosis. Following that, NDRG1 upregulation in pancreatic cancer has been found to be correlated to a significant reduction in primary tumor growth, angiogenesis, and metastasis [59]). In the same year, the Fe chelator, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) with selective antitumor activity was reported to upregulate NDRG1 *in vitro* and *in vivo* in many tumor types, including pancreatic cancers [60].

Later, looking into the mechanism of interaction between NDRG1 and (Dp44mT), The Fe-chelator (DD44mT) hydrochloride was reported in [61] to activate NDRG1 by phosphorylation at the Ser330 and Thr346 sites which are relevant to cancer cell proliferation. Moreover, the Fe chelator increased expression of the cyclin-dependent kinase inhibitor p21 (inhibiting CDK2, see **Figure 4**), whereas decreasing cyclin D1(an effector that regulates cell proliferation by blocking cells at G1 phase) was observed in pancreatic cancer cells. The growth of the pancreatic cancer xenografts was also found to be inhibited by the above Fe chelator, without causing alterations in normal tissue histology [61].

6. New Discoveries of the Hippo Signaling Pathway in Pancreatic Cancer Progression

6.1. Some Basics of the Hippo-YAP Signaling Pathway Relevant to Carcinogenesis of Pancreatic Cancers

Classically, the Hippo pathway is a system of signaling effectors serving as a checkpoint for proper exit from mitosis. The basic function is conserved through evolution, but with some replacements of proteins participating in different species and cell types within the same species [62]. The initial stimulating mechanism is still not fully understood in the human pancreas, but let us introduce some key proteins in the Hippo signaling pathway. The first two key members are: YAP1 (yes-associated protein 1, also known as YAP or YAP65), and TAZ (transcriptional co-activator with a PDZ-binding domain; also known as WW domain-containing transcription regulator 1, or WWTR1). The formal name of the protein encoded by TAZ is Tafazzin, but TAZ is used commonly for both the gene and the protein in literature now. YAP and TAZ do not have DNA-binding

domains, and they are transcriptional coactivators. In normal cells, they are mainly found in the cytoplasm. Upon being translocated into the nucleus, they regulate gene expression through interaction with TEAD1-4 (transcriptional enhanced associate domain protein, with members labelled 1-4), which mediate the main transcriptional output of the Hippo pathway in mammalian cells [63].

In the "un-stimulated state of the Hippo pathway", protein TEAD can also bind to VGLL4 (vestigial-like proteins with four members 1-4) in the nucleus and thus function as transcriptional repressors. VGLL1-4 does not contain a DNA-binding domain and they also impose their transcriptional regulatory functions through binding with TEADs [64]. Though the study pertains to gastric cancer, since the Hippo pathway is conserved in the downstream signaling, such TEAD-VGLL4 interaction holds for a variety of cells. There is no transcription due to the Hippo pathway and the proliferation of the cell is checked at that state (as schematically shown in **Figure 5(a)**), so far as YAP/TAZ is kept in the cytoplasm, because YAP/TZ competes with VGLL to bind TEAD in the nucleus.

On the other hand, under physiological growth conditions, p53 is maintained at a low level by the E3 Ubiquin ligase Mdm2, which together with its family member Mdm4 (in a complex form), mediates partial p53 proteasomal degradation. Some amount of p53 remains in the cytoplasm [65]. Also, the enzyme Large tumor suppressor kinase 1 (LATS1, with LATS2, LATS3, LATS4 in the family) has been found to inhibit both the action of translocation of YAP/TAZ, as well as the action of protein Mdm2 in its negative regulation of p53.

6.2. Agonists of All the Three Proteins—p53, Ptpn14, LATS (Members 1-4), Inhibitors of Furin Are Potent Future Drugs to Treat Pancreatic Cancers Basing Their Inhibition of Translocation of YAP/TAZ to the Nucleus

There are many proteins involved in holding YAP/TAZ in the cytoplasm, depending on cell type, and we shall only bring in the up-stream signaling involving the gene called PTPN14 [66] which encodes the protein called "protein tyrosine phosphatase non-receptor type 14" (Ptpn14). This protein contains an N-terminal noncatalytic domain like that of band 4.1 superfamily cytoskeleton-associated proteins, suggesting that this protein is located in the cytoplasm or cell membrane. In literature, Ptpn14 or PTPN14 has been used to represent this encoded protein.

To briefly describe how p53 would regulate Yap, we need to define the term "TAD", an abbreviation of "topologically associating domain". TAD is a self-interacting genomic region, implying that DNA sequences within a TAD physically interact with each other more frequently than with sequences outside the TAD. There are two TADs found in the gene TP53 [67]. This finding is relevant to the mutations in p53 in the experiment on the analysis of the Hippo pathway specified below.

Note that a knockin mouse is an animal model in which a gene sequence of interest is altered by one-for-one substitution with a transgene, or by adding

gene sequences that are not found within the locus. In a comprehensive study of the Hippo pathway in [68], p53 knockin mouse strains expressing mutants in p53 TADs were prepared according to the technicality described in [69]. Several human and animal (non-pancreatic) cancer cell lines together with human PanIN and PDAC cells, were included in this study. The authors of [68] have found that 1) p53 negatively regulates Yap through Ptpn14 activation; 2) p53-Ptpn14-Yap is a key "tumor suppressive axis"; 3) genes TP53 and PTPN114 mutations are mutually exclusive in human cancer.

Moreover, the ubiquitously expressed serine protease Furin can cleave a plethora (excessive) of proteins at polybasic recognition motifs [70]. Using five human pancreatic cancer cell lines, it has been demonstrated that depletion of the protein Furin would result in a significant reduction of EMT formation, cell proliferation, migration, and invasion of these cancer cells [71]. Thus inhibitors of Furin deserve attention as a therapeutic measure to PDAC.

Summing up the rather sophisticated *in vitro* and *in vivo* analysis (of the mouse model [68]), together with the works of other groups on the Hippo pathway [64] [70] [72], we present a summary in **Figure 5(b)**, the main part of which is based on Figure 7(E) of [68]. In this figure, the Kibra protein is encoded by gene WWC1 and has been inferred to be a negative regulator of the Hippo pathway [73]. In the work of [68] Kibra seems to provide a means of cross-talk between LATS and Ptpn14, both inhibiting the translocation of YAP/TAZ to the nucleus. If either TP53 or PTPN14 is mutated, there is a high chance of translocation of YAP/TAZ to the nucleus. From the clinical point of view, we propose that antagonists of all the three proteins—p53, Ptpn14, LATS (members 1-4), inhibitors of Furin should be considered in further experiments *in vitro* and *in vivo*, for the treatment of pancreatic cancers. The potent inhibitor of Furin, *i.e.*, phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide (15) has been synthesized in the laboratory [74]. The interaction between YAP/TAZ and TEAD1-4 dissociates VGLL4 from TEAD1-4 and thereby activates TEAD-mediated gene transcription to promote tissue growth and inhibit apoptosis.

The c-Jun N-terminal kinases (JNKs) have three members; JNK1 and 2 are found in all tissues so far known, and JNK3 is found in the brain. Using the transgenic mouse model [75], it has been shown that the mere activation of JNK would induce insulin resistance in pancreatic β-cells by inhibition of insulin signaling in these cells, but it is not sufficient to evoke β-cell death. On the other hand, whether JNK plays a role in the carcinogenesis of acinar cells is not clear. In another study reported in [76], using human mammary epithelial cell line MCF10A, when cultured in a soft substrate, protein molecules YAP were mainly located in the cytoplasm, whereas YAP molecules were predominately in the nucleus when the cells were on a stiff substrate. Analyzing the expressions of 1) the proteins JNK, 2) LIMD1(LIM domain-containing protein 1, known to regulate negatively the Hippo signaling pathway and to antagonize phosphorylation of YAP1, 3) LATS1, which take part in the Hippo signaling pathway, it has been

concluded that mechanical regulation alone could translocate YAP to the nucleus via the JNK-mediated inhibition of the Hippo signaling process [76]. In passing, we should note that mechanotransduction has already been reviewed as the upstream triggering signal of the Hippo pathway for cells apart from that of the pancreas [62].

Despite the convincing verification of mechanotransduction being able to trigger translocation of YAP in epithelial and other cells, whether mechanical stimulation at the cell membrane in pancreatic cells is still under research, and we put a question mark in **Figure 5(b)**, a mechanism to be filled in hopefully in the near future.

6.3. Discovery of Synthetic Anti-Cancer Compounds SR1078 That Stabilizes p53 and Naphthofluorescein Disodium Salt That Inhibits the Action of Furin

In 2012, a novel mechanism for the stabilization of p53 protein expression via activation of the orphan nuclear receptor—RORα was introduced [77]. It was demonstrated, employing a human liver cancer cell line HepG2, treatment of synthetic ROR agonist, SR1078, would lead to p53 stabilization and induction of apoptosis. Note that orphan receptor α (RORα) is retinoid-related. Several months ago, gastric cancer cell line MGC803 with RORα-overexpressing and silencing were constructed (for details see Figure S1B, C) of [78]). It has been found that overexpression of RORα could inhibit the proliferation, EMT, and invasion of these cells [78]. It has been pointed out that stimulation of G-protein-coupled receptors by glucagon or epinephrine activates Lats1/2 kinase activity, thereby inhibiting the YAP function [79]. More recently, the drug AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) and its monophosphate derivative ZMP has been shown to be agonists of LAT1,2, producing anticancer effects [80].

Note that an analog of adenosine monophosphate (AMP) can stimulate AMP-dependent protein kinase (AMPK) activity (see **Figure 2**). AICAR has already been used clinically to treat ischemic heart disease [81]. Collagen degradation enzymes and their inhibitors have been implied in carcinogenesis in general. By 2000, however, phase III clinical trials of MT1-MMP inhibitors demonstrated no clinical efficacy in treating cancers (see review of [82]). A revisit to inhibitors of Furin was carried out in 2008 [83]. It has been found that Furin would play role in processing the protease membrane type-1 matrix metalloproteinase (MT1-MMP) and in enhancing cellular motility and invasiveness of COS cell line (fibroblast-like cell lines derived from monkey kidney tissue) and human HT1080 cancer cell line (a fibrosarcoma cell line). A small molecule inhibitor of Furin, which is a naphthofluorescein disodium salt (B3), is a mixture of dihydroxynaphthalene and phthalic anhydride. This salt was prepared and found to inactivate the proprotein convertases of Furins, and lead to anti-invasive effects of the two cell lines stated above [83].

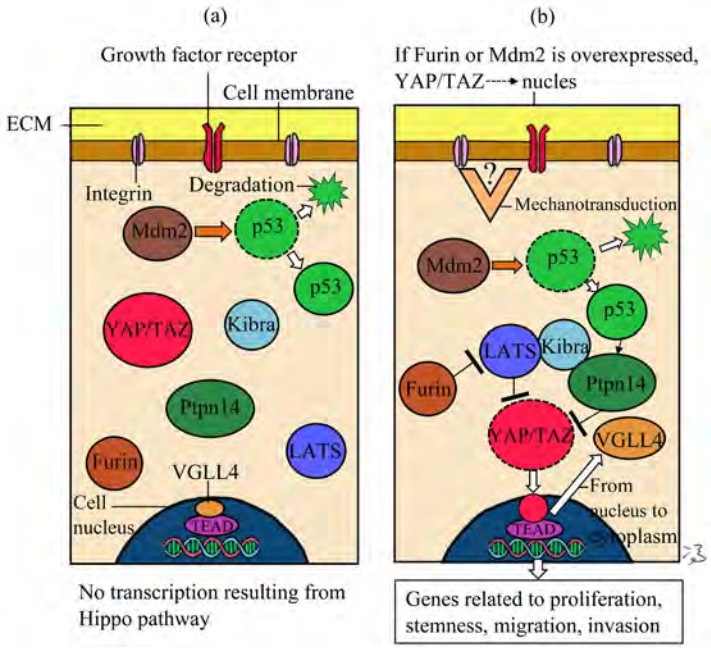

Figure 5. During the normal state, protein Mdm2 induces some of the p53 molecules to go through proteasomal degradation, retaining part of it in the cytoplasm. by proteasomal degradation. VGLL4 does not contain DNA-binding domain, and when it binds with TEAD, there is no transcription resulting from the Hippo pathway as shown schematically in (a). Now p53 protein is a strong activator of Ptpn14, which forms a complex with LATS and Kibra. Both Ptpn14 and LATS are inhibitors of YAP/TAZ (one of the few key proteins in the Hippo pathway), so that YAP/TAZ cannot be translocated to the nucleus. However, if i) p53 is mutated, or ii) there is overexpression of Mdm2, or iii) protein Furin, which is an inhibitor of LATS, is activated, there are situations where YAP/TAZ can no longer be held in the cytoplasm; YAP/TAZ has a strong affinity to the transcriptional factor TEAD and will be translocated to the nucleus, replacing VGLL4, and transcription of genes related to the box below (b) occurs. For pancreatic cancers, mutation of gene TP53 has been found to be correlated to a high percentage of carcinogenesis. Whether the upstream triggering factor arises from mechanotransduction is still under research, but there is evidence that at least for epithelial cells, mechanical stimulation can trigger the translocation of YAP/TAZ via the JNK route. For details, see text. This figure was painted by author PCWF.

7. Over 50% of the Pancreatic Cancer Cells Have Mutation (Loss of Function) of the Gene SMAD4, and It Is Important to Look for Measures to Activate This Gene

7.1. Transforming Growth Factor-β and the SMAD Pathway

The canonical transforming growth factor-Smad (TGF-β-Smad) signaling pathway is initiated by activation of the transforming growth factor receptor (TGF-βR) by its ligand TGF-β, as shown in **Figure 6**. The activated Smad complex is heterotrimeric, composed of two phospho-Smad2 or phospho-Smad3 moieties with a Smad4 molecule to become a site specific transcriptional regulator. Thus, we express the number 2/3 in the "R-Smad pair". This "Smad transcriptional regulator" recruits coactivators or corepressors to regulate target gene expression. For normal cells, the proteins generated via the "canonical

Smad pathway" cause cell cycle arrest, inhibition of immortality, and allow apoptosis. Therefore, activation by TGF-Smad represses carcinogenesis [84]. TGF-β is normally dormant in the ECM and a special mechanism is needed for its release. In this section, we leave out the mechanism on such release, as it will be analyzed in a later section. We start simply by considering the "canonical pathway" initiated by binding of the TGF-β (as a ligand) to its receptor(s). The receptor TGF-β R is built of two dormant units of type I and type II receptors: TGF-β RI and TGF-β RII. A TGF-β ligand "joins" these two units on docking from the extracellular domain, leading to phosphorylation of the type I receptor (**Figure 6(a)**). The combination of (TGF-β with TGF-β RI and TGF-β RII) can be treated as a complex [85].

On the other hand, the protein SarA (or called endosomal protein) interacts directly with the Smad2/3-complex of proteins in the cytosol and recruits it to the activated TGF-β receptor complex stated [86] (**Figure 6(b)**). The complex (Smad2/3) is phosphorylated and leaves the receptor site, attracting the common mediator Smad4 (a well-established process) [87]. The enlarged complex of (Smad4 - Smad2/3) is then translocated to the nucleus; with the help of a transcription factor targeted for a particular gene (plus the help of other supporting proteins), transcription proceeds (**Figure 6(c)**), leading to the synthesis of the associated protein in the cytosol. The protein SarA is degraded, after completing its work. We follow up on the proteins synthesized by this pathway and the effects on carcinogenesis in the next section. Note that TGF-βs signaling regulates the transcription of over 500 genes.

7.2. Evidence of the Anti-Cancer Signature of the TGF-β-Samd4 Signaling Pathway—The Roles of Genes PAI-1, AKAP12, and ITGB6

A large-scale microarray approach has been used [88] with an inducible system to knock down levels of Smad4 in either the human immortalized keratinocyte cell line HaCaT or a pancreatic tumor cell line, Colo-357. Out of the 114 TGF-target genes, 49 were identified as Smad4 dependent and 65 were Smad4 independent. To validate the screen, RT-PCR and RNase protection analysis of representative genes were then carried out and it was reported that at the mRNA level, 1) PAI-1, 2) AKAP12 (protein Akap12), and 3) ITGB6 were confirmed to be Smad4-dependent, TGF-target genes. First, let us analyze the functions of the proteins encoded by these three genes.

Plasminogen activator inhibitor-1 (PAI-1) is one of the key regulators of tumor invasion and metastasis. SW1990, which is a human highly metastasis pancreatic cancer cell line, was transfected with the protein PAI-1(called SW/PAI-1 cell line) in the study of [89]. Cellular invasion, *in vivo* tumorigenesis in xenograft and liver metastasis, were found to be significantly suppressed in SW/PAI-1 cells when compared to control cells. Determination of the microvascular density and the change of expressions in the key angiogenesis factor VEGF as well as TGF-β1, it was concluded that one crucial function of PAI-1,

and hence Smad4, would be suppression of angiogenesis (*i.e.* with anti-cancer action).

The gene AKAP12 encodes protein A-kinase anchor protein 12 (Akap12) which has the function of anchoring protein that mediates the subcellular compartmentation of protein kinase A (PKA) and protein kinase C (PKC). Protein kinase A regulates glycogen, sugar, and lipid metabolism. PKC is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins [90]. In addition to binding PKA and PKC, AKAP12 also binds other signaling mediators such as calmodulin, F-actin, cyclins, Src (a non-receptor protein tyrosine kinase that is involved in the control of cell adhesion, growth, movement, and differentiation). Reduced AKAP12 expression has been consistently observed in liver cancer cell lines [91], and based on its ability to suppress growth rates and promote reorganization of the actin-based cytoskeleton in transformed fibroblasts (as reviewed in [92]), it has been classified as a class II tumor suppressor gene (in general).

With human liver cancer cell lines expressing AKAP12 and/or miR-103 (micro RNA which targeted AKAP12), the anti-cancer nature of AKAP12 was further confirmed in liver cancer. Without detailed data of AKAP12 related to pancreatic cancer at this moment, we accept the microarray analysis (of pancreatic cancer cell line) of [88] and propose that AKAP12 would be an anti-cancer protein depending on the TGF-β-Smad4 pathway in the pancreatic cancer too. The gene ITGB6 encodes the protein integrin $\alpha_v\beta_6$ [93] which takes part in mediating RGD-dependent release of TGF-β1 from the dormant Latency-associated peptide (LAP), thereby initiating the TGF-β1-Smads signaling pathway. Thus, activation of Samd4 leads to the synthesis of proteins that play roles in anti-cancer action. We enter the synthesized proteins in the right-hand lower corner of **Figure 6**.

Over a decade ago, sequencing was carried out in more than 750 million base pairs of DNA from 23, 219 transcripts in a series of adenocarcinomas of the pancreas from 89 patients who underwent pancreaticoduodenectomy. The result indicates that patients with SMAD4 gene inactivation survived a median of 11.5 months, compared with 14.2 months for patients without SMAD4 inactivation [94]. Though the improvement is not large, it supports the notation that inactivation (loss of function) of the gene SMAD4 is a cause of carcinogenesis.

7.3. Attenuation of Cancer Stem Cells' Tumorigenic Ability by TGF-β-Smad4 Signaling

In the study of [95], three human pancreatic cell lines were used: 1) Panc-1, 2) BxPC-3, 3) SUIT-2. RT-PCR analysis revealed that SMAD4 mRNA and Smad4 protein were expressed in 1) & 3). The 4) cell line was constructed by knocking down SMAD4 by shRNA in 1). Cells 1) cultured with shNTC is named cell line 5). Using "RNA knockdown technology", the term shNTC stands for nontarget control (NTC) short hairpin (sh) shRNA. Cells of line 4) were injected into mice

to create the (a) xenograft model group. Cells of line 5) were also injected in the mice model to create the control *in vivo* group, called group (b). The tumors generated in group (a) were found to be larger than those of group (b). It was reported that expressions of cyclin-dependent kinase regulators (such as CDKN1A, CDKN1B, CDKN2A, CDKN2B, MYC) were not different in cells 4) and 5), it was inferred that Smad4 did not affect the cell cycle progression of the cancer cells.

Based on the information that the loss of function of Samd4 occurs in most PDAC, and the established evidence of participation of cancer stem cells in tumorigenesis (evidence will be presented in a later section), authors of [95] looked for markers of the cancer stem cells, though CD44 was known to be one. There was an experiment showing aldehyde dehydrogenase type 1,2,3 (ALDH1,2,3) were also such markers of the stem cells [96]. The isoform ALDH1 was nailed down as more specific and pancreatic cancer cells of the cell lines used were found to contain 3% - 10% of ALDH1+ cells. The regulation abilities of

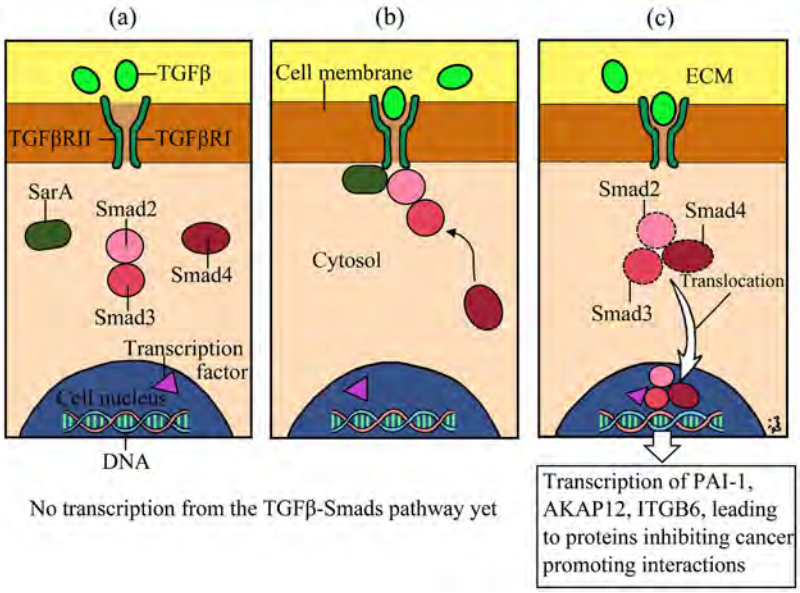

Figure 6. The canonical transforming growth factor-β-Smad pathway. TGF-β (bright green) = transforming growth factor β. TGF-β RI, TGF-β RII (both deep green) = transforming growth factor β receptors type I & II respectively. SarA (greyish) = Smad anchor for receptor activation. The Smads (with 8 members so far known) are a group of intracellular proteins specific for transmitting signals from TGFβ superfamily at the cell surface to the nucleus. When the receptors TGF-β RI, TGF-β RII are activated by TGF-β, protein SarA is recruited from the cytosol, resulting in phosphorylation of first TGF-β RII and then TGF-β RI. The complex Smad2/Samd3 (light and medium red respectively) is also recruited and phosphorylated, attracting the "co-Smad mediator, or Smad4 (deep red)" also, while SarA leaves and be degraded. The bigger complex Smad2/3/4 is translocated to the nucleus. With the help of the specific transcription factor (light purple), transcriptions of specific genes PAI-1, AKAP12, ITGB6 lead to the synthesis of proteins inhibiting cancer-promoting pathways (for details see text). Bone morphogenetic proteins (BMPs) are a group of growth factors that also trigger the TGF-β-Smad pathway, but with other Smad members participating. This figure was painted by author PCWF.

TGF-β and bone morphogenetic protein-4 (BMP-4) on ALDH1 activity were also carried out. The key discoveries were: 1) ALDH1 was enriched only in cancer cells with SAMD4 knockdown. 2) ALDH1 was necessary for the tumorigenic ability of pancreatic cancer cells. 3) TGF-β triggered signaling involving Smad4, attenuated tumorigenic ability of ALDH1(high) cancer cells, and (BMP-4) triggered signaling was much less potent in such attenuation. The overall result is that TGF-Smad4 signaling would suppress cancer stem cells' tumorigenesis.

Note also that activation of the TGF β-1-Smad has cross talks with other cancer-promoting pathways Ras-Raf-Mek-Erk for cell proliferation, and the Rho-Rock pathway (helping Actin chains formation) for migration and invasion, and we will not discuss such interactions in this paper. Information on this, and the last section gives a clear explanation of the reason why the loss of functions Smad4 would promote carcinogenesis, as observed clinically.

8. The EGF-Jak-STAT Signaling Pathway and the Retrograde Transport of EGFR to the Nucleus

8.1. The Epidermal Growth Factor EGF Can Induce Pancreatic Carcinogenesis via the Jak-STAT Pathway

Growth factors are secreted by cells to the interstitial fluid and some are stored in the ECM whereas some find their destinations—their receptors directly. The ones that are in the dormant states in different comparts of the fascia can be released from the ECM by mechanical force/chemical force [2] [37] under specific conditions with an example described in detail in a later section. On reception of these signals (ligands) by the growth factors receptors, specific signaling pathways are in action, some of which have been described in the last few sections. The epidermal growth factor (EGF), the transforming growth factor (TGF) are two key growth factors regulating many cellular functions. For example, through EGF's binding to cell surface receptors, EGF activates an extensive network of signal transduction pathways that include activation of the PI3K/Akt and Ras/Erk pathways, as already described in detail in **Sections (3)** and **(4)**. These pathways predominantly lead to activation or inhibition of transcription factors that regulate expressions related to the synthesis of proteins for proliferation or apoptosis. Off-balance of the expressions of key genes/proteins would lead to pathological state(s). Mutation (aberrant activation) of genes responsible to synthesize growth factor EGF is often detected during carcinogenesis [97].

Four tyrosine kinase transmembrane receptors constitute the EGF receptor family: 1) ErbB1/EGFR, 2) ErbB2/HER2/neu, 3) ErbB3/HER3, and 4) ErbB4/HER4. EGF binds exclusively with ErbB1/EGFR; EGF activates the Janus kinase (Jak 1 or Jak2) of the Jak-STAT signaling pathway. Here Stat stands for signal transducer and activator of transcription protein. The Janus kinase (Jak) is a family of (four) intracellular, nonreceptor tyrosine kinases (Jak1, Jak2, Jak3, Tyk2). Upon IL-6 binding to its receptor called gp130 (a transmembrane glycoprotein), the activated IL-6-gp130 system/complex participates in phosphorylation of Jak protein. The Jak-STAT signaling pathway is therefore also called the IL-6-Jak-STAT

signaling pathway in some literature. For simplicity, we leave out the part played by gp130 and IL-6 in Figure 7 below. We simply consider that two Jak1 proteins are attracted to the (activated) receptor ErbB1, giving up two phosphates (Figure 7(a)). Two "signal transducer and activator of transcription proteins" (called STATs, with label 1 to 6) are attracted to the intracellular part of the receptor, accepting the phosphates (Figure 7(b)).

In doing so, they form a dimer and is translocated to the nucleus after passing through the nuclear pore complex (Figure 7(c)). Again, some factors (represented by one transcription factor, the purple triangle in the figure) together with the STAT1 dimer triggers transcription of genes and eventually synthesis of proteins related to proliferation, cell cycle progression, immunity. Note that a gene called suppressor of cytokine (SOC) is also expressed, leading to the corresponding protein which inhibits the dimerization of the STAT proteins and hence the Jak-STAT pathway, as a negative regulation. In this pathway, the receptor remains in the cell membrane. There is another mechanism where EGFR is translocated to the nucleus in a complicated way; this process is described in the following section.

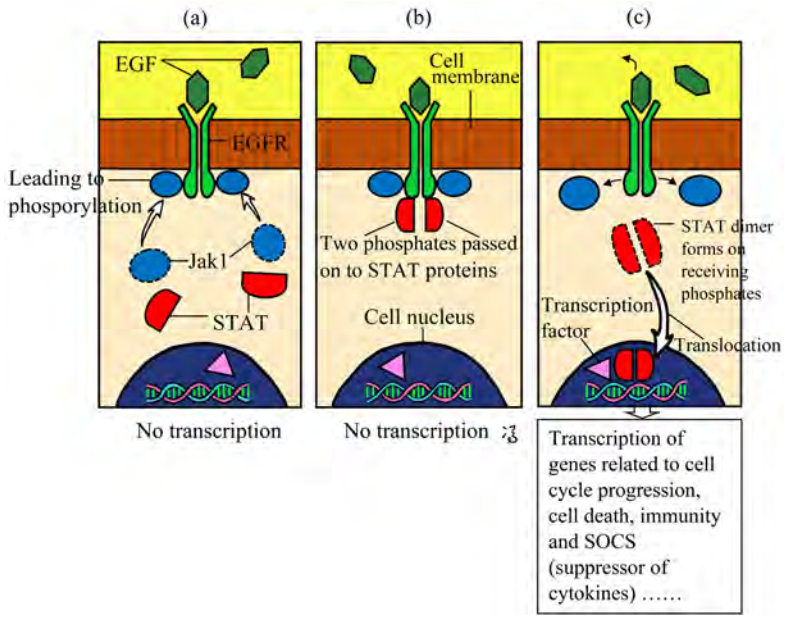

Figure 7. The Jak-STAT pathway was triggered by the epidermal growth factor (EGF). On reception of the EGF, the EGF receptor is phosphorylated, attracting two Jak1 molecules to the internal side of the cell membrane, as shown in (a). Two phosphates are passed on from the receptor complex to two STAT proteins in the cytosol (b) and the STATS form a dimer; such binding allows them to leave the cell membrane domain and the dimer is further translocated to the nucleus, passing through nuclear pore complex, where some proteins participate in helping the transcription of specific genes. (c) The light purple transcription factor represents a group of the stated transcription proteins. Apart from the usual genes leading to synthesis of proteins related to cell growth, differentiation, immunity response, a gene called suppressor of cytokine (SOC) is also expressed, as a (negative) regulator of the dimerization of the STAT proteins. For other details, see text. This figure was painted by author PCWF.

8.2. The Dynein Motor and Retrograde Transport of the EGFR to the Cell Nucleus

In many physiological processes, proteins are synthesized and transported from the ER to the cytoplasm, cell membrane, and some of them might be secreted to the ECM by exocytosis. Such trafficking is called anterograde transport. In contrast, many cell-membrane growth factor receptors (together with the simulating growth factors) have been observed to be translocated to internal departments of the cell first by endocytosis, which is characterized by membrane and vesicular trafficking [98].

Before we describe the mechanism further, we need to introduce the vehicle (like a car, called a molecular motor) which brings the receptor along. Molecular motors are machines that convert free energy, mainly obtained from ATP hydrolysis, into mechanical work. It has been pointed out that the cytoskeletal motor proteins of the myosin and kinesin families, which interact with actin filaments and microtubules, respectively, would lead to intracellular cargo transport [99]. A typical molecular motor is a member of the superfamily of dynein; it is a complex composed of two heavy chains (deep red in **Figure 8**), three intermediate chains (also red), and four light intermediate chains (orange) [100].

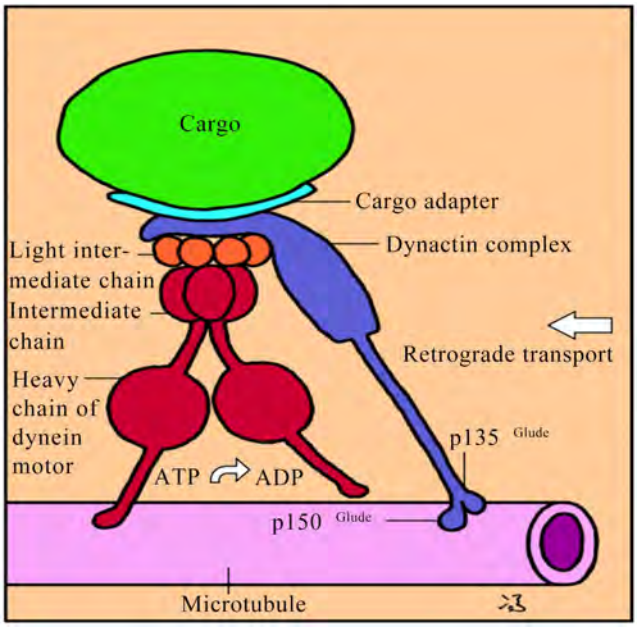

Figure 8. The dynein motor protein is built of two heavy chains, three intermediate chains and four light intermediate chains. The dynactin complex (bluish purple) has 10 subunits as described in the text. The part of the dynactin attached to the dynein protein also attracts a suitable cargo adapter protein (light blue) for a specific cargo (green), such as an epidermal growth factor receptor set. While ATP is converted into ADP in the dynein motor, energy released is converted to mechanical work to transport the motor set with the cargo towards the nucleus direction. The light purple tube is a simplified form of a microtubule. There are many microtubules to keep the integrity of the cell shape. Such a transport towards the nucleus is called retrograde transport in the cytoplasm. This figure was painted by author PCWF.

Each of the two heavy chains has an extension with a binding site attached to a microtubule. To transport a cargo, each dynein must be associated with another complex called dynactin (bluish-purple). The dynactin complex has ten subunits-p150^{Glued}, p135^{Glued}, p.62, dynemitin (p50), actin capping protein β subunit, p27, and p24. The heterodimer formed by (p150^{Glued}-p135^{Glued}) is a side-arm of the dynactin complex, ending with two spherical-shaped heads each of which contains a microtubule binding site [100]. This complex is painted with a simplified structure. The dish-like part (light blue) attached to the "top part" of the dynactin complex is an adapter protein for a specific cargo (green), such as an epidermal growth factor receptor set (EGF plus EGFR). While ATP is converted into ADP in the dynein motor, the energy released is converted to mechanical work to transport the motor set with the cargo towards the nucleus direction.

Microtubules are microscopic hollow tubes made of the protein α and β tubulin (around 24 nm in diameter), forming a network of protein filaments that extends throughout the cell, maintaining the cell shape. They have roles in cell movement, cell division, and intracellular trafficking of proteins/peptides. During the past several years, there have been compiling evidence of such "on the micro-tubule" transport of various types of cargoes (such as vesicles containing proteins, membrane receptors, mitochondrion), both away from the nucleus (anterograde transport) and towards the nucleus (retrograde transport).

8.3. Retrograde Transport of EGFR in Action

During the recent several years, we begin to understand more about the retrograde transport of cell membrane receptor. Now refer to **Figure 9** on retrograde transport of the EGFR set. At site (1), the epidermal growth factor (EGF, dull green) docks at its cell membrane receptor EGFR (also green, but brighter). Upon activation by EGF, the receptor together with the growth factor can be engulfed by the cell by the common endocytosis process (as represented by position (2) in **Figure 9**).

Using Hela cells, after endocytosis, the EGFR-set enclosed by the vesicle, has been found to be carried by the dynein motor protein (with Dynactin and cargo adapter) on route to the Golgi apparatus along a microtubule, as demonstrated by immune-microscopy investigation with gold particles, as well as RNA detection in the Golgi in the investigation of [98]). This part of the transport is represented by site (3), where the dynactin and cargo adapter protein have been neglected, and the motor protein dynein is represented by a figure simplified from **Figure 8**. The EGFR-set is enclosed by a vesicle (the brown circle in the figure). Reaching the membrane of a Golgi apparatus, a protein called soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), member syntaxin6 functions to transport the vesicle through the Golgi membrane by a fusion process [101]. The fusion process is also included in site (3) in the figure. The EGFR-set inside the Golgi apparatus, via a process still not fully understood, is transported to some part near a microtubule, as sche-

matically represented by site (4).

After transporting inside the Golgi apparatus, it has been found to exit to the cytoplasm with a "coat protein complex I" (COPI) vesicle, which consists of a small GTPase ADP-ribosylation factor (ARF) and a "coatomer" (which is simply a protein complex that coats membrane-bound transport vesicles). A coatomer has seven subunits (α, β, β', γ, δ, ε, and ζ) [102]. Using MDA-MB-468 cells (derived from a breast cancer patient), the ARF protein with γ-coatomer called γ-COP has been identified by immunoblotting and revelation by confocal microscopy [103].

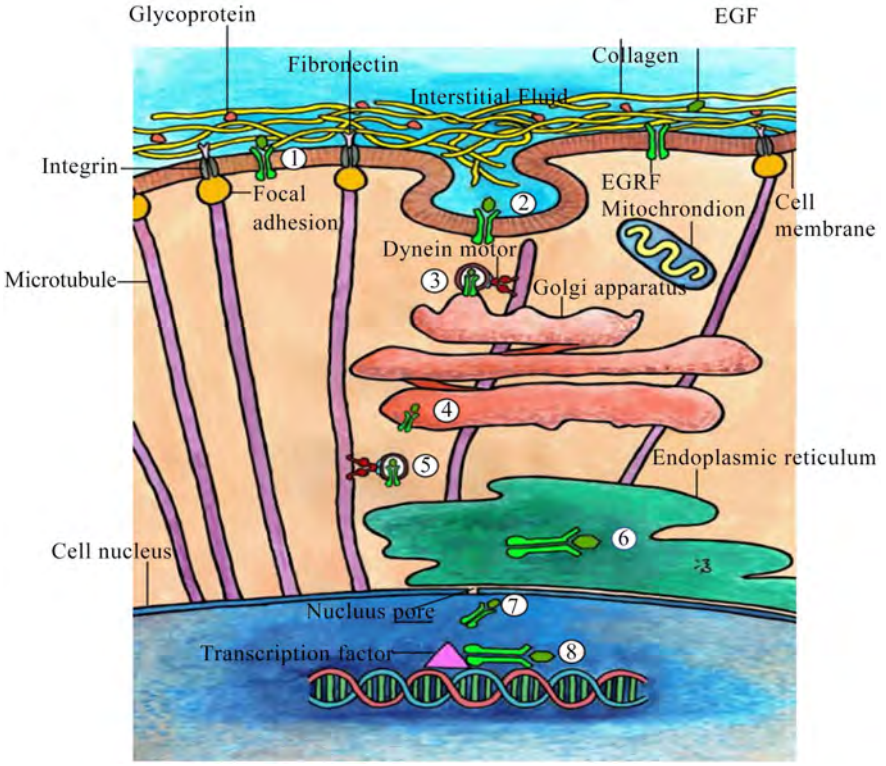

Figure 9. The retrograde transport of EGFR is set to the nucleus. At site (1), the epidermal grow factor (dull green) docks at the membrane receptor EGFR (bright green). At site (2), endocytosis encloses the EGFR-set in a vesicle (boundary was painted in brown). At (3), the dynein motor (red), with the help of the dynactin complex (omitted in this figure, see details in text and **Figure 8**) and the cargo adaptor (light blue) carries this cargo along a microtubule track to the Golgi apparatus. The fusion of the cargo to the Golgi apparatus is governed by a protein called "syntaxin6". The EGFR-set is transported inside the Golgi apparatus to a site near a microtubule (4). Another dynein motor carries the cargo, now enclosed by a vesicle bounded by a "coat protein complex I" (COPI) (painted in deep brown) to the endoplasmic reticulum (ER) as indicated at (5). The cargo fuses through a protein-conducting channel at the membrane of the ER, into ER (6). The cargo is translocated through a pore/slit at the nuclear external membrane, into the nucleus (7). The purple triangle represents a group of proteins participating in choosing the gene(s) specific to the EGFR-set for transcription (8). There is evidence that the EGFR-set can be translocated to the mitochondrion also. Microtubules are generated from focal adhesion complexes attached to integrins. See text for other details. This figure was hand-painted by author PCWF.

Since EGFR sets have been found in the nucleus of a large number of cancer cell lines, it has been proposed already in 2010 [103] that EGFR is further translocated from the ER via the nuclear pore complex to the nucleus by the dynein motor with the cargo enclosed in one type of COPI vesicle. We accept this model and consider that the EGFR-set is enclosed in one type of COPI vesicle (such as γ-COP, painted in deep brown) and is transported also by the dynein motor from the Golgi to the endoplasmic reticulum (ER) in the pancreatic cells.

Following up on the picture of retrograde transport, site (5) represents the process of a dynein motor carrying a COPI vesicle with the EGF-set from the Golgi to ER. The Golgi apparatus was painted in red with shades. There are protein-conducting channels (PCC) at the external "boundary" of ER. The action of the secretory complex called Sec complex (which has subunits α, β, γ) at the PCC would allow the entrance of both hydrophilic and hydrophobic domains of the molecules entering ER. It has been explained that Sec61 protein would allow translocation of membrane insertion [104] and we consider the mechanism is relevant to the translocation of the EGFR-set into the ER in **Figure 9**. Now the EGFR-set is inside the ER (*i.e.* Site (6)). The EGFR-set is translocated eventually from ER to the nucleus (site (7)) via a nucleus pore complex (represented by the slit of the membrane of the cell nucleus). Together with a group of proteins helping to transcribe specific genes, the EGFR-set works at site (8). The anterograde and retrograde transports are well-established evolutionary conserved processes for numerous physiological processes of growth. This retrograde transport has been observed in many cell types [98] and some of the processes have been verified for the pancreatic cancer cells [98] [103] [105]. The fact that the EGFR-set has been found in pancreatic cancer cells supports the notion that the retrograde transport occurs in pancreatic cells also. Microtubules are generated from focal adhesion complexes attached to integrins.

8.4. Evidence That EGFR Is a Transcription Factor of the Cyclin D1 Gene, Shortening the G1 Phase of Cell Cycle

Based on the discussion of the last section, we consider that apart from the classical effects of transcription by the STAT dimer (**Figure 7**) upon activation of EGF, the EGFR-set can be translocated directly from the cell membrane to the nucleus, leading to the transcription of genes responding to the stimulus by EGF. This result provides additional weighting of the influence of EGF on cell growth, differentiation, etc. related to carcinogenesis. On the positive side, as the retrograde transport necessitates the help of proteins such as COPI, dynein motor, dynactin protein, cargo adapter protein, there are more choices of developing inhibitors to stop the retrograde transport of EGF.

Note that long ago, EGFR was already detected in the nuclei of pancreatic cancer cells [106]. It has been pointed out that targeting nuclear EGFR is a crucial step in treating cancer in general in the study of [107]. This important aspect will be followed up in a later section. Note that microtubules form a dense net-

work in most cells. In β cells of the pancreas, they could originate from the Golgi and form a non-radial network. In other cells, they could originate from one side of the cell nucleus and terminate at an integrin-associated protein, which can be taken as part of the focal adhesion [108]. In **Figure 9**, part of the orientation of the microtubules follow this pattern, and with one terminal connected to a focal adhesion.

EGFR contains a transactivation domain, associating with genes and it can activate sequence-specific gene expression [109]. Using specimens of uterus from pregnant mice, mouse embryos, normal human mouth mucosa, and human cancer tissues labeled EGFR was correlated with highly proliferative activity of cells. EGFR was found to bind and activate AT-rich consensus-sequence-dependent transcription, including the consensus site in cyclin D1 promoter, implying EGFR would be a transcription factor of the cyclin D1 gene, overexpression of which was well established to shorten the G1 phase in cell cycle, leading to proliferation at too fast a speed.

8.5. On Antibody-Drug Conjugate (ADC) Drugs Targeting Epidermal Growth Factor Receptor (EGFR), Taking a Potential Compound RC68 as a New Example

Up to the time of writing, four drugs targeting EGFR have been approved by the US FDA to treat other types of cancers (colorectal, esophagus, and lung cancers): 1) cetuximab, 2) panitumumab, 3) nimotuzumab, and 4) necitumumab [110] [111] [112] [113]. We would rather discuss very briefly the working principle of a new class of potential anti-pancreatic-cancer drugs (relevant to targeting EGFR) with one recent example, following the retrograde transport in **Figure 9**, though the potential drug is not even under clinical trial, because we think that targeting EGF/EGFR is particularly important for a number of cancers, including the pancreatic ones.

An antibody-drug conjugate is a substance made up of a monoclonal antibody chemically linked to a drug that is toxic when the ingredient is released inside the target cell. The monoclonal antibody binds to specific proteins or receptors specific for certain cell type. Drugs with such monoclonal attachment/linkage are called ADCs. Last year, a humanized anti-EGFR monoclonal antibody (RC68) was generated by mouse immunization, and the associated technology is called "complementary-determining region grafting" as described in [114]. The highest level of EGFR expression was revealed (after immunofluorescence staining) on the cells of the human pancreatic cancer cell line BXPC-3, whereas the lowest level of EGFR expression was observed on HEK-293 human embryonic kidney cell line HEK-293 (see (Figure 1a) of [114]).

Immunofluorescence staining also indicated that RC68 was able to bind to 99.9% of membrane EGFR of BXPC-3 cells. Internalization of RC68-Based ADCs via Endocytosis was observed, indicating that a process such as that shown in **Figure 9** was responsible for the internalization. The ADCs showed

greater cytotoxicity in BXPC-3 cells (up to 70%) than in another human pancreatic PANC-1 cell line (with cytotoxicity ~40%) (see Figure 5 of [114]), and this difference was interpreted to be due to the difference in EGFR expression level between these two cell lines.

A nude mouse-human pancreatic BXPC-3 xenograft model was used to test the efficacy of this potential drug, with positive result (see Figure 7 of [114]). Note that the BxPC-3 is a human pancreatic cell line for investigating the efficacy of gemcitabine or small molecule tyrosine kinase inhibitors (TKIs), such as erlotinib. This cell line is often used to create the BxPC-3 xenograft mouse model.

9. The Canonical, Non-Canonical NF-κB Action Pathways and Pathogenesis to Pancreatic Cancinogenesis

9.1. The Canonical and Non-Canonical NF-κB Action Pathways

Members of the Nuclear Factor-kappa B (NF-κB) family are transcription factors that regulate the expression of a wide range of genes for inflammatory and immunity responses, cell survival, and cell proliferation. Referring to **Figure 10**, in the first step, when a cytokine receptor registers a ligand (cytokines like tumor necrosis factor-α (TNF-α), interleukin), a signal is sent to a protein complex (called IκB kinase, or IKK) which consists of two kinases (IKKα and IKKβ) and a regulatory subunit named NF-κB essential modulator (NEMO, or IKKγ). Now the NF-κB transcription factor family in mammals consists of five proteins, 1) NF-κB1 (also called p50), 2) NF-κB2 (also called p52 and its precursor is p100), 3) RelA (alias p65, v-rel reticuloendotheliosis viral oncogene homolog A (avian)), 4) RelB, 5) c-Rel. These five members associate with each other to form distinct transcriptionally active homo- and heterodimeric complexes. The "canonical NF-κB members" predominantly form dimers of (p50/RelA) and (p50/c-Rel) [115] [116]. In the dormant state, the dimer (take, for example, a combination of NF-κB1 and RelA), binds to the inhibitor IκBα. The activated IKK phosphorylates IκBα, which is then degraded in the proteasome. Concurrently, the dimer (NF-κB1 and RelA) is translocated to the nucleus and binds to the response element of a specific sequence of the DNA, leading to transcription of the gene into mRNAs. The corresponding protein is then synthesized in the usual way.

For the non-canonical NF-κB pathway, proteins Traf2, Traf3 are attached to the non-canonical receptor at the cell membrane. These two proteins, together with some other supporting proteins form a complex; this complex causes continual degradation of the NF-κB-inducing kinase (NIK), whose activity triggers off the non-canonical pathway. There are several receptors of the non-canonical pathway. We just take one example here. The Lymphotoxin β receptor (LTβR), is a cell surface receptor for lymphotoxin involved in apoptosis and cytokine release [117]. Here Lymphotoxin is a member of the cytokines, whose members are responsible for regulating the function of and cell growth, and are expressed

by a wide variety of cells [118]. When receptor LTβR is docked by the ligand Lymphotoxin, via the action of the Traf2-Traf3 complex, NIK is not degraded and NIK phosphorylates the dimer IKK*α*, as indicated schematically on the right side of Figure 10. IKKα, in turn, phosphorylates the transcription factor dimer formed by NF-κB plus RelB (precursor of NF-κB2(p100)). The dimer (RelB and NF-κB2(p52)) is translocated to the nucleus. As the two NFκB pathways occur in most cells [116], we show the typical products of proteins synthesized in the middle of Figure 10 according to references [116] [119].

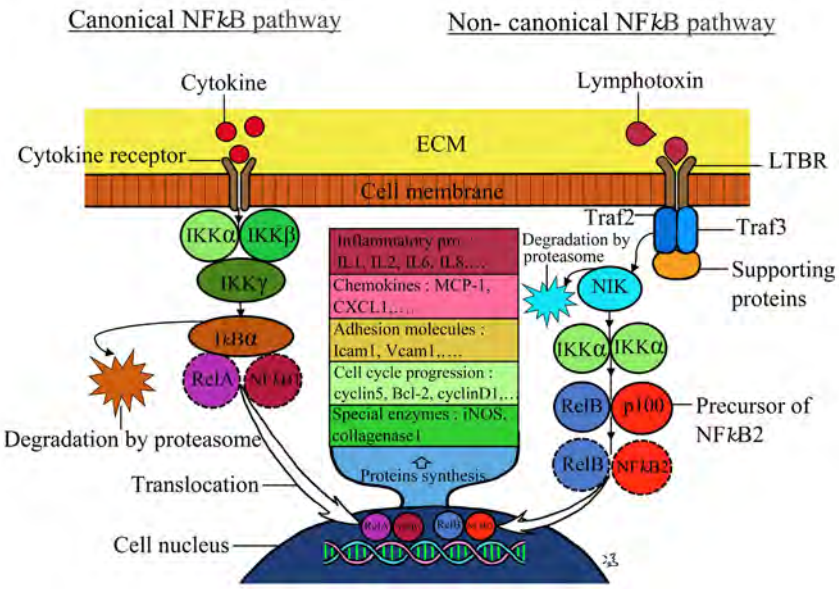

Figure 10. The canonical (left side) and non-canonical (right side) NFκB pathways. IκB kinase α, β, γ = IKKα, IKKβ, IKKγ respectively. Pro. = proteins. IL = interleukin, MCP-1 = monocyte chemoattractant protein 1, CXCL1 = chemokine C-X-C motif ligand-1, Icam1 = (Inter) cellular adhesion protein 1. Vcam1 = vascular cell adhesion protein 1. cyclin D1 = a protein that helps control cell division. Bcl-2 = B-cell lymphoma 2. iNOS = inducible nitric oxide synthase. Collagenase1 degrades collagens of certain types. IκB*α* = inhibitory κ protein type α. NFκB1, 2 = NF-κB transcription factor type1, 2 respectively. NIK = NF-κB-inducing kinase. LT-βR = Lymphotoxin receptor type β. RelA = v-rel reticuloendotheliosis viral oncogene homolog A (avian), is also called p65. RelB = v-rel reticuloendotheliosis viral oncogene homolog B (avian). NFκB1,2 are two members of the NF-κB transcription factor family. p100 is the precursor of NF-κB2. The cytokines include interleukins, TNF-α. For other details, see text. This figure was painted by author PCWF.

9.2. Proteins Relating to Inflammation, Cell Adhesion, Cell Cycle Progression, Collagen Remodeling and Immunity Are Synthesized in Response to Activation of NF-κB

Many proteins can be synthesized by these canonical or non-canonical pathways, even with one type of stimulation. For example, for inflammatory response, interleukins IL-1α, IL2, IL6, IL8, Tumour Necrosis Factor α (TNFα, an inflammatory cytokine produced by macrophages/monocytes), cyclooxygenase-2 (COX-2), an enzyme involved in the process of inflammation, can be

generated [116].

The chemokines monocyte chemoattractant protein 1 (MCP-1), chemokine C-X-C motif-ligand 1 (CXCL1) are produced for recruiting monocyte, neutrophil and other immunity cells during angiogenesis and inflammation. Adhesion molecules such as ICAM1(Intercellular adhesion protein 1) [120], vascular cell adhesion protein 1 (VCAM1) [121], which regulate the vascular permeability, allowing leukocytes transmigration to the ECM for inflammatory action, are also synthesized. There are also proteins related to stress response, such as angiotensin II, a vessel constrictor. Anti-apoptotic factors, such as the B-cell lymphoma 2 (Bcl-2), which is a key member of regulator proteins that regulate apoptosis, is another stress protein. Cyclin D1(a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6) is a protein that helps control cell division (see **Section (V)** on CDNK2A). Transforming growth factor-α is a member of the epidermal growth factor (EGF) family, being a mitogenic polypeptide; it belongs to the group of cell cycle regulation [122]. The Systemic enzyme inducible nitric oxide synthase iNOS (one of the three isoforms of nitric oxide) produces a large amount of nitric oxide during immunity response. Nitric oxide combines with ROS superoxide to become peroxynitrite $(ONOO)^-$ which is detrimental to cell death (of the health cells). Collagenase1 digests collagen during ECM remodeling. All these proteins/peptides (as listed in the middle part of **Figure 10**, can be generated via the two NF-κB pathways [116].

9.3. Involvement of the NF-κB Pathway in the Pathogenesis of Pancreatitis, Angiogenesis, and Pancreatic Ductal Cancer

Using peripheral blood mononuclear cells isolated from patients' blood, it has been found by measurement [123] that Both NF-κB and heat shock factor-1(HSF-1) would be elevated systemically in patients suffering from acute pancreatitis. Based on the finding that HSF-1 is inversely correlated with the Acute Physiology Score (APS) and Acute Physiologic Assessment and Chronic Health Evaluation II (APACHE II) score, it has been inferred that HSF-1 activation could protect the organ against the severity of pancreatitis. In fact, the mechanism of the protective effects of heat shock proteins for general cancer has been analyzed in our previous work, and we agree with this proposal; for details, see [3].

Using genetically engineered mouse models, it has been demonstrated [124] that a pathway linking dual forward loops of IL-1α /p62 through which IKK β/NF-κB is activated by $Kras^{G12D}$ [125]. Here p62 (SQSTM1) is a multifunctional stress-inducible scaffold protein involved in diverse cellular processes. Noting that mutational activation of KRAS has been detected in 80% - 95% of PDAC by the year 2000 [126] and activation of NF-κB in the epithelial cells of the pancreas leads to the synthesis of IL-1α (also called hematopoietin 1) and other inflammatory cytokines, together with antiapoptotic proteins; such result implies that pancreatitis and then pancreatic carcinogenesis could be sustained, once initiated.

In 2009, two pancreatic cancer cell lines BxPC-3 and SW 1990, human umbilical vein endothelial cells (HUVECs) were used, and cell invasion, angiogenesis assays were applied to study pancreatic EMT [127]. It was found that IL-1α mRNA levels in the cancer cells were significantly higher than the control. Vessel tubes formation and migration of the HUVECs were observed when co-cultivated with the cancer cells.

In another investigation, the human pancreatic cell lines PANC-1, BxPC were employed. It was demonstrated that with overexpression of the hypoxia-inducible factor-1α (HIF-1α) (**Figure 2** and **Section (III.2)**), the cells underwent EMT in an NF-κB–dependent manner [128]. It was also noted that inhibition of NF-κB activity would reverse the EMT phenotype. Putting the results reviewed in **Sections (9.1-9.3)** above suggests that there are interactions between the NF-κB pathways and the HIF-1α-VEGF pathway (in **Figure 2**), leading to angiogenesis and concurrent EMT progression towards carcinogenesis, with NF-κB playing a significant role. Looking for inhibitors of NF-κB is desirable.

9.4. Searching for Inhibitors of HIF-1α and NF-κB—APX3330, SLC-0111, Agonist of IL-10

So far, 16 carbonic anhydrases (Cas), which are expressed in human tissues, interact with HIF-1 [129] [130]. Among these 16 anhydrases, CA9 has been shown to be driven by HIF-1 activity. On the other hand, HIF-1 transcriptional activity is enhanced by Apurinic/Apyrimidinic Endonuclease-1-Reduction/oxidation Effector Factor 1(APE1/Ref-1) [131]. The small molecule APX3330 is a potent inhibitor of APE1/Ref-1. This potential drug is about to proceed to Phase II trial. APX3330 has been found to regulate activity not only of HIF-1 α, but also NF-κB and Jak-STAT3 (see **Figure 7**) [132]. Another molecule called SLC-0111, which inhibits the action of CA9 at the cell membrane, is under preclinical experimentation [133].

More than 2 decades ago, a synthetic interleukin-10 agonist IT 9302 was found to reduce the mortality rate (from 60% to 0%) in rabbit models of acute necrotizing pancreatitis. It was suggested that the mechanism of action could be due to inhibition of circulating levels of TNF-α, IL8, which could be generated via the NF-κB pathway just discussed [134].

10. The Hedgehog Pathway and Its Involvement in Human Primary Pancreatic Adenocarcinomas

10.1. The Hedgehog Pathway and the Proteins Synthesized upon Activation

The Hedgehog signaling pathway is a crucial regulator of proliferation and differentiation during embryonic development. The two key transmembrane proteins are: 1) Protein patched homolog 1 (Patch 1) is a member of the Patch protein family; so far two members are found, and Patch 1 is relevant to our discussion. 2) Smoothened protein (Smo). In the quiescent state Patch 1 inhibits the action of Smo. The suppressor of fused homolog (Sufu) forms a complex with

the Gli1 protein in the cytosol. The Gli1 protein contains five successive repeats of a zinc finger which can bind to DNA for transcription [135]. There are five members in the Gli family. The quiescent state is represented schematically by the left side of Figure 11.

We note that three hedgehog ligands have been identified—Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh); these ligands could send signals to receiving cells at a distance greater than cell sizes involved [136]. When a hedgehog ligand (such as Shh) binds to a domain receptor of the Patch protein, the inhibition of the Patch protein on Smo is relieved. The activated Smo binds with Sufu, which then releases the transcriptional factor Zinc finger protein (Gli1), followed by translocation of Gli1 to the cell nucleus, inducing transcription of the following proteins [137]: 1) myc, a proto-oncogene which encodes a nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation; 2) cyc D1, a cyclin that functions as a regulator of a cyclin-dependent kinase (CDK) involved in regulating transcription, mRNA processing, and the differentiation of nerve cells; 3) Patch 1, to replace the degraded one after enzymatic action; 4) Bcl-2 (or B-cell lymphoma 2) is the key member of the Bcl family of regulator proteins that regulate apoptosis; 5) Sox2 (or sex determining region Y-box 2), is a transcription factor essential for maintaining self-renewal stem cells. We thus see that dysregulation of the hedgehog pathway may readily lead to the formation of cancer cells, based on the functions of the proteins produced in response to the activation of a receptor of a hedgehog ligand.

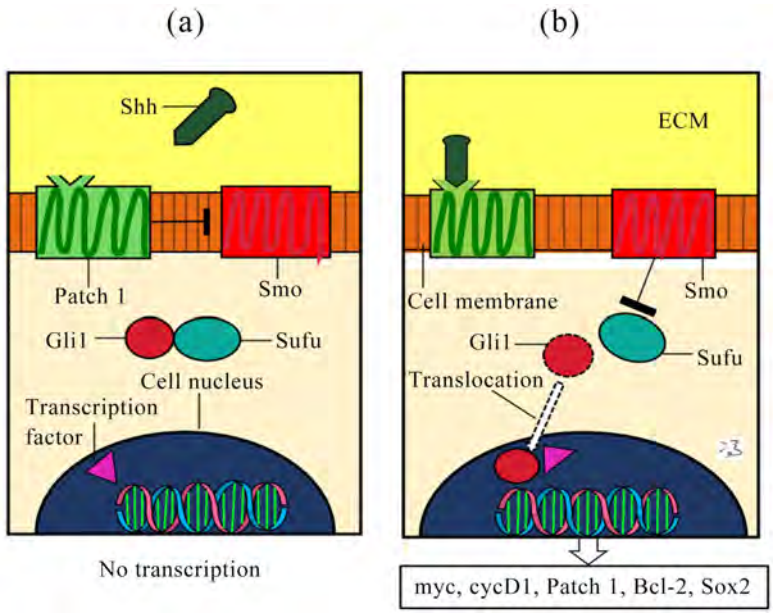

Figure 11. Sufu locks to Gli1 in the quiescent state. When a Hedgehog ligand, such as Shh binds to Patch1, the transmembrane Smo is activated, which then binds to Sufu, releasing the transcriptional factor to the nucleus, leading to transcription of genes (then synthesis of the proteins) as marked at the lower right corner. For other details, see text. This figure was painted by author PCWF.

10.2. Evidence of the Involvement of the Hedgehog Pathway in Progression to Human Primary Pancreatic Adenocarcinomas

It has been reported that the hedgehog pathway can be aberrantly activated in many solid tumors, such as skin cancer [138], prostate cancer [139], medulloblastoma [140], colorectal cancer [141], ovarian cancer [142], pancreatic cancers [143]. A number of inhibitors of the active proteins of the hedgehog pathway have been attempted in *in vitro* and some clinical trials with some success—showing signs of efficacy; the results, in general, are not promising (see e.g. the negative result of using Vismodegib to treat colorectal cancers according to the study of [141]). Since the environment of different cancers can be significantly different, despite the controversial results; even with one positive result, it is worth investigating the mechanism of the successful case.

In the study of [136], the expressions of protein members involved in the hedgehog pathway (particularly the ligand sonic hedgehog (Shh) and receptor Smoothened (Smo)) were analyzed in 1) pancreatic cancer and 2) pancreatitis specimens. Technically, the human pancreatic Nestin-expressing cell line (HPNE) was used as a control cell line; these cells were derived from normal pancreatic tissue and immortalized (with telomerase) using hTERT (a catalytic subunit of the enzyme telomerase, which, together with the telomerase RNA component (TERC), comprises the most important unit of the telomerase complex. The technicality of preparation is described in [144]). Two more human immortalized control fibroblasts cultures (SC2 and SC3) were also established from non-malignant pancreatic tissues. SC2 was derived from an intraductal papillary mucinous neoplasm (IPMN) of the pancreas, and SC3 was derived from resected chronic pancreatitis tissue. Gene expression profiling analyses of 1) the human pancreatic cancer-associated fibroblasts (CAFs or named stellate cells in this paper) and 2) non-neoplastic pancreatic fibroblasts were carried out. Total RNA was isolated from 1) and 2) cell cultures using a commercial miRNA kit.

The expression of the SMO protein was examined by immunohistochemical labeling of eight tissue microarrays containing a total of 156 different surgically resected pancreatic ductal adenocarcinoma. IPMNs, and chronic pancreatitis tissues (*i.e.* SC3) were constructed according to the method of [145].

Analyzing the stated specimens, the findings are: 1) SMO is upregulated in human pancreatic activated stellate cells. 2) Note that Zinc finger protein Gli1 is a transcriptional activator and is found to mediate Shh signaling [135]. The Shh ligand has been found to induce expression of Gli1 mRNA in pancreatic stellate cells. 3) siRNA knockdown of SMO expression blocks GLI1 induction in pancreatic stellate cells. 4) Hedgehog pathway has been confirmed active in pancreatic cancer associated stromal cells *in vivo* using mouse xenografts [136].

10.3. Combination of Hedgehog Inhibitor (Cyclopamine) and Chemotherapy Drug Paclitaxel Delivered by Nanoparticles Demonstrated Efficacy in Treating Patient-Derived Xenograft Model

One major hurdle to clinically treat PDAC, as compared with other solid cancers, is the existence of an extensive desmoplastic stroma, which is a fibrotic deposition produced by stellate cells in the organ [146]. In the study of [147], in order to overcome the stated hurdle, a sonic hedgehog inhibitor, cyclopamine (CPA), and a cytotoxic chemotherapy drug paclitaxel (PTX) with a polymeric micelle formulation (called M-CPA/PTX) was prepared as a drug to treat cancer models. CPA is supposed to deplete the stroma-producing stellate cells (or cancer-associated fibroblasts (CAFs)), while PTX has been known to have efficacy to inhibit tumor proliferation (acinar cells). The stellate cells have been tested to be α-SMA positive (see a later section to describe the stellate cells).

Three (1 - 3) orthotopic xenograft models were used in this investigation on efficacy and related mechanism: 1) Human pancreatic cancer cells MiaPaca-2 were injected into the pancreases of the mouse models to prepare the orthotopic xenografts. 2) Patient-derived xenograft model, which is similar to 1) but preserving many features of tumor heterogeneity and introducing human leukocyte populations [148]. 3) The KPC models, where alteration of the genes KRAS, TP53, Cre are genetically engineered; here Cre is a special tool gene that is used to control where KRAS and TP53 are turned on (see details of the mechanism in [149]). In the three groups 1) - 3) stated above in the investigation of [147], different combinations of treatment settings were adopted: a) untreated (control); treated with b) M-CPA; c) M-PTX; d) M-CPA/PTX. M-CPA/PTX nanoparticles were delivered to the tumor site by blood circulation. The size of the nanoparticles is <150 nm. Due to the natural leakiness of the intratumoral vasculature, and based on the analysis of this issue reported in ref. [150], there was a high chance of M-CPA/PTX being extravasated into the interstitial fluid of the ECM. It is anticipated that both CPA and PTX were released to affect the behavior of the stellate and tumor cells. Since the tumor sizes of the models strank back about to normal after such treatment (Figure 6 of [147]), the combined effects of M-CPA/PTX must have worked to eliminate the proliferating cells. There was evidence that the micro-vessel density was increased, hypoxia was alleviated, matrix stiffness was reduced; there was indirect evidence that the tumor-restraining function of the extracellular matrix was maintained. Based on the variation of the protein expression of the α-SMA of the stellate cells, there was indication that the vicious cycle of mechanotransduction interaction between the stellate and tumor cells was disrupted (detail will be described in a later section). A model of the efficacy is presented in Figure 7 of [147]. We wish to point out that the activation of at least some stellate cells seems to be closely related to the sonic hedgehog pathway; other growth factors/stimulations can activate a stellate cell also.

11. The Importance of Stopping Tumorigenesis at the Early Stage by Inhibiting the Notch Signaling Pathway

11.1. The Notch Signaling Pathway between Adjacent Cells and EMT, Tumorigenesis

The Notch signaling pathway is a highly conserved cell signaling pathway, restricted to neighboring cells in all Mammals [151] [152]. Notch has four different notch receptors (each is a single-pass transmembrane protein): Notch1, Notch2, Notch3, Notch4, [153]. Notch family members control cell fate decisions during development; for that reason, the signal of proliferation can be transmitted to adjacent cells. There are five functional ligands, JAG1, JAG2, DLL1, DLL3, and DLL4 discovered so far known. Notch signaling has also been found to be dysregulated in some cancers, implying such signaling is important in developing anti-cancer target therapy (see e.g. [154]).

In **Figure 12**, we label the Ligand and receptor as Jag and Notch respectively as an example (leaving out the member number). Both the receptor and ligand have their main physical parts in the extracellular domain of the adjacent cells. Up to now, we only know that the receptors tend to be cell-shape dependent (preferably located at the tip) for the ligand-receptor docking in some animals [155]. We can only start from the pathway after the docking process. Stimulated by a signal from the ligand in the signal sending cell (cell 1), two proteolytic enzymes are recruited in the internal side of the membrane of signal receiving cell (cell 2): 1) ADAM10/TACE (of the metalloprotease family); 2) γ-secretase. The bonding causes a conformational change in the extracellular juxtamembrane region of the receptor Notch, rendering Notch to be susceptible to successive cleavages by ADAM metalloproteases and γ-secretase (see Figure 1 of [151]). It has also been inferred that the triggering force is mechanical, arising from ligand endocytosis [156].

After the stated cleavage processes, the intracellular domain of the Notch receptor (called Nicd) is translocated to the nucleus. There the DNA binding protein/(transcriptional regulator) called CSL (or CBF-1) and co-repressor CoR binds to the DNA, keeping DNA in a quiescent state. Nicd enters the nucleus and activates CSL, recruiting the co-activator (CoA), mastermind-like 1 (MAML1) plus others to form a transcriptional activator complex. This complex causes transcription of certain genes, leading to the synthesis of the corresponding proteins. Relevant to pancreatic cancer, they are [157]: 1) C-myc, a proto-oncogene and encodes a nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis, and cellular transformation. This gene is pleiotropic, but the amplification of this gene is commonly detected in numerous human cancers. 2) cyclin D1, a cyclin functions as a regulator of a Cyclin-dependent kinase (CDK) which is involved in regulating transcription, mRNA processing, and the differentiation of nerve cells. Cyclin D1 forms a complex with CDK4 or CDK6, whose activity is required for cell cycle G1/S transition (see **Figure 4**). Overexpression of cyclin D1 is found in many cancers. 3) Transcription factor Hes1 (hairy and en-

hancer of split-1) is encoded by the gene bearing the same name. There are 7 members in the family (Hes1-7). HES genes code nuclear proteins that suppress transcription [158]. 4) The Hairy/enhancer-of-split related with YRPW motif protein 1 (or in short, Hey1) is a transcriptional repressor protein [159]. 5) Cyclooxygenase 2 (COX 2), is an enzyme responsible for the synthesis of prostaglandins which promote inflammation, pain, and fever. 6) Vascular endothelial growth factor (VEGF), as a protein, participates in the growth of (blood) vessels (see **Figure 2**). 7) The enzymes matrix metalloproteinases (MMPs) are involved in the breakdown of collagen in the extracellular matrix, during embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration [2]. The MMPs are necessary for numerous physiological processes. MMP 9 is more specific to the degradation of the basal membrane, relevant to the invasion of cancer cells. Looking down the list 1)-7), the proteins resulting from the Notch signaling is involved in daily physiological processes, including wound healing. However, from the high correlation of over-expression of Notch and the occurrence of tumors, obviously, the quantities of these proteins are not balanced during EMT, to tumorigenesis.

Going back to the Notch signaling process. The Ligand left in cell 1 actually participates in another set of processes, sending a signal back to the nucleus of the signal-sending cell. To simplify matters, we leave the readers to the description of this part of the Notch process to [160].

11.2. The Non-Canonical Notch Pathway and Mirco-Tumorigenesis by Stem Cells; Inhibitors of the Notch Pathway as a Potential Drug—γ Secretase Inhibitor R04929097 Plus Gemcitabine Was under Phase II Trial by 2014

In general, when the signaling process is limited to adjacent cells, it is called canonical. Using actin-based cellular projections, which include the Notch ligands, to deliver activating signals to a Notch process at distant sites is called non-canonical, as demonstrated with the Drosophila model [161]. Malfunctional or dysregulated Notch signal is related to abnormal growth and can be associated to the development of micro-tumorigenesis, in our view.

This cell-cell communication is inferred to occur during the development of the EMT and tumorigenesis states of the acinar cells, which connect to form a ring shape structure and the "outlet" of the ring simply joins the duct cells of the pancreatic duct.

Tissues of primary human pancreatic tumors of 8 patients were used to prepare the (mice) xenografts in the study of [162]. Triple-marker profile CD44+/CD24+/ESA+ (ESA is an epithelial surface antigen) was used to isolate cancer stem cells from cancer cells from the xenograft tissues [163]. Some expression levels of Notch signaling pathway components in cancer stem cells were compared to that of the bulk tumor cells. The gamma secretase inhibitor R04929097, plus gemcitabine were found to have anti-cancer effects to suppress the degree of

carcinogenesis. Note that such a combination was taken to phase I trial for the treatment of advanced solid tumors excluding pancreatic cancer in 2012 [164]. The expression levels of three proteins/RNA, *i.e.* Hes1, Nicd (detected by cleaved Notch), and actin were used as markers since their expressions were more than 1.5 folds compared to the primary tumor. shRNA Hes1 was applied to confirm the result.

In the resectable tumorigenic cases, the high occurrence frequency of distant relapse following successful surgery suggests the spread of cancer cells before surgery. Identification of the activation of the Notch pathway in the pancreatic cancer stem cells, it is thus plausible to treat patients with drugs targeting Notch right at the beginning of the discovery of the disease. Note that the gamma secretase inhibitor R04929097 was taken as the drug for phase II trial in patients previously treated metastatic pancreatic adenocarcinoma by 2014 [165].

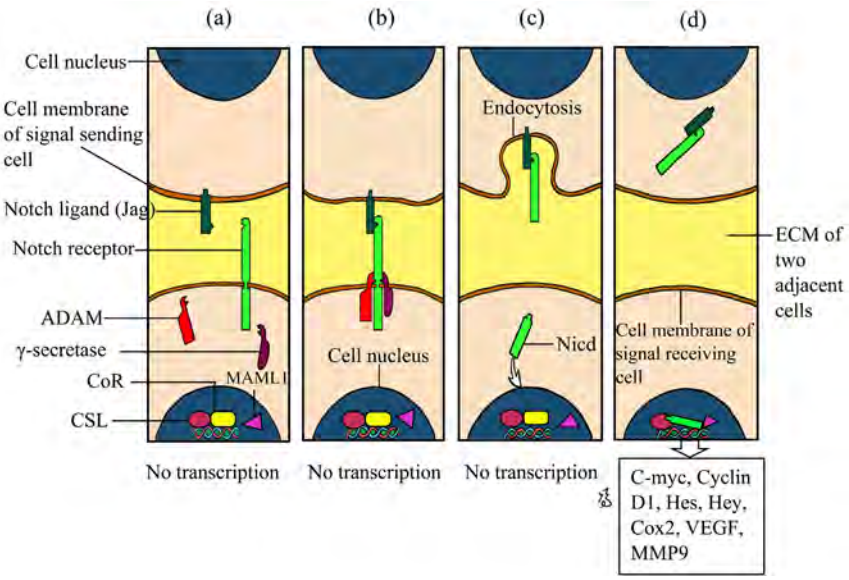

Figure 12. The canonical Notch pathway is restricted to neighboring cells, with parts of the nuclei shown in this figure. Jagged1 (encoded by JAG1) is one of the five cell surface proteins (ligands) that interact with 4 Notch receptors in the mammals. The DNA binding protein called CSL (light red) and co-repressor CoR (yellow) binds to the DNA, keeping it in a quiescent state. The mastermind-like 1 (MAML1, in purple) is also in the dormant state. There is no proliferation resulting in this pathway in (a)-(c). Here Jag1 was painted in deep green and Notch1 in light green. The two cleavage enzymes ADAM (red) and γ-secretase (brown) are inactive when the pathway is not turned on. When Jag1 binds Notch1, signals are sent to the stated enzymes and they begin to cleave the Notch1 receptor into two parts. The intracellular domain of the Notch receptor (called Nicd, light green) is translocated to the nucleus, binding CSL and MAML1. While Jag1 with the extracellular part of Notch1 protein is engulfed by endocytosis in the signal sending cell, transcriptions of C-myc, cyclin D1, Hes, Hey, Cox2, VEGF, and MMP9 are carried out in the nucleus of the receiving cell. See text for the functions of these 7 proteins. The number 1 has been omitted in the key members for simplicity, as other members give similar results in different cells. The extracellular matrix of these two adjacent cells is represented by a yellow background, which contains many constituents as described in Figure 2 of [166].

12. One Major Factor of the Progression of Pancreatic Ductal Cancer: Transformation of the Quiescent Stellate Cells to the Myofibroblast Type Cells

12.1. A Quiescent Stellate Cell Has Numerous ATR Drops in the Cytoplasm

Pancreatic stellate cells (PSCs), amounting to 4% - 7% of the mass of the gland [11], are found to be adjacent to the basolateral aspects of pancreatic acinar cells and around pancreatic ducts and blood vessels [12]. PSCs have been detected around islets too [167].

Based on staining examination for the marker desmin (encoded by DES), which is a cytoskeletal protein, a quiescent PSC appears as an elongated cell, with a central cell body, extending along the base of adjacent acinar cells. Quiescent PSCs have the capability to store retinoids (with their analogs) inside fluid droplets [168]. Retinoid and its metabolites have been shown to inhibit the expression of α-SMA and to decrease activation of relevant signaling pathways involving invasion and cross-talks with the acinar cells [169].

The quiescent cells proliferate at very slow rates and migrate only within the parenchymal tissues of the pancreas. However, when they lose their lipid droplets and begin to show α-SMA tubule structures, they begin to transit to the myofibroblast-like phenotype [170], in response to stimuli like growth factors, inflammatory signals, to be specified in a later section. In **Figure 3**, the proteins forming the focal adhesion painted are Talin, Fak, Paxillin, Zyxin, YASP, α-actinin, and "attachment protein" Vinculin. In a quiescent stellate cell, the focal adhesion is not formed. In **Figure 13** only three separate proteins such as

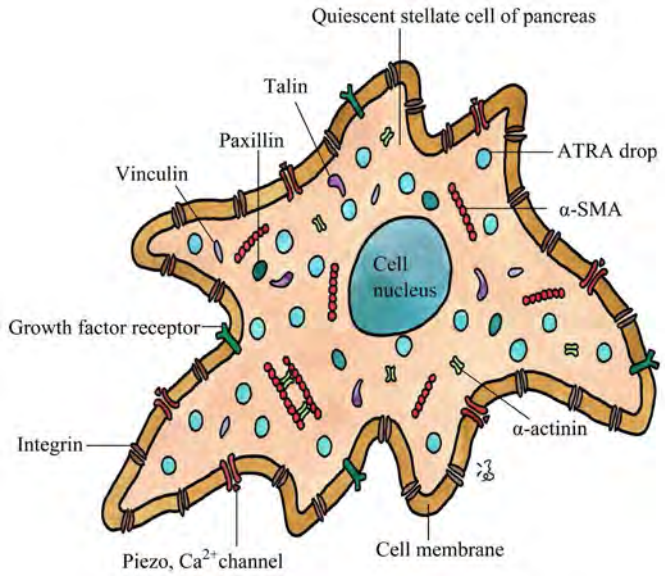

Figure 13. The quiescent state of a stellate cell of the pancreas. There are many ATRA (oil) drops (blue) in the cytoplasm. Paxillin (green), talin (purple), vinculin (light purple) represent some of the proteins forming the focal adhesion and joining (via vinculin) the actin chains, which are built of α-SMA and α-actinin. The green Y-shape proteins are growth factor receptors. This figure was hand-painted by author PCWF.

Talin (purple), Paxillin (green), Vinculin (light purple) in the cytoplasm have been painted to represent such individuals of proteins. The actin chains are formed by α-actin (red small circles), and "glued" by α-actinin (light green), but the chains are not formed yet without the action of Vinculin. The light blue circles are ATRA drops that have been clearly revealed in experiments in the stellate cells (see Figure 2 of [168]).

Integrins (brown), each built of one α unit and one β unit, are transmembrane proteins. Without mechanical (or some chemical) stimulation, they do not act in any manner. The green Y-shape structure represents a growth factor receptor (such as TGF, EGF). There are calcium ion channels Piezo (mainly class1) in the cell membrane too. A "stopper" closes the entrance of extracellular calcium in each one without mechanical traction from the integrin/collagen fibrils of the basal membrane.

12.2. Activation Mechanism of a Stellate Cell by Transforming Growth Factor TGFβ and the Occurrence of Fibrosis around the Activated Stellate Cell

When a pancreatic stellate cell is deprived of vitamin A droplet, a stellate cell can readily be activated. In this section we consider one triggering mechanism to be TGFβ activation as an example.

We first note that in the quiescent state the TGFβ (bright green) binds to the protein LAP (deep blue) and LTBP-1(black), forming a complex (sites (1), (2) of **Figure 14**). LTBP-1 has a strong affinity to fibrillin (orange), a glycoprotein in the ECM, particularly associated with elastin (see Section (1) of [2]). Using lung fibroblasts and vascular smooth muscle cells, it has been found that one end of LTBP-1 protein is attached to fibrillin in the ECM [171]. The other end of LTBP-1 has an affinity to fibronectin fibril (light purple), also a glycoprotein, which is readily attached to unit β (the slightly longer unit of the integrin, whereas the other unit is called α) [171]. The contraction of the cell membrane of a stellate cell would have a larger chance of attracting the LTBP-1 complex. However, at the normal state, the TGFβ-LAP-LTBP-1 complex stays dormant.

Many cytokines would cause the integrin to contract, and TGFβ itself is one of them. A pull from collagen fibers at a distance can also transmit a force to the integrin, and the reaction is the formation of a focal adhesion complex (as represented by the blue ellipsoid here) by the connection of paxillin, talin, and other proteins (already described in detail in **Section 9.1** with **Figure 3**). The focal adhesion complex also causes the α-SMA fibrils (small red circles) to be lined up, with proteins such as α-actinin (green clip-like structure) as "glue". Since the α-SMA fibrils can contract as muscles do, the morphology of the cell can be changed so that the cell can crawl along the collagen fibrils/fibers (yellow) in the ECM according to the rules of durotaxis and chemotaxis (see [2] [172]). As the cell starts to exert a contract force on the ECM, the cell membrane contract via the action of the integrins. At site (3), a TGFβ-LAP-LTBP-1 complex is attracted to an integrin. Further contraction of the cell membrane would "pull"

the LAP protein, releasing TGFβ as shown in the site (4). On the other hand, the contraction force of integrins can also open the newly discovered calcium ion channel Piezo1 (deep red). The entry of Ca^{2+} from an extracellular source (indicated by the dotted arrow on the right side of the site (5)), would trigger off the Rho-Rock pathway leading to the lining up of more α-SMA fibrils, shown in **Figure 13** and **Figure 3**. Both the contraction of the integrin and entry of Ca^{2+} would lead to the formation of focal adhesion.

Now the free TGFβ has an affinity to its own receptor TGFβR, as in site (6). The receptor of TGFβ is in the active state, initiating more activity of the Rho-Rock signaling pathway. Some α-SMA fibrils line up to join the nucleus, initiating the transcription (and hence protein synthesis) of the digestive enzyme MMPs (plus also their regulators, metalloproteinases inhibitor (TIMPs)), represented by the fan-like structure painted in red on one side and white on the other). This is a natural response because the enzymes must be secreted to the ECM to digest the collagen fibrils to leave room for the cell to migrate. The cell is now a myofibroblast, with the intention to do wound healing work. Therefore, procollagen (triple helix in the upper part of the cell (for details see [2]) must be synthesized also. These procollagen molecules are also secreted to form collagen molecules proper and then collagen fibrils. When there are more collagen fibrils/fibers in the ECM, the mechanical tension on the cell membrane is automatically greater. The integrins sense such tension, and the α-SMA reacts more strongly than before. We see that a slight imbalance of the above complicated processes would lead to a vicious cycle, with more accumulation of collagen and a stronger contraction of the stellate cells. The activated stellate cells are communicating with the acinar cells, and β cells of the islets and an epithelial-mesenchymal-transition (EMT) has occurred.

Using lung fibroblasts, and vascular smooth muscle cells, it has been found that LTBP-1 protein is attached to fibrillins in the ECM, whereas to release TGF-β from the latent complex, the LTBP-1 needs to bind to fibronectin also. Note that fibrillin is a glycoprotein being attached to microfibrils associated with elastin in the ECM around the pancreas. Fibronectin is well established as a glycoprotein also, and it has a high affinity to integrins. Although the cells used in the experiments of [171] were not pancreas cells, yet the TGF activation is an evolution conserved process, and we take the above statement as the model to describe the release of TGF in the ECM of pancreatic cells. The stated connection of LTBP-1 is supported by research of other groups (see e.g. [173]).

When a PSC changes from the quiescent to the activated myofibroblast phenotype, the following events occur: 1) loss of ATRA lipid droplets; 2) excessive production of collagen; 3) excessive production of proteoglycans and glycol-proteins—in particular, hyaluronan which is electrically polarised to attract water to form a large volume of think interstitial fluid [37] [166]; 4) overproduction of inhibitors of matrix metalloproteinases (TIMPs) so that the accumulated collagen is not digested; 5) overexpression of HSP47 in PSC. Note that in the

event 1) ATRA keeps PSC quiescent by regulating the target genes by binding to the associated nuclear receptor (for details see [174]). There is evidence that overexpression of HSP47, which is specific in folding the native collagen in the ER [3], is implied in PDAC fibrosis [175].

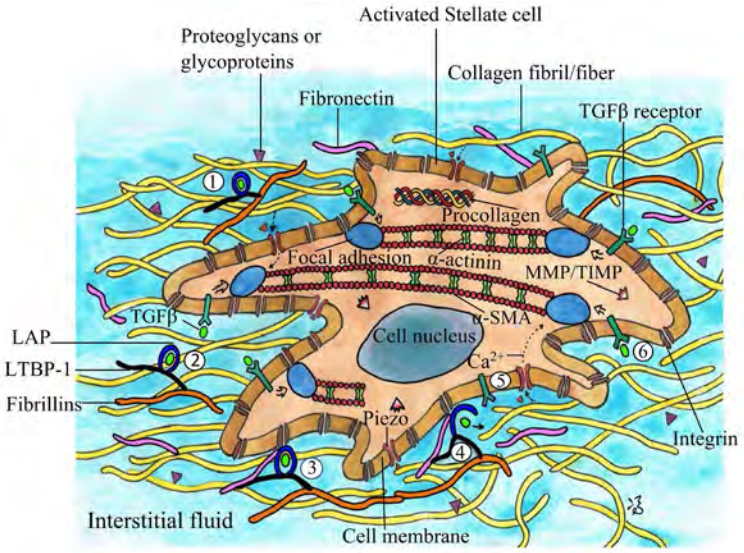

Figure 14. The transforming growth factor TGF-β (bright green) is enclosed by protein LAP and LTBP-1 in the dormant state, with one end of LTBP-1 attached to a glycoprotein fibrillin, as shown in sites (1) and (2). The glycoprotein fibronectin (purple) has affinities to the β unit of integrin at one end and LTBP-1 at the other end (3). Stiff ECM and traction of the stellate cell both give a high chance of docking at (3). Further traction of the cell releases TGF-β (4). The free TGF-β is attracted by its own receptor at the site (6), as an example (Y-shape structure, green). On the other hand, traction of the cell membrane by integrins causes the ion channel Piezo 1 to open its "cover" (5). Both Ca^{2+} and activated TGF-βR triggers the formation of focal adhesion (greyish-blue ellipsoid) and lining up of the actin chains (red small circles with *α*-actinin). The triple-helix is a procollagen which will be secreted to the ECM, to become collagen fibril (see [2]). MMP is an enzyme to digest collagen and TIMP is a regulator of MMP; both are secreted to the ECM. Some purple triangles represent the proteoglycans and other glycoproteins in the ECM. An activated stellate cell becomes a myofibroblast; it migrates and invades with the cancer cells of the pancreas. This figure was hand-painted by author PCWF.

12.3. Factors Triggering the Transformation from Quiescent PSCs to Myofibroblast-Like PSCs

1) Transforming growth factor

Using rat models of fibrosis and surgical specimens from patients with chronic pancreatitis, the crucial expressions of proteins (such as α-SMA, TGF-β1) were determined in both human tissues (from necessary surgery operations) and rat specimens [176]. Pancreatic stellate cells were found to be the main source of collagen I (adjacent to the injured acinar cells) in pancreatic fibrosis in both human tissue specimens and that of experimental rat model. There was also evidence that TGF-β1 activation of stellate cells would lead to the excessive expression of procollagen alpha$_1$(I) mRNA [176]. More details have been included in

the previous subsection.

2) Connective tissue growth factor

Connective tissue growth factor (CTGF, also called CCN2) is a member of the CCN family of extracellular matrix-associated heparin-binding proteins. CTGF has important roles in many biological processes, including cell adhesion, migration, proliferation, angiogenesis, skeletal development, tissue wound repair, fibrosis, and pathogenesis of several types of cancers [177]. It is known that CCN is produced at the injury site by acinar or pancreatic stellate cells, which were inferred to include a major portion of dormant PSC plus some activated PSCs also [177]. In a mouse model of alcoholic chronic pancreatitis, CTGF has been found to be secreted by activated pancreatic stellate cells [178]. There is evidence that CTGF can be activated by TGF-β. Downregulation of CTGF by the miRNA method (described in a later section) led to a decrease of activated PSC proliferation also [179].

3) Platelet-derived growth factor (PDGF)

PDGF (having 5 members in the family) is secreted by the inflammatory cells and has been shown to stimulate the activation of PSCs [180] [181]. Two PDGF receptors α and β have been found in many cell types. *In vitro* experiments using PSCs of the rat provides evidence that the activation of PDGF receptors α and β by the associated growth factors would activate the PI3K-Erk pathway (see **Figure 2** and **Figure 3**), suggesting PDGF to be a potent mitogen for PSCs [179].

4) Interleukin-10 (IL-10)

This cytokine, which is secreted by Th2 cells, is anti-inflammatory and anti-fibrotic; it has been found that this cytokine can reach the PSCs sites. It is also found that IL-10 takes part in downregulating procollagen-1 but upregulating collagenase gene expression; the consequence is the downregulation of fibrosis [182]. IL-10 can block NF-κB activity [183], and is involved in the regulation of the JAK-STAT signaling pathway [184]. Over 2 decades ago, a synthetic interleukin-10 agonist was found to partly relieve acute lung injury in rabbit models with acute necrotizing pancreatitis [Osman 1998].

5) External pressure applied on PSCs induces generation of ROS, driving these cells towards the state of inducing fibrosis

The pancreas of the rat model was digested with a mixture of collagenase, and pronase which is a commercially available mixture of proteases isolated from the extracellular fluid of Streptomyces griseus [185].

The isolated PSCs were cultured and subjected to a quantifiable pressure-loading apparatus. PSCs were cultured (A) with or (B) without antioxidants (green tea polyphenol epigallocatechin gallate (EGCG) and N-acetyl cysteine) under (C) normal or (D) under high pressure of 80 mmHg. The common oxidants generated "internally" under chronic inflammation/injury are superoxide(O_2^-), hydrogen peroxide (H_2O_2), oxygen molecules in the excited state; in the worst condition is a large amount of nitric oxide generated by the inducible enzyme iNOS [26] which can bind superoxide to form the cytotoxic pe-

roxy-nitrite $(ONOO)^-$. Superoxide dismutase (SOD) and catalase are synthesized intracellularly to scavenge superoxide and H_2O_2 in the healthy state. During inflammation, endogenous antioxidants are deficient and a measure of the typical ROS level (s) is a good indication of the degree of injury roughly in general.

The main findings of [185] were: 1) Application of 80 mmHg pressure for 1 hr reduced SOD activity to 86.4% of control; at 2 hr after releasing the external pressure, SOD activity was restored to 93% of control. 2) Using hydroxyphenyl fluorescein (HPF) as a detector of intracellular ROS, PSCs showed ROS even 30s after application of the stated amount of pressure. The number of HPF labeled PSCs reached a sustained maximum around 1 hr after pressure application. 3) Application of pressure led to a significant increase in gene expressions of TGF-β, α-SMA, and procollagen type I. 4) PSC cultured with antioxidant EGCG one hour before application of pressure practically nullified the increase in gene expressions stated in 3).

13. Cells in the Islets Can Also Be Cancerous

The Angiogenetic Switch of the Islets

Both cells in the acinar cells and cells in the islet can be cancerous. The following series of diagrams in **Figure 15** indicates schematically the progression of the β and the α cells of islet during four stages of carcinogenesis according to the 4-stage model of [27], while the acinar/ductal cells also progress along the process of tumorigenesis. Some of the β (bright green) and α cells (light brown) in the normal islet cells have undergone dysplasia to part (b), called hyperplastic islet, with a slight change in colors of the cells in our presentation.

Angiogenesis begins at stage (c), where cross-sections of aberrant blood vessels are grown. The transition of the stage (b) to stage (c) is called the "angiogenic switch"—this phenomenon is intuitively true because a tumor grows must be accompanied by angiogenesis. These new blood vessels supply oxygen and nutrients for further proliferation of cells in the islet, forming a large tumor with many other pathological islets in the environments (stage (d)). The α cells (secreting glucagon, some of them were painted in deeper red) were found to be colocalized with some proteoglycans, and become cancerous, a phenomenon discovered over half a century [186]. Whether the rates of cancer progression of islet cells and acinar/ductal cells are the same has not been investigated in detail, yet angiogenesis is common for all types of cancers. Since islet cells constitute only ~5% of the organ mass (**Section (II)**), we infer that the islet cells progress with that of the acinar/ductal cells and are embedded within a lump of a tumor at the fourth stage of **Figure 15**, surrounded by a large stroma which contains a large amount of collagen together with their associates—proteoglycans and glycoproteins. The few δ cells (yellow), which supply somatostatin are assumed to remain normal here, as their population is only a few % of the islet in mass and we are not aware of concrete experimental work on their way to carcinogenesis.

The very few γ cells, which secrete pancreatic peptide, are not shown here. Clinically, when the β cells are progressing along a pathological path, diabetic symptoms would emerge.

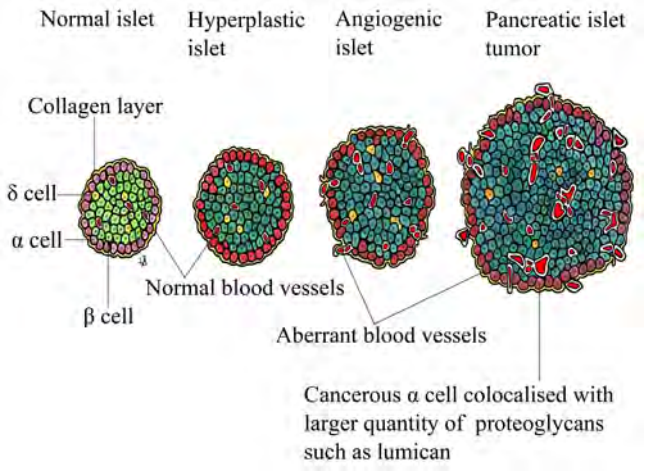

Figure 15. Progression of Angiogenic switch carcinogenesis of islet cells in the pancreas. We adapt the four-stage model of [27], with a hand-painted figure, during the progressing from a normal islet to a cancerous islet. The normal β cells (bright green) and α cells (light brown) have changed to the "hyperplastic state" in (b) already, with β cells in deeper green and α cells in bright red. The islet in stage (b) may be called or hyperplastic. Angiogenesis begins at stage (c), where cross-sections of aberrant blood vessels are grown. A large tumor is formed in stage (d), where the α cells were found to be colocalized with some proteoglycans. The α cells, which secret glucagon, were found over half a century ago capable to become cancerous also [186], and some of them were painted in deeper red at the cancerous stage. The few δ cells (yellow), which supply somatostatin are assumed to remain normal here, as their population is only a few % of the islet and we are not aware of concrete experimental work on their way to carcinogenesis. When the cancer cells begin to invade, the boundary layer of collagen (yellow) is degraded. The very few γ cells, which secrete pancreatic peptide, are not shown here. The figure was hand-painted by author PCWF.

14. The Small Population of Stem-Like Cells in the Pancreas Could Well Be the Earliest Metastatic Cells

Participation of the Stem-Like Cells in Pancreatic Cancer Formation and Metastasis in an Early Phase

Note that aldehyde dehydrogenase (ALDH1) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, has been identified as markers of stem cells of pancreatic adenocarcinomas [187]. A small population of human mammary cells was found to have a high level of ALDH1 [188]. CD24 is a mucin-like cell surface glycoprotein which may have a pivotal role in cell differentiation of different cell types [189]. CD44, a non-kinase transmembrane glycoprotein, is overexpressed in some cell types including cancer stem cells. The proteoglycan hyaluronan is the main ligand for CD44, binds to and activates CD44. In the investigation of [190], expressions of CD44, CD24 were discovered to vary with the characteristic of the local microenvironment in human pancrea-

tic adenocarcinoma cell lines. Later, human pancreatic cancer cell lines Panc-1, BxPC3, and HPAC were used in the study of [191], and two groups of small cell populations were found to be characterized by ALDH+, CD24+, and CD44+ (called group (i) cells) and ALDH−, CD24−, and CD44− (group (ii)). Group (i) cells were found to express higher levels of phosphorylated STAT3, together with translocation of STAT to the nucleus; group (i) cells also had greater tumorsphere-forming ability than group (ii) cells. This result suggests that in group (i) cells, the EGFR pathway as schematically represented by **Figure 7** (EGF-Jak-STAT signaling) was active. Note that EGF can also trigger other pathways such as Akt-mTOR 1,2 signaling pathway, the LKP1-AMPK-(TSC1/TSC2) pathway (**Section (3)** with **Figure 2**), Ras-Raf-Mek-Erk signal transduction cascade (**Section (4)** with **Figure 3**), the pathway involving translocation of EGFR to the nucleus by the dynein motor (**Section (8.3)** with **Figure 9**), plus the Hippo-YAP signaling pathway (**Section (5)** with **Figure 5**). Hence other tumorigenesis processes in addition to the EGF-Jak-STAT pathway might have been involved also. In a study using oral adenosquamous carcinoma cell line CAL 27, CD44 and EGFR were found to be colocalization by laser scanning confocal microscopy, and CD44 in fact was found to activate EGFR [192]. A few months ago, using human non-small-cell lung cancer cells, it has been found that CD44 also effectively activates EGFR in these cells [193], resulting in the activation of cell signaling pathways that induce cell proliferation, increases cell survival, modulates cytoskeletal changes, and enhances cellular motility.

In fact, more than a decade ago, three special clinical observations have been noted in the treatment of cancers of various types: 1) the appearance of metastatic lesions years after resection of small tumors with no clinically evident of metastases at diagnosis [194]; 2) detection of metastases of unknown primary tumors—such cases account for 4% - 5% of all clinical metastases [195]; 3) it has been pointed out that only a small fraction of cells from a primary tumor enter the circulation, and less than 0.01% of these cells/cell groups develop into metastases [196]. Taken all these results together, there is ample evidence to suggest that a small population of stem-like cells play roles in pancreatic carcinogenesis. Cancer stem cells are not the only type of cells that invade. The process of invasion is of prime importance. This aspect is followed in the next several sections.

15. Epithelial-Mesenchymal Transition and Micro-Metastasis Progression in Pancreatic Cancer

15.1. The Pathology of Epithelial-Mesenchymal Transition (EMT)

Epithelial-like cells must have an organized apical-basal polarity maintained by the precise arrangement of actin filaments and adhesive molecules, such that the integrity of the organ in concern is maintained. The adhesive molecules include integrins, adherins (of which cadherin is an important example), tight junctions, other cell surface proteins, and desmosomes (structures by which two adjacent

cells are attached, formed from protein plaques in the cell membranes which are linked by filaments) [197]. Disruption of just one or some of these surface proteins would trigger off a redistribution of molecules attached to the cell surface, including those attached to the internal surface of the membrane. There can be a disruption of the cell polarity, cytoskeletal reorganization, due to mechanotransduction [198]. The consequence is that cells are detached to become small groups or even individual cells, some of which acquire invasive power.

A similar phenomenon happens for basal cells (cells situated at the base of a multilayered tissue, as at the lowest layer of the epidermis). Thus, it is not surprising that research recognizes that, during metastasis, the cancer cells from solid tumors would disseminate into micro-metastasis entities. There is a progressive loss of epithelial cell polarity and cell-cell adhesion, and the concurrent acquisition of mesenchymal features that subsequently develop into clinically detectable metastases. This process has now coined the name "epithelial-mesenchymal transition" (EMT). Since EMT has also been found to be implicated in therapy resistance, immune escape, and maintenance of cancer stem cell properties, intermediate states might exist, as proposed in [199].

As an exocrine, the pancreatic acinar cells (PACs) produce zymogen granules which release a digestive enzyme, but the pancreatic ductal cells (PDCs) secrete water and electrolytic (HCO_3^-). These two types of secretions become the alkaline pancreatic juice [7]. When an injury occurs, acinar cells may transdifferentiate into PDCs, a transition called acinar-to-ductal metaphasic (ADM). The reverse transition is called DAM. If ADM is under the condition of recurrent inflammation, overexpression of KRAS or other oncogenes may bring the metaphasic state into the pancreatic intraepithelial neoplasia state (PanIN) [7]. This state is considered benign because the cells do not invade. Under a further genetic aberration such as one or more of the oncogenic pathways discussed in the previous sections, PanIN cells may develop via an EMT into pancreatic ductal adenocarcinoma PDAC [200].

15.2. The Correlation of Solitary Cell Infiltration with EMT Using KNOWLEDGE LEARNED about EMT from Mouse Models with Mutations of the KRAS and TP53 Genes

More recently, a special mouse model of PDAC, with pancreas-specific mutations in KRAS and TP53 genes, was used to track the fate of the pancreatic epithelial cells during various stages of pathogenesis in time [201].

One effective method to track the invasive cells was to use special lag to label an epithelial marker (such as E-cadherin, a calcium-regulated adhesion molecule that is expressed in normal epithelial tissues, including that of the pancreas; E-cadherin proteins bind cells of the same type [202]). Note that PDX1 (pancreatic and duodenal homeobox 1), which is a transcription factor, is necessary for pancreatic development, including β-cell maturation, and duodenal differentiation. $Pdx1^+$ cells are almost completely apancreatic and following the track of

Pdx1, it provides a good way to track pancreatic cancer cell invasion to other tissues [203].

Similarly, another tag was used to label a mesenchymal marker, such as ZEB1, a transcriptional repressor that has been identified as an inducer of EMT. Cells labeled with the stated two markers are "biphenotypic cells". Via the stated lineage tracing, it has been demonstrated that pancreatic cells could cross the basement membrane before invasive behavior could be detectable by standard histology, and invasive mesenchymal cells could even enter the bloodstream, as detected by flow cytometry [204]. It has been noted in another clinical study [205] that microscopic revelation shows a wide variety of intratumor glandular differentiation, with solitary infiltrating cancer cells in 114 resected pancreatic ductal adenocarcinoma specimens. A high degree of solitary cell infiltration has been correlated with reduced E-cadherin and increased vimentin expression in these specimens. Since E-cadherin is a typical adherent junction protein of epithelial cell-cell contact, and vimentin is a type III intermediate filament protein expressed in mesenchymal cells, the result highly suggests that solitary cell infiltration is correlated with EMT. The authors conclude that solitary cell infiltration would be a morphological clue to EMT and could be considered as a significant prognostic indicator of pancreatic cancer progression [205].

15.3. Evidence of Glycoprotein Adhesion Receptor CD44 as an Inducer of Progression of EMT Based on Mouse Model Investigation

Cells participating in EMT become mesenchymal stem cells with migratory properties; they are also considered as multipotent stromal cells that can differentiate into different phenotypes during cancer metastasis. CD44 (considered briefly in **Section (14.1)**), with a few isoforms, is a transmembrane glycoprotein adhesion receptor that binds hyaluronic acid in the interstitial fluid; it is involved in many cellular processes such as adhesion, growth, survival, and cell migration [206]. The primary cell lines of pancreatic tumors in iKras*p53* mice (a model that mimics the progression of human diseases) were employed in [207]. The findings of such study were: 1) CD44 expression in mouse pancreatic cancer correlates with EMT, with a causative link established by qRT-PCR analysis for the EMT genes (see Figure 2 of [207]). In other words, CD44s induced EMT in mice pancreatic cancer cells. 2) Short hairpin RNA (shRNA) is an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference. Mouse pancreatic cancer cells iKras*p53*#1b were infected with either (a) Control vector or (b) CD44 shRNA. Then (a) and (b) were separately injected to the pancreatic tail of NOD/SCID mice, which is a brand of immunodeficient laboratory mice, lacking mature T cells, B cells, and natural killer (NK) cells. All the animals in group (b) had large primary tumors at the site of injection. Moreover, group (a) mice showed extensive liver metastasis. 3) Expressions of CD44s were analyzed in RNA extracted from (A) re-

sected human pancreatic cancers and (B) matched adjacent uninvolved pancreas tissues. CD44s was significantly overexpressed in the tumor specimens (see Figure 3D of [207]). 4) MT1-MMP expression was detected only in the two human cell lines that overexpressed CD44s (Figure 3E of [207]). Moreover, the application of MT1-MMP inhibition significantly decreased the invasion of the pancreatic cells on Matrigel substrate (Figure 3F of [207]). 5) Based on transcription expression analysis, the authors provided evidence that CD44s was a regulator of Snail expression. Note that Snail is a family of transcription factors that promote the inhibition of the adhesion molecule E-cadherin to regulate epithelial to mesenchymal transition (EMT) during development. The results indicate that CD44 participates in carcinogenesis at the site, most likely to be associated with the development of a favorable environment for cancer formation and metastasis. Thus, we have to be alert on the presence/activity of CD44 in cancer stem cells (**Section (14)**) as well as acinar cells, islet cells in the organ.

16. The General Micro-Mechanism of Invasion of Pancreatic Cancer Cells, Stellate Cells, and Other Pancreatic Cells by the Process of Invadopodium

The Formation of Invadosomes, Secretion of Membrane-Anchored Degrading Enzymes, Activations of Several Signaling Pathways Are Necessary for Cells to Invade through the Basal Membrane and Fascia Compartment

The basal membrane is a physical barrier between non-invasive (*i.e.*, carcinoma in situ) and invasive types of cancer [208]. Three processes have been identified to be controlling factors as to whether cancer cells can invade through this barrier: 1) epithelial cytoskeletal contractility, 2) stromal stiffening and 3) growth factor/cytokine signaling (see Figure 1 of [208]).

The invasion of a cancer cell through the basement membrane requires the cell to acquire a motile phenotype. This cell develops actin-rich protrusive structures, called invadosomes which can degrade the first basal membrane and then the collagen fibers around the basal membrane. There are several groups of proteins/peptides involved in this complex process.

Referring to **Figure 16**, as an example of excitation, on the reception of epidermal growth factors (EGF, bright green hexagon) by it receptor EGFR (deep green Y-shape structure), the integrin (α and β units, brown) is activated, as indicated by the light-greyish curved arrows. Since the integrin is also attached to the basal membrane collagen by the protein fibronectin fibril (purple), mechanical stimulation can also trigger off the action of the integrin units. There are many integrins during migration/invasion in the figure. Focal adhesions are then built up around these integrin units (see **Figure 3** and **Figure 16**).

Some key members of the focal adhesions are 1) vinculin, 2) talin, 3) paxillin, 4) Tks4/Tks5, 5) Rho A/C TPase, with 3) 4) acting as adaptor proteins. Referring to **Figure 3**, in brief, part of the focal adhesion organizes the polymerization of

the actin fibers which can be attached to other parts of the cell membrane, or directly to the cell nucleus causing the synthesis of more proteins for migration. In Figure 16, we use a bluish ellipsoid to represent the focal adhesion, and a few actin chains together with the connecting proteins (*i.e.* the "glues") are presented simply by red tubes connecting from one focal adhesion complex to another or the cell nucleus. The calcium channel Piezo was painted in deep red. When mechanical force is applied to the integrins with focal adhesion and actin channels formed, the signal can be transmitted to the nucleus by mechanotransduction, leading to transcription of gene expressions that are associated with proliferation via the Ras/Raf/Mek/Erk and Ras/PI3K/PTEN/Akt/mTOR signaling pathways (see **Sections (3)** and **(4)**).

For cancer cells of organs (such as the pancreas) to invade, enzymes called MMPs must be in action to degrade the constituents of the ECM, leaving room for migration, in the first place. The family of metalloproteinases (MMPs) has at least 14 members. One or a small group of MMPs cooperatively degrade a variety of extracellular proteins, including the stated tightly structured collagens which are resistant to other proteases [209]. The functions of the matrix metalloproteinases (MMPs) are discussed in [2] with a summary in Table 2 of that paper.

Among these MMPs, MMP-14 is called membrane-anchored membrane-type-1 matrix metalloproteinase (MT1-MMP). The prototype protein MT1/MMP is translocated to the endoplasmic reticulum (green) after native protein transcription for "protein folding checking" with the help of chaperons (see Section (2) of [3]). It is then released from the Golgi Apparatus; an MT1-MMP complex is painted in reddish color with a fan shape structure having a zig-zag boundary.

Research in the past several years indicates that translocation of MT1/MMP is carried out by endosomes [210] [211]. Very recent evidence has suggested that the MT1-MMP complex is transported from the endoplasmic reticulum (ER) to the Golgi apparatus. There is evidence to suggest that the mode of trafficking to the cell membrane is via the anterograde transportation [212] (same as retrograde transport in Figure 9, but in the opposite direction).

Note that the protein that controls the anterograde transportation of the endosome, which carries the MT1-MMP, is found to be protrudin which is located at the ER membrane [213]. To show a simplified picture, we present a dynein motor carrying the cargo—an endosome (light yellow) containing an MT1-MMP enzyme molecule (without showing other molecules for simplicity) at site (1). The cargo is carried along one microtubule, many of those often extend from some region(s) of the cell nucleus to focal adhesions. At site (2), MT1-MMP is transported towards a focal adhesion at about the central domain of an invadosome. At site (3), a group of proteins at the focal adhesion, to be called "exocyst complex" (deep blue) that helped to expose the degrading enzyme with the active part outside the cell membrane; the MT1-MMP molecule then begins to degrade the collagen fibril (mainly collagen IV and I). Two other exposed

MT1-MMP complexes are also shown (without painting the associated microtubules) at the tip of the invadosome, and the white zig-zag patterns represent the action of collagen degradation. Different groups of researchers found out different members of this exocyst complex; we will not review all these works, but just note that there is ample evidence that the cell membrane-anchored MT1-MMP does play a key role to degrade collagen fibrils at the basal membrane and collagen I in the ECM, leaving room for invasive cells (of which pancreatic cells are examples) to metastasize [214]. During an invasion, mechanotransduction leads to the synthesis of proteins playing roles in proliferation and invasion, as marked in the cell nucleus in the figure.

The readers are referred to different members of this complex such as N-WASH, ARF6 together with c-Jun NH2-terminal kinase-interacting protein 3 and 4 (JIP3 and JIP4) [211], GFP bound-ARF6 protein [212], TKS5, cortactin [214].

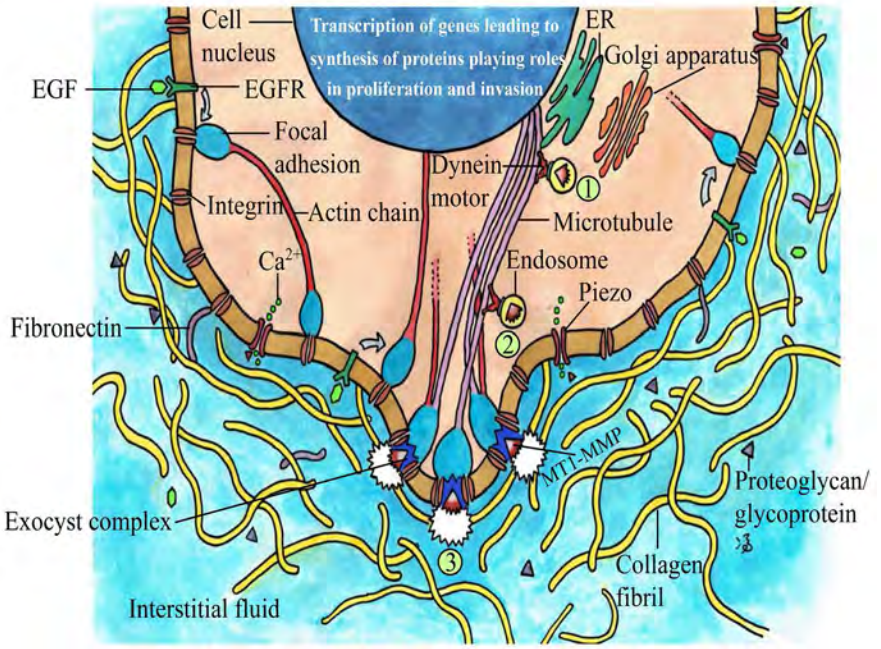

Figure 16. An invadosome is a protrusion part of a cell during an invasion. The integrins are painted in brown, the α-SMA (F-actin) chains in red, including the proteins (such as *α*-actinin) that connect the chains. The endoplasmic reticulum (green) and Golgi apparatus (orange) are labeled. The hexagon (bright green) is an epidermal growth factor (ligand) and the Y-shaped structure is its receptor. The MT1-MMP complex is enclosed by an endosome (light yellow) at site (1), after exiting from the membrane of ER. The MT1-MMP complex is carried by the molecular motor dynein along a microtubule (light purple) to site (2), towards a focal adhesion at about the central domain of an invadosome. At site (3), a group of proteins at the focal adhesion, to be called "exocyst complex" (deep blue) that helped to expose the degrading enzyme with the active part outside the cell membrane; the MT1-MMP molecule begins to degrade the collagen fibril (mainly collagen IV & I). The degrading action is represented by white patches with the zig-zag boundary. The purple line in ECM represents a fibronectin fibril that has affinity to integrin. For other details, see text. This figure was hand-painted by author PCWF.

In fact, the concept of invadopodium was recognized over a decade ago; see e.g. [215]. The process of invadopodium is at work when stellate cells and invasive cancer cells are in action.

17. The Overall Picture of Progression from Normal Acinar Cells via EMT to PDAC

The Progression of a Pancreatic Ductal Tumor with a Huge Stroma with Thick Interstitial Fluid in the Facia Compartment

We have provided the crucial updated information based on experimental evidence on the progression of normal pancreatic ductal cells via EMT to PDAC. The progression of the islets has been explained in **Section (13)**. We summarize the result schematically in **Figure 17**, starting with 7 normal ductal cells on the left, without painting the islet cells. The stellate cell (orange) is in the quiescent state, and the collagen fibrils form the normal basal connective tissues with interstitial fluid (blue on the "external side" of the duct). The normal blood vessel is shown with a few red blood cells to represent schematically the normal cells found in the blood. When EMT begins to proceed, there is a change in morphology of the ductal cells, and the stellate cells start to lose the oil droplets and become activated as explained in **Section (12)**. There are more collagen molecules synthesized and excreted to the ECM.

The pancreatic duct is under inflammation, and two macrophages are painted there to represent the presence of immunity cells in action. The development region barred by two vertical dotted lines represents the progression of the PanIN-1a, PanIN-1b, PanIN-2 to PanIN-3 states in EMT.

PanIN-3 state/stage is often called in situ carcinoma, as the cancer cells, though already formed, are not invasive. However, during this stage, some caner-stem-like cells (or simply called cancer stem cells) could have entered the upper branch of the blood vessel, as indicated by an arrow with the tip pointing to a cell (with rugged boundary). This type of metastasis is supported by the clinical finding that soon after a PDAC tumor has been resected, cancer (with evidence of pancreatic carcinoma origin) could be detected in organs at a far distance inside the body (see remarks in **(Section (14.1)**). We call this early metastasis, and the cancer cells are not acinar nor islet cells.

When the primary PDAC is formed, it is surrounded by abundant collagen fibrils (fibrosis), embedded in thick/viscous interstitial fluid (**Section (12.2)**). Antigen-presenting cells and other immunity cells are difficult to carry their duties (not painted in the figure) as they can hardly reach the core of the cancer-stroma domain. Ductal cancer cells are detached from the tumor and migrate to the fascia compart of that region, with some enter the blood vessel, as indicated by the arrow on the right-lower part of the figure.

There are proteoglycans and glycoproteins in the fascia compart, and they are represented by greyish-purple triangles.

The bright green circles represent the drug molecules (e.g. chemotherapeutic)

which can only physically reach the outside boundary of the stroma, as the interstitial fluid pressure inside the stroma is very high—this is a well-known fact expressed as drug resistance in clinical treatment. Radiotherapy sends electromagnetic energy which is absorbed by the stroma and healthy cells on route to the supposed target. In Figure 17, the white small circles in the interstitial fluid, blood are vesicles containing important micro-RNA information of the sending cells; this issue will be discussed in later Sections.

By the way, the purplish-blue domain above the ductal cells represent the digestive enzymes passing towards the duodenum, and the greenish patch at the upper right-hand corner represents some bile, as both the pancreatic duct and bile duct enters the duodenum in the close neighborhood.

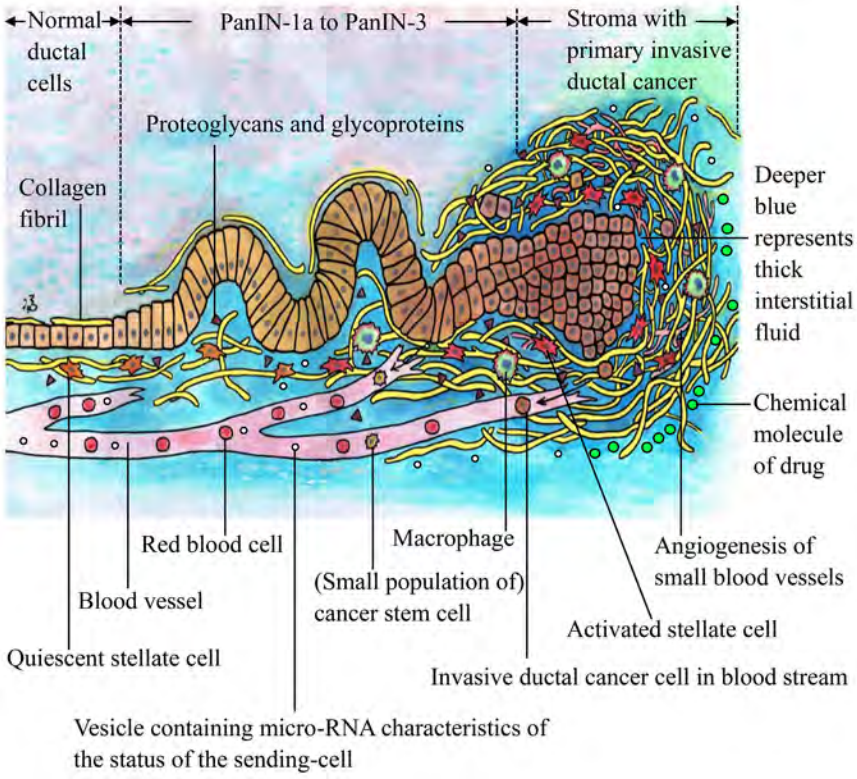

Figure 17. The overall picture of carcinogenesis of PDAC. The brownish cells are ductal (acinar) cells that progress from normal structure to a lump of tumor surrounded by dense collagen fibrils (fibrosis) and viscous interstitial fluid (deep blue). Several macrophages represent the presence of immunity cells. The quiescent stellate cells (orange) change to myofibroblast-type of activated stellate cells (red). There is a small population (<5% in body mass of the organ) of cancer-stem-like cells that could migrate to the bloodstream during the PanIN-3 stage. Losing physical connection with the ductal cells, individual ductal cancer cells can migrate via the blood, interstitial fluid to other internal organs. Drug molecules (green circles) and radiotherapeutic energy cannot reach the primary tumor because of the high interstitial fluid pressure and dense collagen fibrils around the stoma. The white circles are vesicles containing information of non-coding micro-RNAs sent by all cells for cell-cell communication via the fluid system of the body—this important aspect will be followed up later. This figure was hand-painted by author PCWF.

18. Having Analyzed the Pathogenesis of Pancreatic Cancers, in Addition to Those Considered at the Ends of Previous Sections Related to Signaling Pathways, the Following Are Examples of Therapeutic Measures, Including Examples of Non-Coding RNAs, as Potent Treatments

18.1. Prevention of Stellate Cell Activation and Efficacy of Treating Pancreatic and Gastrointestinal Xenografts with ATRA, the Main Constituent of Vitamin A

Retinoids are a class of biomolecules having similar structural with respect to chemical activity of vitamin A. All-trans-retinoic acid (ATRA) is an oxidation product of retinol which exists only in small amounts in food. Taken orally, ATRA is rapidly absorbed in the digestive system, bound to serum albumin in the blood. Whereas vitamin A from food is first stored in the liver, ATRA is rapidly metabolized and excreted in the bile. This type of metabolism avoids the side effects of taking too much Vitamin A (hypervitaminosis A), leading to the symptoms of skin desquamation, vomiting, and diarrhea [216].

To study the effect of ATRA on human pancreatic cancer as the main goal, two human pancreatic cancer cell lines were cultured: 1) Panc-1, 2) MIA-PaCa-2, as well as 3) carcinoid cell line (BON)—derived from the gastrointestinal tract, plus 4) the rat insulinoma cell line RIN-5mF (a clone derived from the RIN-m rat islet line (ATCC CRL-2057)). Different amounts of ATRA were cultured with these cell-lines [217].

BON cells were injected into the healthy mice model to introduce carcinogenesis as *in vivo* study. The resulting tumor xenografts were then harvested and the number of viable tumor cells was counted. In the *in vitro* investigation, a large amount of ATRA (100 micro-M) had to be used to reduce the viable cancer number to that of the control, whereas an amount of 0.5 to 1.0 micro-M of ATRA was needed to reduce the viable cancer cells to the control for cell lines 1), 2) and 4) (see Figure 1 of [217]). The size of the BON-induced tumor of the *in vivo* model was also found to decrease in size (from 175 mm^2 to 70 mm^2) after 37 days of treatment (see Figure 4 of [217]). This section deals with the beneficial result of treatment of cancer cells with ATRA.

In another study, human pancreatic stellate cells (PSCs) were cultured on 3D Matrigel and the magnetic tweezer micro-rheology technique (with fibronectin-coated beads) was employed to analyze the contractility ability of PSCs by measuring the displacements of the magnetic beads [218]. Through the detection of the expressions of alpha-smooth muscle and vimentin, which are two basic markers of myofibroblasts activation, it has been reported that 1) these two expressions were decreased when PSCs were treated with ATRA (see Supplementary Figure 4 of [218]. 2) The expression of another protein desmin, which is an established marker of PSC quiescence, was found to decrease. These two results suggested that ATRA would suppress the ability of the PSCs to sense the mechanical cues of the ECM, so that the PSCs would remain in the quiescent state,

avoiding the mechanical vicious cycle mentioned in **Section (12.2)**.

Note that myosin light chain-II (MLC-2) is part of the functional motor protein myosin. Activation of MLC-2 is necessary to produce traction force. The mechanism of ATRA action in keeping PSCs in the quiescent state was inferred to be due to transcriptional suppression of MLC-2 [218].

Human tumor tissues were obtained from PDAC patients and mouse xenograft models with the injection of human cells Panc02 were established in [Bleul 2015]. The concentrations of ATRA and levels of ATRA receptors (RAR α and RAR β) were reduced in tumor tissues as compared to normal.

In a recent investigation, using nano-gold particles to deliver ATRA to PSCs, it has been reported that activated PSCs could be brought back to the quiescent state, suggesting that such a method might be used as a therapeutic treatment in the future [174].

18.2. Three Members of Tocotrienols (a Subfamily of Vitamin E) Were Found to Suppress the Activity of NF-kB in Pancreatic Cancer Cells

Tocopherols, found commonly in food, function as antioxidants by donating H atoms to radicals. The four members of the tocotrienols, a subclass of vitamin E, are also antioxidants and found to be derived mainly from palm oil. Tocotrienol rich fraction (TRF) is an extract of palm oil, which consists of 25% alpha tocopherol (α-TCP) and 75% tocotrienols (with sub-types α-, β-, δ-, γ) [219] [220].

The tocotrienols are similar to the tocopherols in chemical structure but with carbon bonding shifts in three sites; these two sub-classes of Vitamin E have homologs named according to the degree of methylation at R1 and R2.

Tocotrienols of types α-, γ- and δ were detected in both plasma and lipoproteins with α-tocotrienol as the major homolog after administration of TRF. It was remarked particularly that tocotrienols were mainly detected in the HDL cholesterol at 4 to 8 hours before clearance from the body [220]. The small HDL particles selectively distributed α-tocotrienol to organs and tissues with high adipose content. On the other hand, tocopherols were distributed equally in all the lipoprotein fractions; since LDL receptors are present in all tissues, tocopherols are found in all tissues and for a time longer than 8 hours. It is thus not surprising that a special delivery system is needed to bring tocotrienols to cancer sites.

When administered orally, the amount of absorption of vitamin E depends on the degree of lipolysis and food intake, as it is water insoluble. With tocotrienols embedded in nano-carriers aimed at targeting tumor, intravenous administration via tail vein injections confirmed that the modality of administration did affect significantly the degree of absorption [221]. Using hydrophilic polymers (such as cyclodextrin) and emulsifiers (such as polysorbate 80, polyoxyethylene sorbitan monooleate (Tween 80)) as "drug delivery systems", it has been shown that the concentration of γ-tocotrienols in plasma would be increased in rat

models (see e.g. [222]). Employing murine xenografts created by injection of human cancer cells, there is evidence that tocotrienols therapy can be a very promising one to eradicate breast, pancreatic, and prostate cancers when encapsulated in a tumor-targeted vesicle system [223].

In another study, human pancreatic ductal epithelial cells (HPDE6 C7) and HPDE6C7-KRas cells were used in [224]. These two cell lines were grown in keratinocyte growth media (Invitrogen) supplemented with human epidermal growth factor and bovine pituitary extract. Using the Cell proliferation MTT assay on human pancreatic cancer MiaPaCa2 and AsPc-1 cell line *in vitro*, it has been demonstrated that natural tocotrienols with d-tocotrienol and γ-tocotrienol caused cancer cell viability reduction up to 60% with 50 mmol/L; β-tocotrienol has a moderate anti-cancer effect with cancer cell viability reduction up to 40% with 50 mmol/L (Figure 1B of [224]). With human pancreatic cells AsPc-1 injected into the mice *in vivo* models, it was found that d-tocotrienol significantly reduced tumor volume by 50% ($p < 0.001$), γ-tocotrienol reduced tumor volume by 42% ($P < 0.01$), and β-tocotrienol reduced tumor volume by 32% ($p < 0.01$), whereas α-tocotrienol did not decrease tumor size growth significantly. The *in vitro* (human cells) and *in vivo* (mice) studies substantiated each other in general.

The authors particularly pointed out that in the *in vitro* investigation, 1) γ-and d-tocotrienols inhibited the NF-kB activity in the nuclear extract, 2) whereas γ-, δ- and β- tocotrienols inhibited the NF-kB activity in the extract obtained from the cytosol (see Figure 3A of [224]), but 3) α-tocotrienol and α-tocopherol were found to be ineffective in reducing NF-kB activity. More recently, using human pancreatic cell lines L3.6pl and MiaPaCa-2, plus orthotopic xenograft mouse models, both cancer volume growth and cancer stem cells (with CD44 as a marker) activity have been suppressed by the natural form of tocotrienols, preventing metastasis [225].

18.3. Agonists of Vitamin D Receptor Reduce Crosstalk between Pancreatic Stellate and Pancreatic Cells—VDR Ligand Calcipotriol (Cal) Treat Pancreatitis and Pancreatic Cancer

The pancreatic stellate cell was discovered as peri-acinar cells in the rat with a stellate shape when activated [168]. Based on the heatmaps representing selected genes from RNA-sequence analysis of 1) quiescent mouse PSCs and 2) activated mouse PSCs (isolated from the animals), the authors have discovered that certain genes are overexpressed in 2). These genes correspond to the proteins participating in forming focal adhesion, gap junction formation, matrix remodelling, inflammation [226]. Specimens of PSCs were obtained during resection surgery of non-cancer associated and cancer-associated patients in the same study. A comparison of the transcriptomes of non-cancerous human specimens and cancerous human specimens gave similar results, pointing to the fact that PSCs actively participate in matrix remodelling during carcinogenesis and me-

tastasis of pancreatic cancer.

These findings substantiate the suggestions/conclusions of other studies, pointing to the fact that PSCs actively participate in matrix remodelling during carcinogenesis and metastasis of pancreatic cancer.

The more important discovery in [226] is the revelation of vitamin receptor (VDR) in the stroma of pancreatic cancer, rather than in acinar cells, with a minimal amount in the islet, based on quantitative RT-PCR investigation. Human pancreatic cell lines (MiaPaCa-2, BxPC-3, HPAC, Panc1, APC1) study demonstrated that VDR activation drives the reversion of PSCs to a more quiescent state. More precisely, the application of VDR ligand calcipotriol (Cal, as a non-hyper calcemic vitamin D analog) caused a decrease in markers of inflammation, markers of fibrosis in pancreatitis, and human pancreatic tumor stroma samples. Cal treatment was found to induce liquid droplet formation in 19/27 primary cancer samples from patients. The authors consider that as VDR behaved like a master transcriptional regulator of PSC activation network; targeting the receptor with vitamin D as a ligand would drive the activated PSCs to their rest state [226]. The authors remarked also that the addition of Calcipotriol to gemcitabine treatment enhanced survival of the "KPC mice" model by 58%. The KPC mice model has KRAS, TP53 mutations to promote chromosome instability with widely metastatic ability [227].

In recent work, 3 immortalized stellate cell lines were generated from pancreatic patients [228]. Exosomes released by stellate cells were labeled with a fluorescent dye and incubated with human pancreatic cell lines PANC-1 and SW1990. The exosomes received by the cancer cells with efficiency > 90%. Exosomes from different stellate cells showed similar miRNA expression profile with each other. Five most abundant miRNA in exosomes were miR-10a-5p, 92a-3p, 181a-5p, 191-5p, 92b-3p. Vitamin D receptor signaling (supply of Vitamin D to the stellate cells) could suppress secretion such as miR-10a-5p, implying that messages from the stellate cells to the cancer cells were influenced.

18.4. Drugs to Decrease the Concentration of the Interstitial Fluid in the Stroma—Pegvorhyaluronidase Alfa (PEGPH20), together with Other Anti-Cancer Drugs Aiming to Treat PDAC

Note that hyaluronan (hyaluronic acid, HA), which is the most abundant and ubiquitous member of the glycosaminoglycans (GAGs, a member of proteoglycan), forms negatively charged long chains in the fascia. The hydronium ions together with layers of water (originated from the bloodstream) are then bound to the hyaluronan chains, forming a thick interstitial fluid (IF) [2]. HA, secreted by cancer and stromal cells, binds to and interacts with specific cell surface receptors including CD44 (a surface marker for cancer stem cell), and receptor forming HA-mediated motility (receptor) (abbreviated RHAMM). It is not surprising that HA, which is also secreted by cancer cells, has been shown to be abundantly accumulated in the surrounding stroma of tumor. The concentration

of HA is about 12-fold in PDAC as compared with that of normal pancreas. HA takes part in strong tumor cell-stroma interaction. Thus we can view the concentration of HA in the ECM of the pancreas as a sign of cancer initiation and progression [229]. Stromal modifying agents, such as Pegvorhyaluronidase alfa (PEGPH20), which can (temporarily) degrade HA, in combination with nab-paclitaxel plus gemcitabine (NCT01839487) together with modified FOLFIRINOX, is in clinical trials for patients with metastatic PDAC [230] (see also the review of [229]). Though we have not heard about positive results by 2019, the general idea is scientifically sound. The reader is also referred to the work on enzymatic targeting HA—it was in preclinical animal trials by 2010 [231].

19. MicroRNA Therapy to Treat Pancreatic Cancers in General up to 2015

19.1. How Does the microRNA Therapy Work?

A microRNA (abbreviated miRNA) is a small non-coding RNA molecule (containing about 20 nucleotides) which functions in RNA silencing and post-transcriptional regulation of gene expression via DNA interference.

The interaction of miRNAs with their target genes is dynamic and dependent on many factors, such as the subcellular location of miRNAs, the amounts of miRNAs and target mRNAs present, plus the affinity of miRNA-mRNA interactions. The miRNAs can be secreted into interstitial fluids and transported to target cells via vesicles, such as exosomes. Thus cell-cell communication can be mediated by miRNAs that function as chemical messengers through the ECM [232].

The role played by miRNA has drawn much attention during carcinogenesis. For example, miR-320 has been reported to have an inhibitory effect on gastric cancer progression and being taken clinically as a novel biomarker for its diagnosis and prognosis [233]. As another example, transcriptome profiling of the epithelial components of micro-dissected ovarian tumor (as an example of epithelial cancer) samples isolated from long- and short-term survivors has been carried out. Then it was found, via a miRNA transfection process, that there was a direct binding of miR-320 to the 3' untranslated region of mRNA of the transcription factor called "E74-like factor 3 (ELF3)", arriving at the "silencing effect". The authors concluded that the protein ELF3 would be a negative regulator of epithelial-mesenchymal-transition (EMT) of the ovarian tumorigenesis [234]. Furthermore, using specimens from 52 breast cancer (BC) patients, it has just been demonstrated [235] that miR-320 targeted ELF3 would suppress BC cell progression via regulation of PI3K/Akt signaling, the details of which have been reviewed in **Section (3)**. Since all of the above cancers are epithelial in nature, it would be fruitful to look for the same/similar miRNA silencing mechanism in pancreatic cancers.

The detailed mechanisms of the interactions between miRNAs and the identi-

fication of the targeted transcriptors/proteins which are involved in tumorigenesis are certainly useful in developing therapeutic measures. In this new process, first, using the transcriptome profiling technique, scientists look for one or more transcription factor-encoding genes whose expressions are high in certain cancer samples. With the relevant cancer cell lines, the miRNAs are applied to silence such transcription factors and analyze the progress of carcinogenesis in these cell lines; after that, it is fruitful to establish xenograft *in vivo* models to analyze the anti-cancer effects.

19.2. MiRNA96 and the Ras-Mek-Erk Pathway

The three human RAS genes have members KRAS, NRAS, and HRAS. KRAS is the most frequently mutated oncogene, appearing in 90% of pancreatic cancers [236] and is one of the earliest genetic alterations detected in pancreatic cancer development. KRAS encodes small GTPases that are involved in cellular signal transduction. Oncogenic KRAS has been established to promote pancreatic tumorigenesis through activation of (multiple) downstream pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt [237], extracellular signal-regulated kinase (Erk, see **Figure 3**), and NF-κB [238]. The protein Ras has been observed in rat's pancreatic ductal cell line to elicit mitogenic and morphogenic responses, with the result of increasing S-phase cell population [239].

In the study of [240], specimens of tissues were obtained from patients receiving surgical operation for pancreatic cancer. Three pancreatic cancer cell lines 1) MIA PaCa-2, 2) PANC-1, 3) BxPC-3 and 4) the well-known cervical adenocarcinoma cell line Hela were used in the *in vitro* investigation. Total RNA was extracted from the cells and tissues. Real-time reverse transcription PCR assay was conducted to detect the mRNA levels of KRAS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Human KRAS mRNA was cloned using a PCR-generated fragment.

The findings of this investigation [240] using the above preparations were; a) Northern blotting analysis was conducted in 10 pairs of pancreatic cancer tissues and matched adjacent normal tissue samples indicated that the expression of miR-96 was lower in all the pancreatic cancer tissues than in normal tissues. b) Expression miR-96 was downregulated in tumor cell lines 1) to 3).

c) adopting three bioinformatic algorithms, a large number of potential target genes of miR-96 was identified. Among these candidates, a binding site of miR-96 was observed in the sequence 3'-UTR of KRAS mRNA, with perfect base pairing. d) Using "CCK-8 proliferation essay" (for details see [240]), overexpression of miRNA in cell lines 1) and 2) inhibited cell proliferation compared to control (untreated cells transfected with "miRNA scramble" sample). e) Pre-miR-96 treatment induced cell cycle arrest in G1 phase, with detected decrease in population of the S phase. f) Using artificial wound model *in vitro*, the introduction of miR-96 into cell lines 1) and 2) resulted in a reduction of cell migration inclosing the artificial wound cells. We note that the Ras-Mek-Erk

pathway in **Figure 3** is related to the Akt/mTOR pathway (**Figure 2**) via cross-talks.

19.3. Promise of micro-RNA34 Mimics to Treat Pancreatic Cancers with TP53 Mutation by Targeting the Notch Pathway and Anti-Apoptotic Bcl-2 Protein

In a normal pancreas, the Notch signaling pathway has been shown, using the mice model, to be an important regulator of the balance between self-renewal and differentiation, involving both the endocrine (islet) and exocrine (acinar) cells [12]. Activation of the Notch pathway prevents the differentiation of pancreatic acinar cells and attenuates endocrine development [241]. The result of suggested the Notch Pathway is necessary to maintain the cancer stem cell population in pancreatic cancer cells as explained in [162].

On the other hand, the tumor suppressor gene p53 was found to regulate directly each of the three family members of the micro-RNA miR-34a,b,c (see e.g. [242]), which targets Notch pathway proteins. Moreover, there is crosstalk between the Notch pathway the Bcl-2 proteins which are the regulators (anti-apoptotic) of (intrinsic) apoptosis [243].

Given the above logical connection, the effects of this mi-RNA-34 on cancer cells became the focus of study in [244]. Five pancreatic cancer cell lines with p53 mutations were used in [244]: 1) Mia PaCa2; 2) BxPC3; 3) Capan1; 4) Capan2; 5) Panc-1. A normal human lung fibroblast cell line WI-38 was also employed.

Further, mice models transfected with a) cells of 1), and b) cells of 1) plus miR-34(s) were established. Their findings were i) The levels of miR-34 in cancer cells were in general lower compared to lung fibroblast cells. ii) Tumorsphere-forming and tumor-initiating cells (with surface markers CD44 and CD133), called cancer stem cells, were identified in 1). These stem cells showed high levels of Notch/Bcl-2, but a loss of miR-34s. iii) Transfection of miR-34 mimics to 1) and 2) inhibited expression levels of Bcl-2, Notch1, Notch2. iv) Restoration of miR-34 to cells 1) and 2) showed significant inhibition of clonogenic cell growth (forming colony of cells) and invasion, accompanied by more apoptosis and cell cycle arrest. v) Restoration of miR-34 to cells 1) led to an 87% reduction of cancer stem cells. vi) miR-34 mimics would partly restore the tumor-suppressing function of p53 in cells 1)-4) with deficient p53. vii) In the *in vivo* mice model b) stated above, restoration of miR-34 inhibited tumor formation.

19.4. Using Inhibitors of microRNA21 and microRNA 221 to Treat Highly Metastatic Pancreatic Human Cancer Cell Lines, Resistant to Gemcitabine Using *in Vitro* and *in Vivo* Mouse Models

By the early 21st century, it was confirmed that a small population (SP) of cancer stem cells existed in cancers [245]. In [246], a highly metastatic human pancrea-

tic cancer cell line 1) L13.6pl (which contained the cancer stem cells); 2) a small population of cancer cells were isolated following Hoechst 33342 staining of 1); 3) L13.6pl cells with cancer stem cells removed—called non-SP cells; and 4) L3.6pl cells$_{Gres}$ (gemcitabine resistant cells cultured with increasing concentrations of the drug gemcitabine). Note that an earlier study already indicated that anti-sense oligonucleotides (ASOs) of microRNA-221 and microRNA-21 would produce antiproliferative effects on human pancreatic carcinoma cell lines [247]. Other cell lines were constructed readily: 5) L13.6pl cells transfected with anti-sense miR-21; 6) L13.6pl cells transfected with anti-sense miR-221; 7) L13.6pl cells transfected with anti-sense miR-221 plus anti-sense miR-21. For *in vivo* study, cells of specimens 1), 4), 5), 6), 7) were injected into mouse models to investigate the development of cancer. The following results were highlighted: a) Expressions of miR-21and miR-221 were high in sample 1) compared with that of the sample 3). b) Expressions of miR-21and miR-221 were significantly upregulated in the sample 2) as compared to 3). c) The content of SP in 1) was 5%; that of the sample 5) was reduced to 4.7%; that of the specimen 6) was reduced to about 3%; that of the specimen 7) was further reduced to about 2%. d) There was no change in viability cell number in the specimen 5) as compared to 1). e) The viability number of 6) was reduced to about 85% as compared to 1). The viability number of 7) was reduced further to about 50% as compared to 1). The *in vivo* study indicated that the combined anti-sense therapy of (miR-21 plus miR-221) gave the best result in tumor size reduction. The above experimental result indicates that inhibitors of miRNA21 and miRNA221 together might be an effective therapy to treat pancreatic cancers which are resistant to gemcitabine.

19.5. Evidence of Suppressing Cancer Progression Employing miRNA 146a, 3,3'-Diinolyl-Methane, Natural Products Isoflavone G-2523 Based on Human Cell Lines Study—Leading to Downregulation of EGFR, Downregulation of NF-kB, Upregulation of PTEN

Two human pancreatic cancer cell lines 1) Colo357, 2) Panc-1, 3) human pancreatic ductal epithelial cells (HPDE) were used in [248]. Two types of treatment were tried: a) 3,3'-diinolyl-methane (DIM), a natural product derived from vegetables like broccoli, Brussels sprouts, cabbage; b) isoflavone G-2535 (Genistein), a soy phytoestrogen from soy, green peas, alfalfa, peanut. The findings of [248] are: i) Both a) and b) activate miR-146a. ii) Levels of miR-146a in HPDE are higher than that in each of the cancer cell lines. iii) Treatment of 1) and 2) cells with a) and b) increases expressions of miR-146a. iv) When cells 1) and 2) were transfected with miR-146a pre-micro-RNA, the expression of epidermal growth factor receptor (EGFR) was downregulated at mRNA and protein levels. v) Anti-miR-146a was transfected into 1) and 2); the EGFR level was found to increase after such inhibition of miR-146a. vi) When cells in 1) and 2) were transfected with miR-146a, there was a decrease of the expression level of NF-kB. vii)

Moreover, the expression of metastasis-associated protein MTA-1 was downregulated after the stated transfection in v).

Note that there is recent research reporting that overexpression of MTA 2 was detected in 64 PDAC tissues [249]. Through an *in vitro* study using human pancreatic cell lines MiaPaCa-2, PANC-1, and mouse xenograft *in vivo* investigation, MTA2 was negatively correlated with the anticancer protein PTEN (see **Figure 2**). Benzyl isothiocyanate (BITC) which is found in cruciferous vegetables; BITC has been used as a chemoprotective agent against carcinogenesis. There was evidence showing MTA2 and PTEN played roles in the regulation of the PI3K-Akt pathway in BTIC-mediated cancer suppression [249]. Collecting the above information, we can forth the notion as written in the title of this section.

20. Early Non-Invasive Detection of Pancreatic Cancer in Body Fluids—From Micro Non-Coding RNAs, Circular Non-Coding RNAs to Long Non-Coding RNAs

20.1. Introduction to Three Types of Non-Coding RNAs—They Are Enclosed by Vesicles, Carrying Physiological/Pathological Signals, and Should Be Found in Body Fluids

Almost all cell types known (including cancer cells) abundantly release small "particles" known as extracellular vesicles (EVs or called exosomes) into the extracellular milieu for cell-cell communication, at near and far distances through the largest organ of the body—the fascia. The "messages" in these EVs contain nucleic acids (including microRNAs), proteins, lipids, and carbohydrates, characteristics of the cell type, and status. The term exosomes are now defined as extracellular vesicles that are released from cells upon fusion of an intermediate endocytic compartment. In fact, transport of exosomes is like the artificial nano-particle drug delivery system that has recently been developed.

Several years ago, using high-throughput RNA sequencing (RNA-Seq), an in-depth bioinformatic analysis of non-coding RNAs (ncRNAs) in human cell-free saliva (CFS) from healthy individuals was conducted [250]. A "common ncRNA" (*i.e.* miRNA) has only up to about 20 nucleic acids, having their free 3' and 5' ends exposed and can be degraded by nucleases. Note that circular RNA (circRNA) is a segment of (single-stranded) RNA that forms a covalently closed continuous loop. Through binding with miRNAs, and other gene expression regulators, together with circRNA regulate various biological processes in the human tissues (see e.g. [251]). Note that a circRNA cannot be digested readily by nucleases due to its ring structure. Since a circRNA is stable in the body for a longer time, it is of interest to detect such units for diagnostic purposes, specifically for various diseases. The circRNAs are also called "sponge" RNAs which interact with the common microRNAs due to their loop structures [252]. The piwi-interacting RNAs (piRNAs) which are available in embryonic stem and skin cells were also detected. These RNAs are also of interest clinically in the

early stages of an epithelial type of cancers, of which pancreatic and breast cancers are typical ones. A non-coding RNA composed of more than 20 nucleic acids is called lncRNA, the research on which has also become a hot topic in the recent few years.

Note that the non-coding RNAs have been detected in the cerebrospinal fluid also [253]. Most studies so far have been focussed on their detection in the bloodstream.

Four years ago, we have provided evidence that the interstitial fluids, cerebrospinal fluid, glymphatic fluid, lymphatic fluid, blood, and other fluids form a circulating fluid system of the body [166]. Saliva and sweat are in fact parts of the interstitial fluid. We predict that various types of non-coding RNAs will be detected in the interstitial fluids in different compartments of the body, because the fluids mentioned form a circulation system, with input as drinks through the mouth, and discharged as urine. Indeed, several months ago, for renal transplant patients with acute T cell-mediated allograft rejection, it has been reported that the circular RNA "has_circ_0001334" is enhanced in the urine of these patients [254]. Non-invasive detection of such a circular RNA becomes a biomarker of acute kidney rejection; the technicality of the method of measurement is described in that paper.

In an "earlier" study, the expression profile of circRNAs in six PDAC cancer specimens and associated adjacent normal tissues using microarray has been analyzed [255]. A high-throughout circRNA microarray was used to identify dysregulated circular RNAs in specimens of these six PDAC patients and the authors have concluded that clusters of circRNAs are aberrantly expressed in PDAC compared with the normal specimens [255].

20.2. The Non-Coding Circular RNA IARS Enclosed within Exosomes Regulates Endothelial Monolayer Permeability to Promote Pancreatic Cancer Metastasis Based on Human Pancreatic Cell Line and Xenograft Investigation—Looking for Antisense circRNA IARS

In the study of [256], RNA was isolated from 85 fresh frozen tissues and 16 peritumoral normal tissues of 92 patients underwent pancreaticoduodenectomy surgery. The human pancreatic cancer cell lines AsPC-1, Hs 766 T, plus the human microvascular vein endothelial cells (HUVEC) were also used. qRT-PCR was employed to measure the "circulating" Circular RNA (circRNA) IARS (enclosed by vesicles) secreted by the pancreatic cancer cells. Expressions of proteins related to focal adhesion and F-actin were also determined in the relevant specimens. The endothelial monolayer permeability was estimated. Human pancreatic cells were injected into the mouse model for *in vivo* study of cancer development. They found that [256]: 1) The circulating vesicles enclosing microRNA increased endothelial monolayer permeability (by enhancing expressions of the F-actin and focal adhesion proteins) and the vesicles entered the venous blood. 2) Based on the *in vivo* investigation, the circRNA IARS was

found to be correlated to tumor invasion and metastasis. 3) Overexpression of circRNA IARS significantly down-regulated miR-122 and ZO-1 levels. We would remark that ZO-1 is a tight-junction protein acting for cell-cell adhesion [257]. For a group of cancer cells to invade, the migrating cell must be detached, by breaking cell-cell adhesion.

There is evidence that the expression of miR-122 mediated by adenoviral vector induces apoptosis and cell cycle arrest of (liver) cancer cells [252]. Intuitively the "absorption" of miR-122 and ZO-1 by the circRNA IARS would promote cancer micro-metastasis, as observed in [256]. Given the above analysis, the application of anti-sense non-coding circular RNA IARS would reduce endothelial permeability to decrease the ability of invasion of cancer cells.

20.3. The Expressions of Non-Coding Circular RNA LDLRAD3 Are Upregulated in Human Tissues, Plasma Samples, and Human Pancreatic Cell Lines

Expression levels of circ-LDLRAD3 were determined in normal pancreatic cell lines (HPC-Y5 and HPDE6-C7) and pancreatic cancer cell lines (Capan-2, Panc-1, SW1990, and AsPC-1) [258]. Cancerous tissues and nearby non-cancerous pancreas tissues were obtained from 30 patients. Thirty-one sets of blood samples were obtained from these patients and a group of healthy subjects. Analysis of these specimens indicated that the expression levels of a specific circular RNA (circ-LDLRAD3) were higher in pancreatic cancer cell lines, pancreatic cancer specimens, plasma samples from pancreatic patients than in normal pancreatic cell lines, non-cancerous specimens, plasma samples from healthy normal respectively ($p < 0.01$) [258]. Anti-sense non-coding circular RNA LDLRAD3 might be a potent treatment of PDAC.

20.4. circRNA_100782 Regulates PDAC Cell Growth by Affecting IL6-Jak-STAT3 Signaling Pathway

In the study of [259], the authors have used the PDAC cell lines BxPC3 and HPDE and HEK293T cells, plus pancreatic cancer mice models to study tumor status *in vivo*. Based on xenografts analysis, they have found that circRNA_100782 regulates PDAC cell growth by affecting IL6-Jak-STAT3 signaling pathway and this pathway promotes tumor growth as briefly explained in **Section (8)** with **Figure 7**. circRNA_100782 knocked down models repressed tumor growth. Therefore, inhibitor of circRNA_100782 is a potential treatment for PDAC.

20.5. Non-Coding Circular RNA circ_0007534 Plays an Oncogenic Role

In another recent study [260], real-time (RT-qPCR) was used to detect the expression of circ_0007534 in 60-paired PDAC tissue samples of subjects. 5 human PDAC cell lines (BxPC3, Capan-2, AsPC-1, PANC1, and SW1990) were used to detect cell proliferation, apoptosis, and metastatic properties affected by circ_0007534. Mice xenografts injected with human pancreatic cells were used to

detect the status of tumor growth. The results were: 1) In PANC1 cells, silencing of circ_0007534 attenuated cell proliferation, migration, and invasive ability but promoted cell apoptosis. 2) Circ_0007534 was upregulated in PDAC patients' tissues (undergoing radical surgery) and cancer cells. 3) Circ_0007534 could inhibit cell apoptosis partly via a Bcl-2/caspase-3 pathway (see details of this pathway in Figure 3 of [3]), noting that the Bax protein is a member of the Bcl-2 family. 4) Based on the xenograft study, it was found that miR‐625 and miR‐892b were sponged by circ_0007534. The results of this investigation suggest that anti-sense of circ_0007534 is a potent therapeutic treatment; mimics of either one (or a combination) of miR-626, miR-892b are also plausible anti-PDAC progression measures.

20.6. The Potential Benefits of microRNA 205 Mimics and Inhibitors of the Long Non-Coding RNA ADPGK-AS1 in Treating Pancreatic Cancers

Aberrant expressions of miRNAs can induce carcinogenesis by enhancing the function of proto-oncogenes or inhibiting the tumor-suppressing genes (such as TP53). LncRNA can affect the cellular functions at three levels: 1) transcription; 2) post-transcription; 3) epigenetic—meaning the influence arises from non-genetic influences on gene expression [261]. Note also that lncRNA can function as competitive endogenous RNA interacting with encoding genes as well as non-coding miRNA.

The Zinc finger E-box binding homeobox 1 (ZEB1) is a transcription factor involved in cell differentiation and tissue development [262]. ZEB1 is also called E-cadherin transcriptional repressor. Intuitively, without adhesion proteins like E-cadherin, cells are detached, breaking the normal morphology of an organ. On the other hand, it has been found over ten years ago [263] that all five members of the microRNA-200 family (miR-200a, miR-200b, miR-200c, miR-205 miR-141, and miR-429) were markedly downregulated in cells that had undergone EMT in response to transforming growth factor (TGF)-β. Together, these microRNAs cooperatively regulate expression of the E-cadherin transcriptional repressors ZEB1 and ZEB2, factors previously implicated in EMT and tumor metastasis. Inhibition of the stated five microRNAs was shown to induce EMT in a process accompanied by upregulation of ZEB1 and/or ZEB2. More recently, the issue that ZEB1 could drive the EMT process has also been revisited and confirmed [264].

With such background, in the study of [265], an analysis of the interaction among non-coding RNA ADPGK-AS1, miR205, ZEB1 was carried out with *in vitro* and *in vivo* experimentation. Normal pancreatic ductal epithelial line HPDEC, human pancreatic cancer cell lines PANC-1, SW-1990, and embryonic kidney HEK-293T were employed. In the *in vivo* experiment, human pancreatic cells transfected with 1) miR-205-5p mimics, or 2) ADPGK-AS1 were injected in the mice models. The development of cancer was investigated in real-time. The findings of their project were a) ADPGK-AS1 and ZEB1 were upregulated in the

cancer cells, leading to the promotion of migration and invasion. b) ADPGK-AS1 was found to target miR-205-5p directly, as ceRNA (competing endogenous RNA) to promote the expression of ZEB. c) ADPGK-AS1 was found to suppress apoptosis. d) *In vivo* experimental result indicated that the average tumor weight is minimum when treated with miR-205 mimics. This weight became heavier when treated with a mixture of ADPGK-AS1 and miR-205 mimics. The average tumor weight became heaviest when treated with ADPGK-AS1. Mimics of antisense lncRNA ADPGK-AS1 as well as mimics of miRNA-205 are desirable for further preclinical trials.

20.7. Detection of Exosomes Containing miR-196-3p and miR-204-3p as Markers of Intraductal Papillary Mucinous Neoplasms, Mucinous Cystic Neoplasms, and Pancreatic Cancer

In the study of [266], exosomes were isolated from four cell lines 1) BxPC-3, 2) Capan-2, 3) PANC-1, 4) MIA PaPe-2. Exosomes (containing some micro mRNAs, called exo-mRNA) were also isolated from sera of 16 patients undergoing pancreatectomy. The tissues of their pancreas were kept for analysis. Based on levels of E-cadherin and vimentin, cells in lines 1) and 2) were found to be the epithelial type, whereas cells in 3) and 4) were of the mesenchymal type. Applying a genome-wide analysis of the exo-mRNA, miR-196b-3p, and miR-636 were identified as downregulated in the epithelial cells, whereas miR-204-3p, miR-3648, & miR-1497 were identified to be downregulated in mesenchymal type cells. Using the resected tissues and sera from patients with diagnosed pathology, a further analysis showed that miR-196-3p and miR-204-3p were differentially expressed in exosomes of intraductal papillary mucinous neoplasms (IPMN), mucinous cystic neoplasms (MCN), and pancreatic cancer (PC). The exosomes containing these two micro-RNAs may then be taken as markers of the three stages of the pathogenesis of EMT towards pancreatic cancer.

20.8. Inhibitors of lncRNA SNHG14 and lncMALAT-1 Which Targets the Protein EZH2 That Inhibits the Synthesis of Adhesion Protein E-Cadherin

In the investigation of [267] on the effects of the long non-coding RNA SNHG14, 58 pairs of pancreatic cancer tissues and adjacent normal tissues from patients undergoing surgical resection were obtained and measured by qRT-PCR. Quantities representing the capacities of cell proliferation and cell invasion were determined. Four human PDAC cell lines Panc1, Panc28, AsPC1, BxPC3, and a human pancreatic ductal epithelial cell line HPDE were used for *in vitro* studies. The main results were: 1) lncRNA SNHG14 expression was significantly upregulated in PDAC tissues. 2) lncRNA SNHG14 expression was associated with positive lymph node metastasis in patients (Table 1 of [267]). 3) lncRNA SNHG14 expression was also higher in the stated four PDAC cell lines compared

to that in the HPDE. 4) lncRNA SNHG14 was knocked down by transfection of Panc1 & Panc28 cells with interference RNAs *i.e.* si-SNHG14-1 and si-SNHG14-2. There was a significant increase of E-cadherin expression (implying cells are attached, tending to form an organ as normal), but a decrease of vimentin expression in the lncRNA SNHG14 knockdown cells. Since vimentin is an intermediate filament protein and the major cytoskeletal component of mesenchymal cells, low expression of vimentin implies the cells are progressing along anti-EMT direction. 5) Note that Enhancer of zeste homolog 2 (EZH2) is an essential component of the polycomb repressive complex 2 (PRC2), Remark that PRC2 has a role in X chromosome inactivation, in the maintenance of stem cell fate [268]. Aberrant expression of PRC2 has been observed in cancers [269]. There was evidence that EZH2 was recruited to the E-cadherin gene promotor by lncRN MALAT-1 in the study of 2016 [270]. In [267], there was further evidence that another long non-coding RNA SNHG14 would also interact with EZH2, in addition to the effects mentioned in 1) - 4). lncRNA SNHG14 and lncMALAT-1 (presumably detectable in body fluids) are early markers of pancreatic cancer progression, and antisense mimics of these two long non-coding RNAs can be considered as therapeutic measures of the disease.

21. Concluding Remarks

1) The proteins encoded by genes, in general, are members participating in signaling pathways involved in many physiological processes, including proliferation, differentiation, apoptosis, cell migration. During embryonic development, organ homeostasis and wound healing processes, cells migrate and collagen and proteins in the fascia are degraded and replaced; new blood vessels are grown; cells die and multiply. In healthy states, all these signaling pathways are regulated in the right time scale and right levels. Off-balance of some signaling pathways would lead to pathological states that are beyond homeostasis and repair. Mutations of the genes related to members of these pathways are the common causes of carcinogenesis.

2) The four major mutations in pancreatic cancers are aberrant activation of the gene KRAS, loss of functions of CDKN2A, SMAD4, TP53. The functions of the signaling pathways associated with such proteins encoded by these genes are discussed in **Sections (3, 4, 5, 6, and 7)**. One such protein, e.g. p53, can be related to more than one pathway. There are 8 pathways in **Sections (3-5)** alone. Moreover, disorders in other signaling pathways can lead to the progression of the disease—from inflammation, via different stages of endothelial-mesenchymal transition (**Section (15)**). More than ten pathways have been effectively discussed in order to understand the pathology and look for detection and treatment. Apart from the pathways related to the four frequently mutated genes mentioned above, other pathways discussed in this paper include the Hippo (**Section (6)**), Jak-STAT (**Section (8)**), NF-κB (**Section (9)**), Hedgehog (**Section (10)**), Notch (**Section (11)**), There can be cross-talks among proteins in differ-

ent pathways. All these 13 pathways can participate in pancreatic carcinogenesis.

3) As a mixed exocrine-autocrine organ, the pancreas is built of cells of different nature. During development, the ductal cells change their phenotype when they migrate to the duodenum and become the endothelial cells of the digestive system. Inherently, there are i) stem-like cells and ii) stellate cells in the organ. The high occurrence frequency of distant relapse following successful surgery suggests there is spread of cancer cells prior to surgery. We have discussed the early invasion of such cancer stem cells (cells i)) in **Sections (14, 17).** The metastasis of acinar type of ductal cancer cells is summarized in **Figure 17**. The invasion mechanism of PSCs and cancer cells is via invandopodium (**Section (16)** with **Figure 16**).

4) The stellate cells (PSC) play key pathological roles in the progression of tumorigenesis. They secrete the cell membrane-anchored digestive enzyme to degrade the collagen in the basal membrane of the pancreas and connective tissues surrounding, leaving room for cancer cells to migrate. PSCs synthesize excessive collagen as if doing a wound healing work; such "scarring" produces one main part of the stroma. PSCs also secrete abundant hyaluronan which attracts water to become a viscous interstitial fluid with high fluid pressure—this fluid together with the dense collagen fibrils is the main cause of drug resistance (**Figure 17**). The existence of the newly discovered calcium channel Piezo in PSC, acinar, islet cells strengthen the vicious cycle action initiated by PSCs and their cross-talks with the cancer cells of the organ.

5) The discovery of many non-coding micro RNA or their inhibitors that target the proteins or genes of the pathological pathways and advent in the delivery of pharmaceutical drugs (including such future RNA mimics) by nanoparticles gives us hope to deliver the treatment agents to the tumor site. We have discussed only a small portion of such discovery in the past decade or so.

6) The detection discovery of non-coding RNA, particularly the "sponge micro RNAs" enclosed within vesicles in body fluids is of prime importance. During the past several years, exosomal and non-exosomal micro RNAs have been detected body fluids such as i) blood [271] [272]; ii) saliva [273]; iii) cerebrospinal fluid [253]; iv) urine [274], and v) bile [275].

7) In the future, it is important to comply a database on the interaction characteristics between the micro RNAs (contained in exosomes detected in body fluids) and other RNAs of the healthy normal and that of the patients. Such information is not only useful for designing therapeutic treatments to target the relevant pathways at different stages, for pancreatic cancers, but is also useful for other diseases.

8) Following the above analysis and remarks, we can now state that manifestation of successive pathological states of pancreatic carcinogenesis can be found in compartments of the largest organ of the human body—the fascia. The following table (**Table 1**) gives a non-exclusive summary of some used drugs,

potential chemicals, vitamins, micro RNA mimics as early detection of the disease in body fluids and plausible therapeutic treatments of pancreatic cancers.

Table 1. Potential therapeutic agents for treatment or early detection of pancreatic cancers.

Drugs, chemicals, non-coding RNAs or their inhibitors as potential treatments, or as diagnostic markers	Proposed Basic action in brief	Sections in paper and related figures
Irisin (synthetic compound of this hormone has been achieved in small amount)	Target the AMPK-mTOR1 pathway	**(3.3)**
Gemcitabine and nimotuzumab	As monoclonal antibody of epidermal growth factor	**(4.5)**
Phenols, sulfonamides, and their analogs	Target Ras protein in Ras-Raf-Erk pathway	**(4.5)**
Abemaciclib	To treat hormone receptor-positive, human epidermal growth factor receptor 2-negative breast cancer; drug also applied to treat pancreatic cancers	**(5.2)**
DD44mT (di-2-pyridylketone 4,4-dimthyl-3-thiosemicarbazone), Fe chelator	Activates protein Ndrg1 and hence cyclic-dependent kinase inhibitor p21	**(5.3)**, Figure 7, Figure 9
DFO (desferrioxamine), Fe chelator	As above	As above
311(2-hydroxy-1-naphthylal dehydro-isonicotinoyl hydroazone)	As above	As above
RC68, a humanized anti-EGFR monoclonal antibody, as an antibody-drug conjugate	A monoclonal antibody chemically linked to a drug that is toxic when the ingredient is released inside the target cell.	**(8.5)**, Figure 7, Figure 9
APX3330, compound	Regulate activities of hypoxia-inducible factor 1 α (HIF-1 α), NF-κB, and function of Jak-STAT3 pathway	**(9.4)**, Figure 2, Figure 7, Figure 10
SLC-0111, compound	As above	**(9.4)**
Combination of cyclopamine and paclitaxel delivered by nan-particles	As an inhibitor of the Hedgehog pathway	**(10.3)**, Figure 11
RO4929097, compound	As an inhibitor of γ-secretase in Notch pathway	**(3.2)**, Figure 12
ATRA (Vitamin A)	Attenuate endothelial-mesenchymal transition of stellate cell	**(18.1)**, Figure 13
Tocotrienols β, γ, δ (sub-family of vitamin E)	Suppress NF-κB mediated inflammation	**(18.2)**, Figure 10
Calcipotriol (Ca), Vitamin D receptor ligand	Reduce inflammation and fibrosis	**(18.3)**
PEGPH20 (Pegvorhyaluronidase alfa) together with other anti-cancer drugs	Reduce the concentration of interstitial fluid in tumor stroma	**(18.4)**, Figure 17
Micro RNA 96 mimics	Target Ras-Raf-Mek-Erk pathway	**(19.2)**, Figure 3
Micro RNA 34 mimics	Target Notch pathway and target anti-apoptotic Bcl-2 protein	**(19.3)**, Figure 12
Micro RNAs 21 plus 221 mimics	Treat metastatic pancreatic cancer cells in general	**(19.4)**
Micro RNA 146a mimics	Downregulates EGFR, downregulates NF-κB, upregulates PTEN	**(19.5)**, Figure 2, Figure 7, Figure 9, Figure 10
Antisense non-coding circular RNA IARS mimics	Reducing endothelial permeability to attenuate cancer invasion	**(20.2)**
Antisense non-coding circular RNA LDLRAD3 mimics	Treats pancreatic cancer cells in general	**(20.3)**
Antisense non-coding circular RNA_100782	Targets IL6-Jak-STAT3 pathway	**(20.4)**, Figure 7

Continued

Antisense non-coding circular RNA circ_0007534 mimics	Treat PDAC by releasing the inhibition of Bcl-2-Caspase pathway	**(20.5)**, **Figure 3** of [3] noting Bax is a member of Bcl family
Micro non-coding RNA 205 mimics	Targets protein ZEB1 to reduce cancer cells detachment	**(20.6)**
Antisense long non-coding RNA ADPGK-AS1 mimics	Promotes function of Micro non-coding RNA 205 directly	**(20.6)**
Detection in body fluids the exosomes containing miR-196-3p and miR-204-3p	as markers of intraductal papillary mucinous neoplasms, mucinous cystic neoplasms, and pancreatic cancer	**(20.7)**
Detection of long non-coding RNA SNHG14 and MALAT-1	These RNAs promote the function of protein EZH2 that inhibits synthesis of adhesion protein E-cadherin (condition of metastasis)	**(20.8)**
Antisense of long non-coding RNAs SNHG14 and MALAT-1 mimics	Downregulate function of protein EZH2 which inhibits the synthesis of adhesion protein E-cadherin	**(20.8)**

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

Section (1) - Section (5)

PDAC = pancreatic ductal adenocarcinoma

KRAS = Kirsten rat sarcoma viral oncogene homolog

CDKN2A = cyclin-dependent kinase inhibitor 2A, or p16

TP53 = tumor protein 53; in fact, the encoded protein p53 is a strong tumor suppressor

SMAD4 = mothers against decapentaplegic homlog-4

RTKs = receptor tyrosine kinases

PI3K = Phosphatidylinositol-3 kinase

PIP_2 ($PI(4,5)P_2$) = Phosphatidylinositol 4,5-bisphosphate

PDK1 = phosphoinositide-dependent protein kinase-1, or the master kinase

ER = endoplasmic reticulum

S6K1 = ribosomal protein kinase β-1 P70-S6K

Akt (PKB) = protein kinase B (PKB), a serine-threonine protein kinase

mTOR1,2 = mammalian target of rapamycin complex 1,2

Rheb = Ras homolog enriched in brain

S6K1 = ribosomal protein kinase β-1 P70-S6K

HSP = heat shock protein; HSF = heat shock factor

AMPK = 5' adenosine monophosphate-activated protein kinase

Tuberous TSC1, 2 = scherosis complex (unit 1 or 2); TSC1 is also called harmartin and TSC2 is also named tuberin

FOXO = Forkhead box protein (with members 1,2,3)

PTEN = phosphatase and tensin homolog protein; phosphatase and tensin homologue deleted on chromosome-10

LKB1 = liver kinase B1 protein

VEGF = vascular endothelial growth factor

4E-BP1 = eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1, encoded by the gene EIF4EBP1

HIF-1α = hypoxia-inducible factor-1α

eNOS = endothelial nitric oxide synthase

NO = nitric oxide

FNDC5 = fibronectin type III domain-containing protein 5

PPAR-γ = peroxisome proliferator-activated receptor-γ

UCP1 = Uncoupling protein 1

KAHA = ketoacid-hydroxylamine

PLC-epsilon1 = pholipase enzyme, type epsilon1

DAG = diacylglycerol

Ras = (encoded by the gene KRAS) which is a member of a class of proteins called small GTPase

Raf = Rapidly Accelerated Fibrosarcoma, a member of serine/threonine-specific protein kinases related to retroviral oncogene

Mek = Mitogen-activated protein kinase

Erk = Extracellular signal-regulated kinase

RSK = Ribosomal s6 family kinases

PanIN = intraepithelial neoplasia

α-SMA = α-smooth muscle actin

Fak = focal adhesion kinase

VASP = Vasodilator-stimulated phosphoprotein

Rho A = ρ factor, type A, is a prokaryotic protein

Rock = Rho-associated protein kinase

MLCK = Myosin light-chain kinase

Piezo1,2 = Piezo-type mechanosensitive ion channel component 1,2

PSCs = pancreatic stellate cells

VASP = Vasodilator-stimulated phosphoprotein

CDKN2A = cyclin-dependent kinase inhibitor 2A

CDK2,4,6 = cyclin dependent kinase 2,4,6

Hdm2 = human double minute 2

p21 = cyclin-dependent kinase inhibitor 1

pRB = retinoblastoma protein, the key "check-point" protein for cell cycle

E2F = protein encoded one of the genes that codifies a family of transcription factors in higher eukaryotes. Three of them are activators: E2F1, 2, and E2F3a. Six others act as suppressors: E2F3b, E2F4-8

DFO = desferrioxamine

311 = 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone

NDRG1 = N-myc downstream regulated gene 1

Dp44mT = di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone

Section (6) - Section (10)

YAP1 = yes-associated protein 1, also known as YAP or YAP65

TAZ = (transcriptional co-activator with a PDZ-binding domain; also known as WW domain containing transcription regulator 1, or WWTR1.The "classical formal name" is Tafezzin

TEAD protein = transcriptional enhanced associate domain protein

VGLL4 = Transcription cofactor vestigial-like protein 4

Mdm2 = a member of the E3 Ubiquin ligase

LATS1 = Large tumor suppressor kinase 1 (an enzyme with four members in the family, labeled 1-4)

Ptpn14/PTPN14 = protein tyrosine phosphatase non-receptor type 14

PanIN = intraepithelial neoplasia

Furin = a serine protease which can cleave a plethora of proteins at polybasic recognition motifs

Kibra = kidney and brain expressed protein (KIBRA) or WW domain-containing protein 1 (WWC1)

c-JNKs = c-Jun N-terminal kinases

LIMID1 = LIM domain-containing protein 1

RORα = orphan nuclear receptor α

AICAR = 5-Aminoimidazole-4-carboxamide ribonucleotide

SMAD2,3,4 = mothers against decapentaplegic homlog-2,3,4

PAI-1 = Plasminogen activator inhibitor-1

Akap12 = protein A-kinase anchor protein 12

ITGB6 = Integrin beta-6, the β6 subunit of the integrin αvβ6

LAP = Latency-associated peptide

ALDH1,2,3 = aldehyde dehydrogenase type 1,2,3

BMP-4 = bone morphogenetic protein, type 4

GLIS3 = Zn-finger transcription factor GLI-similar-3

EGF = epidermal growth factor

Jak = Janus kinase, with four members: Jak1, Jak2, Jak3, Tyk2

Stat protein = signal transducer and activator of transcription protein

SOC = suppressor of cytokine

SNARE = soluble N-ethylmaleimide-sensitive fusion attachment protein receptor and syntaxin6 is a member of this family

COPI = coat protein complex I", vesicle

ARF = GTPase ADP-ribosylation factor

NF-κB = Nuclear Factor-kappa B

MCP-1(CCL2) = chemokines monocyte chemoattractant protein 1

CXCL1 = chemokine C-X-C motif-ligand 1

Lymphotoxin = a member of the Tumor Necrosis Factor (TNF) family of cytokines

IKK = IκB kinase, consists of two members (IKKα and IKKβ)

NEMO/IKKγ = a regulatory subunit named NF-κB essential modulator

IKKγ = inhibitor of nuclear factor kappa-B kinase subunit γ

NIK = NF-κB-inducing kinase, whose activity triggers off the non-canonical pathway.

LTβR = Lymphotoxin β receptor

TNFα = Tumour Necrosis Factor α, an inflammatory cytokine produced by macrophages/monocytes

COX-2 = cyclooxygenase-2

MCP-1 = chemokines monocyte chemoattractant protein 1

CXCL1 = chemokine (C-X-C motif) ligand 1, recruiting immunity cells during angiogenesis and inflammation

Icam1 = (Inter) cellular adhesion protein 1

Vcam1 = vascular cell adhesion protein 1

iNOS = inducible nitric oxide synthase iNOS

Bcl-2 = B-cell lymphoma 2

iNOS = inducible nitric oxide synthase

Collagenase1 degrades collagens of certain types

IκBα = inhibitory κ protein type α

NFκB1, 2 = NF-κB transcription factor type1, 2 respectively

NIK = NF-κB-inducing kinase

LT-βR = Lymphotoxin receptor type β

RelA = v-rel reticuloendotheliosis viral oncogene homolog A (avian), is also called p65

RelB = v-rel reticuloendotheliosis viral oncogene homolog B (avian)

Ca = carbonic anhydrase, Ca9 is driven by HIF-1 activity

APE1/Ref-1 = Apurinic/Apyrimidinic Endonuclease-1-Reduction/oxidation Effector Factor 1

APACHE II = Acute Physiologic Assessment and Chronic Health Evaluation II

Patch 1,2 = Protein patched homolog 1,2

Smo = Smoothened protein in the hedgehog pathway

Sufu = The suppressor of fused homolog, involved in the hedgehog pathway

Gli1 = protein containing five successive repeats of a zinc finger which can bind to DNA for transcription

Shh = Sonic hedgehog

Ihh = Indian hedgehog

Dhh = Desert hedgehog

Myc = member of the family first established after the discovery of homologybetween an oncogene carried by the Avian virus, MyelocytomatosisBcl-2 = B-cell lymphoma 2, a regulator of apoptosis

Sox2 = sex-determining region Y-box 2, a transcription factor that is essential for maintaining self-renewal stem cells

IPMN = intraductal papillary mucinous neoplasm

M-CPA/PTX = a polymeric micelle formulation containing cyclopamine (CPA), and a cytotoxic chemotherapy drug paclitaxel (PTX)

Section (11) - Section (15)

ADAM10 = Disintegrin And Metalloproteinase Domain-Containing Protein 10

Nicd = intracellular domain of the Notch receptor

CSL(CBF-1) = DNA binding protein or transcriptional regulator, alias Suppressor of Hairless, Lag-2

MAML1 = mastermind-like protein 1

Hes1 = hairy and enhancer of split-1, a transcription factor

Hey1 = Hairy/enhancer-of-split related with YRPW motif protein 1

COX 2 = Cyclooxygenase 2, is an enzyme responsible for the synthesis of prostaglandins

MMPs = The enzymes matrix metalloproteinases

ESA = epithelial surface antigen

CTGF = Connective tissue growth factor

PDGF = Platelet-derived growth factor

EGCG = green tea polyphenol epigallocatechin gallate

PDX1 = pancreatic and duodenal homeobox 1

ZEB1 = Zinc Finger E-Box Binding Homeobox 1, a transcriptional repressor

that has been identified as an inducer of EMT

ALDH1 = aldehyde dehydrogenase, member 1

PACs = pancreatic acinar cells

PDCs = pancreatic ductal cells

PanIN = intraepithelial neoplasia state

PDX1 = pancreatic and duodenal homeobox 1

shRNA = short hairpin RNA, an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference

Snail = a member of a family of transcription factors that promote the inhibition of the adhesion molecule E-cadherin to regulate epithelial to mesenchymal transition (EMT)

Section (16) - Section (21)

ATRA = All-trans-retinoic acid

TRF = tocotrienol rich fraction

RHAMM = Hyaluronan mediated motility receptor (motility)

VDR = Vitamin D receptor

PEGPH20 = Pegvorhyaluronidase alfa

miRNA = microRNA

ELF3 = transcription factor E74-like factor 3

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

SP = small population

HPDE = human pancreatic ductal epithelial cells

MTA-1,2 = metastasis-associated protein MTA-1,2

BITC = Benzyl isothiocyanate

EVs = extracellular vesicles

ncRNA = noncoding RNA

circRNA = circular RNA

piRNAs = piwi-interacting RNAs

ceRNA = competing endogenous RNA

lncRNA = long non-coding RNA

IPMN = intraductal papillary mucinous neoplasms

MCN = mucinous cystic neoplasms

ZEB1,2 = Zinc finger E-box binding homeobox 1,2

EZH2 = Enhancer of zeste homolog 2

PRC2 = Polycomb repressive complex 2

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