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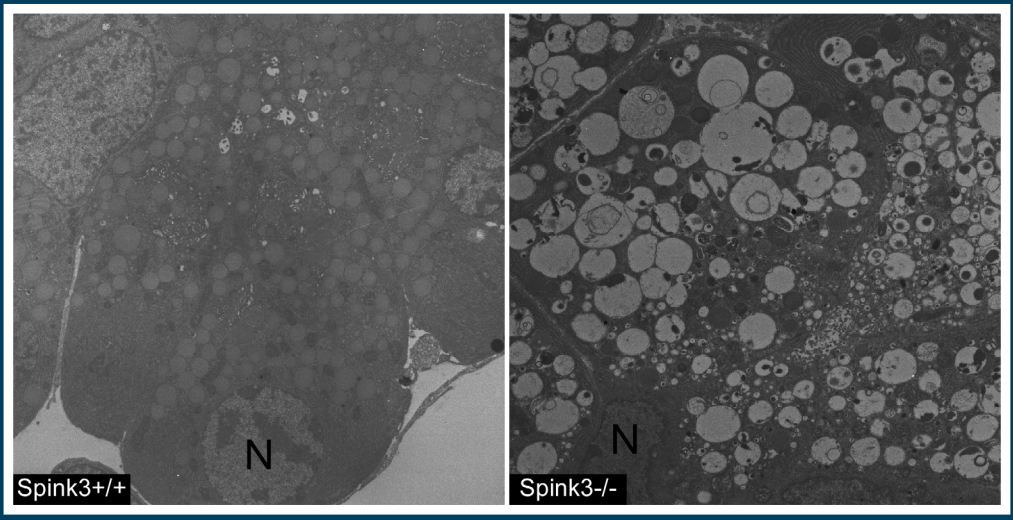

RECENT ADVANCES IN PANCREATOLOGY

Topic Editor
Atsushi Masamune

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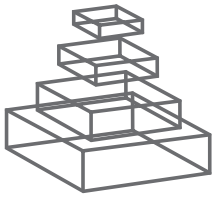

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RECENT ADVANCES IN PANCREATOLOGY

Topic Editor:
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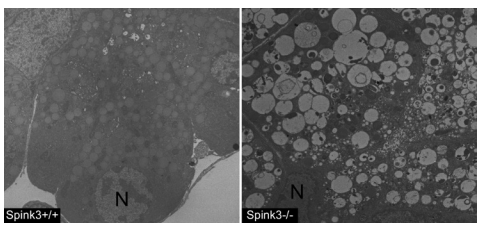

The pancreatic acinar cells of Spink3 deficient mice. Many vacuoles, autophagosomes, are appeared in Spink3 deficient pancreatic acinar cells (Right panel). N, nucleus of the acinar cell.

Figure taken from: Ohmuraya M, Sugano A, Hirota M, Takaoka Y and Yamamura K (2012) Role of intrapancreatic SPINK1/Spink3 expression in the development of pancreatitis. Front. Physio. 3:126. doi: 10.3389/fphys.2012.00126

Pancreatic diseases include intractable ones including acute and chronic pancreatitis, and pancreatic cancer. In recent years, great advances have been made in the field of pancreatology, including the pathogenesis, diagnostic modalities, and development of novel therapeutic interventions.

It has been established that pancreatic stellate cells play a pivotal role in the development of pancreatic fibrosis in chronic pancreatitis as well as in pancreatic cancer known as desmoplastic reaction. Although it might be still controversial, accumulating evidence has shown that interaction between pancreatic stellate cells-cancer cells contribute to the progression of pancreatic cancer through the increased proliferation and migration, and production of cytokines and extracellular matrix components. In addition, pancreatic stellate cells lead to the resistance to chemotherapy and radiation therapy. Pancreatic stellate cells attract the researchers as a novel therapeutic target of pancreatic cancer.

Genetic studies have shown that mutations in the trypsin-related genes such as cationic trypsinogen (*PRSS1*) gene and the serine protease inhibitor, Kazal type 1 (*SPINK1*) gene are associated with pancreatitis. In general, each of these factors appears to limit trypsin activation or enhance inactivation, and is believed to increase intrapancreatic trypsin activity and predispose to pancreatitis when the gene is mutated. These results have supported a concept that pancreatic protease/anti-protease plays pivotal roles in the pathogenesis of pancreatitis. In addition, genetic studies focusing on phenotypic variances would provide us with important information how genetic variants would affect the phenotypic variances.

Autophagy is an intracellular bulk degradation system in which cytoplasmic components are directed to the lysosome/vacuole by a membrane-mediated process. Recent studies have highlighted a role of autophagy in acute pancreatitis. Using a conditional knockout mouse that lacks the autophagy-related (Atg) gene *Atg5* in the pancreatic acinar cells, autophagy exerts a detrimental effect in pancreatic acinar cells by activation of trypsinogen to trypsin. A theory in which autophagy accelerates trypsinogen activation by lysosomal hydrolases under acidic conditions, thus triggering acute pancreatitis in its early stage.

The epithelial-mesenchymal transition is a developmental process that allows a polarized epithelial cell to undergo multiple biochemical changes that enable it to assume a mesenchymal phenotype. The phenotype associated with epithelial-mesenchymal transition includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix components. In addition to its role in development, tissue regeneration, and fibrosis, epithelial-mesenchymal transition is now considered as a critical process in cancer progression. Induction of epithelial-mesenchymal transition in cancer cells results in the acquisition of invasive and metastatic properties. Epithelial-mesenchymal transition could be an important mechanism in the progression of pancreatic cancer and its poor prognosis.

Autoimmune pancreatitis is a unique form of pancreatitis in which autoimmune mechanisms are suspected to be involved in the pathogenesis. There is accumulating study to deal with this new disease concept. In addition to these topics, we have selected several topics in pancreatology, focusing on recent studies increasingly deepening our knowledge in both basic and clinical researches.

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Recent advances in pancreatology

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Keywords: autoimmune pancreatitis, cystic fibrosis transmembrane conductance regulator, epithelial-mesenchymal transition, fibrosis, pancreatic cancer, pancreatic stellate cells, pancreatitis, trypsin

Pancreatic diseases, including acute and chronic pancreatitis (CP) and pancreatic cancer, are intractable. In recent years, great advances have been made in the field of pancreatology: the pathogenesis, diagnostic modalities, and development of novel therapeutic interventions. This E-Book is derived from the *Frontiers in Physiology section Gastrointestinal Sciences* Research Topic entitled "Recent Advances in Pancreatology." Its goal is to bring established experts to present state-of-art studies in pancreatic physiology, and to provide ideas on different approaches useful to research challenges. This book presents nine contributions, in the form of reviews, hypothesis and theory article, and original article.

The articles can be mainly classified into three categories: pancreatitis, autoimmune pancreatitis (AIP), and pancreatic cancer. To date, several pancreatitis-associated genes have been identified such as the cationic trypsinogen (*PRSS1*) gene and the serine protease inhibitor, Kazal type 1 (*SPINK1*) gene have been identified. The review article by Whitcomb (2012), who had originally identified the mutations in the *PRSS1* gene as a cause of hereditary pancreatitis, presented a new framework for the interpretation of genetic variants in patients with CP based on modeling and simulation of physiological processes with or without genetic, metabolic, and environmental variables. This framework is especially important when we deal with billions of sequencing data obtained by the next-generation sequencers. Ohmuraya et al. (2012) reviewed the old and new roles of the intrapancreatic *SPINK1/Spink3* expression in the development of pancreatitis. In addition to the established roles as a trypsin inhibitor, SPINK1 is involved in autophagy, cell growth, and cell death in pancreatic acinar cells and cancer cells. The precise molecular mechanisms of intraductal pancreatic stone formation in CP are largely unknown. Ko et al. (2012) reported that the mislocalization of the cystic fibrosis transmembrane conductance regulator (CFTR) is a cause of protein plug formation, leading to the formation of pancreatic stones in CP. CFTR was largely mislocalized to the cytoplasm of pancreatic duct cells in CP, including AIP. Because corticosteroids normalized the localization of CFTR to the proper atypical membrane, Ko et al. concluded that corticosteroids might be useful to prevent protein plug and stone formation in patients with CP.

Pancreatic stellate cells (PSCs) have attracted increasing attention from researchers. Apte et al. (2012), who originally identified PSCs in rats, reviewed the current knowledge about the roles of PSCs in normal and diseased pancreas. In healthy pancreas, PSCs may maintain normal tissue architecture and act as progenitor cells, immune cells, and an intermediary in exocrine secretion in the pancreas. It has been established that PSCs play a critical role in pancreatic fibrosis, a consistent histological feature of CP and pancreatic cancer. PSCs interact closely with pancreatic cancer cells facilitating cancer progression. Several therapeutic strategies targeting PSCs have been examined in experimental models of CP and pancreatic cancer, although their clinical usefulness remains a challenge.

AIP has been increasingly recognized as a distinctive type of pancreatitis with a presumed autoimmune etiology. The molecular mechanisms responsible for the development of AIP are largely unknown. As reviewed by Haruta et al. (2012), the induction of AIP-like pancreatic lesions by viral and bacterial components in mice suggests a role of commensal flora in the development of AIP. From the clinical point of view, Kamisawa et al. (2012) gives an overview of AIP including its concept, the international consensus diagnostic criteria (ICDC) and standard therapeutic regimen. The goals of the ICDC for AIP are to develop criteria that can be applied worldwide, taking marked differences in practice patterns into consideration, to safely diagnose AIP and avoid misdiagnosis of pancreatic cancer as AIP. According to the ICDC, AIP has been classified into two subtypes: type 1 related with IgG4 (lymphoplasmacytic sclerosing pancreatitis) and type 2 with granulocytic epithelial lesion (idiopathic duct-centric CP). The ICDC would contribute to further clarification of the clinical features, pathogenesis, and natural history of AIP around the world.

Lastly, three articles focus on pancreatic cancer. The epithelial-mesenchymal transition (EMT) is a developmental process that allows a polarized epithelial cell to undergo multiple biochemical changes that enable it to assume a mesenchymal phenotype. The phenotype associated with EMT includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix components. Thus, EMT plays a critical role in cancer progression. Hamada et al. (2012) reviewed the regulators of EMT in pancreatic cancer. In addition to multiple cytokines, growth factors and downstream transcriptional factors, non-coding RNA including microRNA contributes to EMT. Satoh et al. (2012) focus on MSX2, a member of the homeobox genes family, as an inducer of EMT in pancreatic cancer. MSX2 enhances the malignant phenotypes of pancreatic cancer, and evaluating MSX2 levels might be useful to differentiate pancreatic cancer from CP. Mizuno et al. (2013) describe that leucine-rich-repeat-containing G-protein-coupled receptor 5 (LRG5), a marker of intestinal stem cells, was expressed in the

cytoplasm of pancreatic cancer cells. LRG5 was not co-localized with CD133, a cancer stem cell marker, in either neoplastic or non-neoplastic tissues. Further studies are required whether LRG5 expression is useful as an indicator of the prognosis.

In summary, the articles in this E-book will contribute to deepening our knowledge in both basic and clinical research in the field of pancreatology. Further understanding will underpin rational approaches to the treatment of intractable pancreatic diseases.

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Framework for interpretation of genetic variations in pancreatitis patients

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Chronic pancreatitis (CP) is defined by irreversible damage to the pancreas as a result of inflammation-driven pancreatic tissue destruction and fibrosis occurring over many years. The disorder is complex, with multiple etiologies leading to the same tissue pathology, and unpredictable clinical courses with variable pain, exocrine and endocrine organ dysfunction, and cancer. Underlying genetic variants are central CP susceptibility and progression. Three genes, with Mendelian genetic biology (*PRSS1, CFTR,* and *SPINK1*) have been recognized for over a decade, and little progress has been made since then. Furthermore, application of high-throughput genetic techniques, including genome-wide association studies (GWAS) and next generation sequencing (NGS) will provide a large volume of new genetic variants that are associated with CP, but with small independent effect that are impossible to apply in the clinic. The problem of interpretation is using the old framework of the germ theory of disease to understand complex genetic disorders. To understand these variants and translate them into clinically useful information requires a new framework based on modeling and simulation of physiological processes with or without genetic, metabolic and environmental variables considered at the cellular and organ levels, with integration of the immune system, nervous system, tissue injury and repair system, and DNA repair system. The North American Pancreatitis Study 2 (NAPS2) study was designed to capture this type of date and construct a time line to understand and later predict rates of disease progression from the initial symptom to end-stage disease. This effort is needed to target the etiology of pancreatic dysfunction beginning at the first signs of disease and thereby prevent the development of irreversible damage and the complications of CP. The need for a new framework and the rational for implementing it into clinical practice are described.

Keywords: pancreatitis, cystic fibrosis, genetics, next generation sequencing, GWAS, systems biology, inflammation

INTRODUCTION

Classic Mendelian genetics plays a small but significant role in chronic pancreatitis (CP). Three syndromes are well described including autosomal dominant hereditary pancreatitis (HP), autosomal recessive cystic fibrosis (CF), and autosomal recessive familial pancreatitis from homozygous or compound heterozygous *SPINK1* mutations. The biology and pathology of these genes, plus lower risk genes chymotrypsin C (*CTRC*) and calcium sensing receptor (*CASR*), have recently been reviewed (Teich and Mossner, 2008; Chen and Ferec, 2009; Whitcomb, 2010; Larusch and Whitcomb, 2011; Chen and Ferec, 2012). However, these syndromes make up less than 10% of CP cases in most clinical populations.

It is now recognized that non-Mendelian, complex genetic conditions are far more common and therefore of greater relevance. Complex genetics include gene-environment or gene–gene interactions, or more complex combinations and variable interactions. Any one of these disease-associated factors is neither sufficient nor necessary to cause pancreatitis alone, but can contribute to the disease or its complications when present within the right context. Patients with complex diseases rarely come from large families. Rather, the disease appears to be sporadic or occurring in only one or two other family members. Demonstrating the etiologic basis of complex genetic disorders is much more difficult than Mendelian disorders.

The initial excitement of the discovery of three major pancreatitis susceptibility genes between 1996 and 2000 was followed by slow progress in understanding pancreatic genetics, which reflects the depth of the problem and the large number of patients necessary to understand these complex interactions. Two major genome-wide association studies (GWAS) have been completed (one in Germany, and another in the United States) and the results will soon be reported. What is clear is that the results will either apply only to a small subset of patients, or will be important as cofactors or modifiers in more complex interactions. However, what is needed is a new framework from which to interpret this data. The focus of this article is to describe the old framework and its' limitations, provide rationale for a new framework, and give

examples of how this new framework can now be applied to clinical care.

THE OLD FRAMEWORK FOR INTERPRETING INFLAMMATORY DISORDERS

In science and medicine, a framework, or paradigm, is a theoretical or conceptual structure for defining and organizing information and relationships within a system. Rules and models within a framework are used to understand the relationship and interaction between the components, and these lead to predictions about processes and outcomes within the larger framework.

The paradigm for western medicine in the twentieth century is the germ theory of disease. The premise is that a single pathologic factor causes complex disorders. The germ theory was developed following technical advances of the compound microscope (allowing bacteria to be observed), culture and sterilization techniques (e.g., work of Lister and Pasture), epidemiologic evidence of infections causing disease (e.g., John Snow and the cholera epidemic in London coming from the Broad street pump), and the work of Koch to define the process of proving that an agent causes a disease (Koch's postulates).

Twentieth century Western medicine was built on the germ theory framework. Definitions of various diseases relied on tissue pathology which was expected to reveal the underlying infectious or parasitic agent causing inflammation or cancer. If there was inflammation without infection, then the disorder was defined by the type and duration of inflammation, with the expectation that research, using Koch's postulates, would eventually reveal the etiologic factor. From a clinical setting, combinations of signs and symptoms were used as surrogate markers of underlying pathology, and the idea of "functional" syndromes described clinical complaints when there was obvious tissue pathology. Thus, most medical disorders are classified by pathology rather than etiology, and this framework is the basis of modern disease taxonomy (e.g., ICD-9, ICD-10 codes).

Twentieth century biomedical research was also built on the germ theory framework. The scientific method taught in medical schools following the Flexner Report of 1910 (Flexner, 1910) was developed for identifying a *single* factor that caused a complex disease. The conceptual framework led to the process of rapidly evaluating a series of potential independent factors that were either included or excluded as the cause of disease based on *simple statistical tests* (null-hypothesis significance testing). The problem of experimental variance was addressed by increasing study size so that the effect of the primary etiologic factor within a *population* of subjects could be clearly identified. The result was a rapid progress in understanding, defining, and organizing infectious diseases, toxic agents, and Mendelian genetic traits. In each of these cases, a single factor was responsible for a complex disease syndrome.

The optimism of twentieth century Western medicine and the "scientific method" following the Flexner report diminished in the latter decades of the twentieth century when the simplistic approach failed to identify single etiologic factors for chronic inflammatory diseases, functional disorders, and cancers. Four examples of these failures have been highlighted elsewhere (Whitcomb, 2012), and are summarized here.

TISSUE IS THE ISSUE

A major thrust of twentieth century Western medicine was the development and improvement of minimally invasive techniques to obtain tissue samples in living patients since this was the basis of disease diagnosis and treatment. Indeed, methods to obtain biopsies by endoscopic techniques, fine needle aspirates guided by CT, ultrasound and other techniques, laparoscopy and high-resolution imaging techniques were perfected. However, sophisticated methods of getting a tissue biopsy that were interpreted with early twentieth century criteria *did not* lead to significant improvement in medical management.

FAILED REPRODUCIBILITY

A second problem was identified when larger and more sophisticated clinical studies were conducted to define the etiology of more complex chronic diseases. The results of small and medium sized studies were often noted to be conflicting or non-reproducible. It was suspected that the epidemiological techniques that were used in many of the studies were flawed, and experimental design questions were raised. Evidence-based medicine (EBM) was added to the scientific approach to address these issues (Timmermans and Mauck, 2005). Among the many problems of EBM is the fact that it relies on data that was collected in previous trials that were designed based on theories that were often 15–20 years out of date. Furthermore, the strict criteria that are necessary for developing EBM guidelines were found to exclude large numbers of patients and those disorders that fell outside of the mean of the population without insight. And, depending on the available data and criteria, different groups who use EBM to develop guidelines often come to different conclusions. In reality, EBM is really more of a medical literacy exercise than a way to provide new insights into complex diseases (Wyer and Silva, 2009). EBM that remains within the germ-theory paradigm will primarily be of value in simple diseases, where it rarely provides any new insights.

MINIMAL EFFECTS OF COMMON SNPs

There has been great hope that mapping, and then sequencing the human genome would identify the gene that causes "your-favorite-disease". A common approach was the GWAS, which was developed to quickly identify the genetic variants causing a variety of disorders and diseases (Witte, 2010). The approach, however, was developed within the framework of the germ theory of disease, and the scientific method of null-hypothesis significance testing (i.e., the frequency of each genetic variant is compared between cases and controls using a simple chi square or exact test, with "significance" based on a study power calculation, adjusted for the number of other SNPs tested). However, it was discovered that complex diseases have *many* genetic variants that are statistically associated with disease, but they only have a very small effects, and the presence of absence of a SNP in a patient usually has no clinical relevance. Furthermore, to determine these small effect genetic variants required huge numbers of patients, with the expectation of a minimum of 1000 cases and 1000 controls (Ioannidis et al., 2001), and still suffers from false discovery (Benjamini and Hochberg, 1995). In more complex common diseases, tens of thousands of patients

are being included in each arm of the study (Nettleton et al., 2010). However, the additional data is not bringing further insight into the disease in a way that provides clinically fashionable insights.

INTERPRETING DATA WITHOUT STATISTICS

The final technological breakthrough is next generation sequencing (NGS). This technology has the potential of rapidly sequencing an individual's entire DNA sequence for a few thousand dollars. The problem with NGS is that hundreds to thousands of unexpected genetic variants are discovered in each person's DNA sequence, and it is impossible to demonstrate the effect of each variant based on statistical methods. Together, these technology breakthroughs illustrate the inadequacy of the twentieth century western medicine disease paradigm interpretation of complex disorders in a germ theory model.

THE NEW FRAMEWORK FOR TWENTY-FIRST CENTURY MEDICINE

The great frontier in CP is applied physiology. The new framework needed for medicine is based on integrative physiology, cell biology, systems modeling, and simulation of biological processes in individuals where multiple variables associated with various components of a system, or the external forces that influence them, are considered in individual patients (i.e., individualized or personalized medicine). The need to move away from research based on null-hypothesis significance testing and toward modeling is being recognized (Rodgers, 2010), but the current approaches of systems biology at a molecular level are likely unnecessary in disease modeling (Whitcomb, 2012). Furthermore, the germ theory of disease does not need to be abandoned. It needs to be placed in the context of the new framework as a situation where the number of variants resulting in disease equals one. A personalized medicine approach is needed when a syndrome is complex such that multiple etiologies or combination of factors lead to the same pathology, when the same pathology leads to multiple outcomes and/or when the results of interventions are unpredictable. Therefore, they are needed for chronic inflammatory diseases such as CP, functional disorders such as chronic pain in minimal change pancreatitis, and cancers including pancreatic cancer. Personalized medicine focuses on disease mechanism rather than association; it relies on modeling and simulation rather than classification, but it will be able to provide guidance for individuals rather than for subsets of a population.

NORTH AMERICAN PANCREATITIS STUDY 2 (NAPS2)

North American Pancreatitis Study 2 (NAPS2) is multicenter study that was designed by the author in the late 1990s in anticipation of future modeling in simulation approaches that might *prevent* CP (Whitcomb et al., 2008). Rather than using traditional classification approaches to CP the NAPS2 program took a broad view, envisioning pre-existing risk, stochastic events initiating an inflammatory process that was manifest clinically by episodes of recurrent acute pancreatitis or recurrent pain [i.e., the sentinel acute pancreatitis event (SAPE) hypothesis model (Whitcomb, 1999; Yadav and Whitcomb, 2010)]. Continuation and variations of inflammatory progresses then resulted in a constellation of variations in specialized cell and systems with dysfunctions recognized as of different clinical complications. Activation of pancreatic stellate cells leads to fibrosis. Acinar cell loss or dysfunction results in diminished digestive enzyme production with maldigestion. Islet cell dysfunction leads to endocrine failure with diabetes. Nerve injury and pathologic adaptation leads to chronic pain syndrome, and abnormal transition of inflamed pancreatic acinar-duct cells leads to pancreatic cancer (**Figure 1**).

Prior to NAPS2, there was no systematic way to classify susceptibility factors, other risk factors or combinations of factors. An etiologic-based classification system had to be invented which is known as the TIGAR-O system (Etemad and Whitcomb, 2001), which classifies factors as either Toxic-metabolic (e.g., alcohol, smoking), Idiopathic (e.g., tropical pancreatitis, early or late onset), Genetic, Autoimmune, Recurrent-acute or severe (e.g., 95% pancreatic necrosis in acute pancreatitis) or Obstructive. This is contrast to the definitions of the Marseille classification system that defines acute pancreatitis and CP by traditional clinical and pathologic criteria (Sarles, 1965; Singer et al., 1985) and the Cambridge classification system (Sarner and Cotton, 1984) which defines ages of progressive destruction but provides no insight into the mechanism of disease.

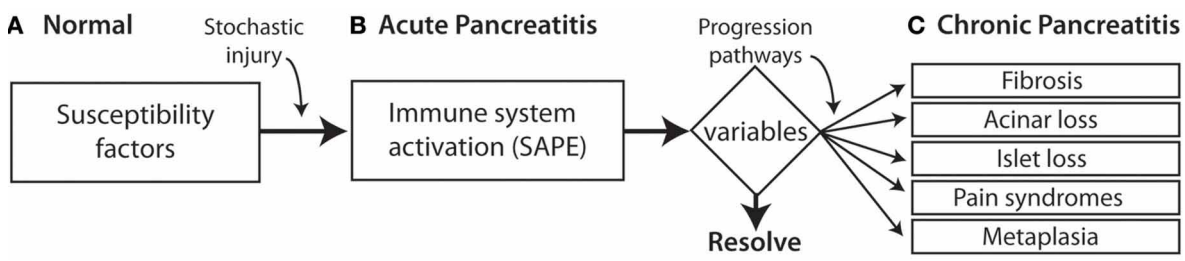

FIGURE 1 | SAPE progression model. (A) Normal histology. Patients may have genetic risk factor and alcoholism but without pancreatic inflammation. **(B)** Acute pancreatitis is triggered by a stochastic injury (e.g., gallstone) leading to acute pancreatitis with activation of the innate immunes system a recruitment of inflammatory cells. A variety of modifying factors and variables (triangle) determine the resolution of acute pancreatitis, or contribute to a variety of pathways that lead to the recognized components of the chronic pancreatitis syndrome. **(C)** Chronic pancreatitis reflects irreversible damage manifests by the response of multiple cell types.

The rate of progression from first symptom to the diagnosis of CP or evidence of exocrine or endocrine failure and cancer was considered to be important. The NAPS2 questionnaires were designed to facilitate construction of timelines, with the dates of key events recorded so that the CP could be modeled as a disease process rather than a diagnosis, and the effect of interventions evaluated. This was put within the framework of the SAPE hypothesis in contrast to using a diagnosis ICD9 577.1 alone. By modeling pancreatitis as an evolving process, susceptibility factors and the types of stochastic events that initiate the process could be identified and quantified, and the role of an acute pancreatitis event and other variables that initiate and drive the progression to CP could be organized, measured, and studied in a series of individual patients. Thus, multiple variables could easily be classified as risk factors, biomarkers, endpoints, or surrogate endpoints and used for constructing predictive models which anticipated the development of complications and allow for etiology based treatments to prevent the progression of diseases before the symptoms develop. In addition, biological samples from consecutive patients were collected and processed for DNA and serum and/or plasma for biomarker studies.

The utility of this approach has been remarkable. It has allowed the North American Pancreatic Study Group to subdivide CP into etiology-based processes that all have the same clinical appearance and pathologic features. This allows for early recognition process and targeting the etiology rather than the symptoms. Much of the data from the first 1000 patients has now been published. Surprisingly, there appear to be a threshold for risk of alcoholic pancreatitis at five more drinks per day (60 ounces of alcohol per day), and only 15% of total patients of all patients drank at this level (Yadav et al., 2009; Cote et al., 2010). The majority of these patients were in the CP group and very few were in the recurrent acute pancreatitis group, suggesting that alcohol also caused rapid progression from recurrent acute to CP so that in a cross sectional study, the pancreatitis category was markedly enriched. Smoking was also found to be a strong, independent, and synergistic risk factor for CP which is often not recognized by general practitioners as well as experts in CP (Yadav et al., 2011). Genetic etiologies (*CFTR*, *SPINK1*, and *PRSS1*) contributed to about 25% of the total cases (Whitcomb, 2011). More interesting was that about 40% of patients were idiopathic. These are the ones for whom we believe complex and environmental factors play a more dominate role. We expect that GWAS will bring further insight into this category.

MODELING CHRONIC PANCREATITIS

The framework for beginning to build models of pancreatic disease includes classifying patients as combinations of factors that occur together, in distinction to factors that are only seen together by chance (Aoun et al., 2008). **Figure 2** is a working model of etiology-based pathway in which two factors are required to drive the macrophages and pancreatic stellate cells to cause fibrosis.

As seen in the figure, alcohol plus a second factor markedly increases the risk of fibrosis. Trypsin-related pathways can begin either in the acinar cell or in the duct and these appear to converge with high risk being linked to secondary factors such as *SPINK1*. Lipase and lipid metabolism disorders may represent a separate pathway with liptoxicity or other factors directly causing pancreatic injury and CP as well as other mechanisms that are

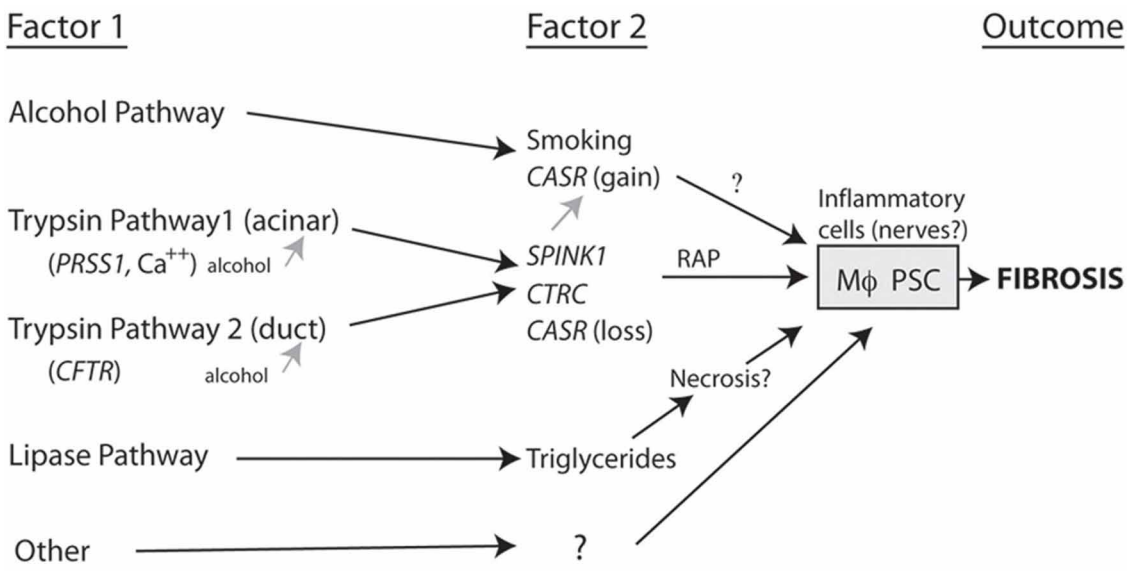

FIGURE 2 | Complex pathways to fibrosis. A combination of two or more factors that together markedly increase the likelihood that fibrosis will develop. For fibrosis to occur the risk factor combination must converge on the inflammatory cells (e.g., macrophages, Mϕ; pancreatic stellate cells, PSC). Note that alcohol can increase susceptibility to pancreatitis by acting on the acinar cell and/or duct cell, but appears to drive CP through another *SPINK1*-independent pathway (Aoun et al., 2008).

yet to be fully defined. These organization diagrams can set the stage for assigning relative effect strengths to various components of primary and secondary factors as well as other environmental and metabolic effects that may alter the rate of progression from the onset of pancreatitis to fibrosis. The most important of these to-be-named variables are genetic variants.

NGS is an enabling technology for rapidly revealing all variants in the genome of an individual patient. The complexity of the human genome has been recognized, and it is further recognized that it will be nearly impossible to evaluate variants of entire genome of an individual statistically. The challenges in analysis of NGS for most disorders, especially those with confounding environmental variables, are almost insurmountable. However, this is not true for the pancreas.

The advantage of developing methods for NGS analysis in the pancreas is that it is a very simple organ in which the exocrine pancreas has two primary cell types, the duct cell and the acinar cell. Each of the two cell types has a primary function (bicarbonate secretion or enzyme synthesis). The mechanism of injury is trypsin activation in most cases. The time of initial injury is often known, and the immune response is fairly stereotypic. Thus, for trypsin related susceptibility factors, five major genes have been identified that have been associated with patients with CP. In addition to *PRSS1*, *CFTR*, and *SPINK1*, the *CTRC* and *CASR* genes are additional risk factors that appear to increase risk of pancreatitis in the context of one of three primary susceptibility factors (Larusch and Whitcomb, 2011; Schneider et al., 2011; Rosendahl et al., 2012). As noted in **Figure 2**, the CTRC is envisioned to be linked to the trypsin pathway where as the CASR gain of function mutations are found in alcoholic patients, and CASR loss of function mutations are found in trypsin-associated pathways. The advantage of using NGS is that it is less expensive to use whole exome sequencing for the entire 30,000 + genes than it is to sequent CFTR using standard sanger sequencing technology. We already demonstrated the utility in a family with idiopathic HP (Larusch et al., 2012). This case demonstrated that four risk factors combined in different patients within the family tree in complex ways to cause pancreatitis from slightly different etiologies in each of the four affected individuals. This was done by focusing on the five known susceptibility genes rather than analyzing the entire human genome. What was amazing is that unexpected variants were found in these patients including a copy number variant of the *PRSS1* gene in one patient, a rare *SPINK1* mutation that had only been described in two French patients in early 2004 (Le Marechal et al., 2004), a strong effect of smoking, and a *CFTR* variant that is considered mild variable may be associated with pancreatitis disease (Larusch et al., 2012). This is a powerful proof of principle to illustrate a practical approach to the use of NGS for pancreatic disease. Of note, caution must be taken when evaluating *PRSS1* variants using NGS since disease-causing mutations are often gene conversion mutations from different forms of trypsin or trypsin pseudo-genes (Chen and Ferec, 2000) so that there is a high risk of variants in the trypsin genes being identified in these patients. Therefore, we always use very specific methods to confirm true mutations in the *PRSS1* genes when analyzing patients at risk for pancreatitis.

The final question is whether or not this new framework is compatible with clinical practice. At the University of Pittsburgh we have reorganized our pancreas clinic so that genetic testing occurs very early in the workup rather than at the end (Whitcomb, 2012). In our experience and in the NAPS2 study (unpublished, Whitcomb et al., 2012) we have found that there is often a delay of 6–10 years between the onset of symptoms and the development of CP to the point where a diagnosis could be made. The diagnosis of CP requires irreversible damage to the pancreas, which is exactly what we are trying to avoid! The 6 year delay does not mean that there is no disease; it means that the criteria of the old paradigm have not been met. Use of genetic testing identifies what part of normal physiology is likely to be disrupted by genetic variation and has implications for which cell type is likely to be the culprit in initiating the pathology. Therefore, attention to either the large ducts, the small pancreatic ducts, the acinar cell, or the immune system before the destruction of the pancreas allows targeted therapies to be initiated that address the etiology rather than covering the symptoms. We believe that this is a new paradigm for approaching complex inflammatory disease with late complications that should be avoided at all costs.

How can physicians receive and interpret the flood of information that is expected to come with whole genome sequencing and new "omics" biomarkers? The answer is that this is clearly impossible. What is needed is the development of decision support tools that are able to rapidly scan all of the information from an individual patients genome and biomarker studies, structure it in an organized way, perform calculations and simulations, and provide the physician with a few options, their likelihood of being successful, and how to best monitor the patient if interventions are made. The role of the physician will also be to use their own training and clinical experience to help guide the treatment of patients in whom there are no helpful predictions. Thus, there will be a lot to discover and apply in twenty-first century medicine.

SUMMARY

We believe that genetics will be the foundation of clinical management of pancreatic diseases in the future. New recognition that the development of CP is associated with a limited number of etiologies, and recognition that there may be several years between the first symptoms and organ destruction is a call to develop early and effective interventions that are based on the *etiology* rather than symptoms and complications. While the full spectrum of genetic variants that is linked to pancreatic disease or have not yet been described, the new framework that is necessary for their interpretation is already here.

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Role of intrapancreatic *SPINK1/Spink3* expression in the development of pancreatitis

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Studies on hereditary pancreatitis have provided evidence in favor of central role for trypsin activity in the disease. Identification of genetic variants of trypsinogen linked the protease to the onset of pancreatitis, and biochemical characterization proposed an enzymatic gain of function as the initiating mechanism. Mutations of serine protease inhibitor Kazal type 1 gene (*SPINK1*) are shown to be associated with hereditary pancreatitis. We previously reported that *Spink3* (a mouse homolog gene of human *SPINK1*) deficient mice showed excessive autophagy, followed by inappropriate trypsinogen activation in the exocrine pancreas. These data indicate that the role of *SPINK1/Spink3* is not only trypsin inhibitor, but also negative regulator of autophagy. On the other hand, recent studies showed that high levels of SPINK1 protein detected in a serum or urine were associated with adverse outcome in various cancer types. It has been suggested that expression of SPINK1 and trypsin is balanced in normal tissue, but this balance could be disrupted during tumor progression. Based on the structural similarity between SPINK1 and epidermal growth factor (EGF), we showed that SPINK1 protein binds and activates EGF receptor, thus acting as a growth factor on tumor cell lines. In this review, we summarize the old and new roles of *SPINK1/Spink3* in trypsin inhibition, autophagy, and cancer cell growth. These new functions of *SPINK1/Spink3* may be related to the development of chronic pancreatitis.

Keywords: chronic pancreatitis, hereditary pancreatitis, trypsinogen, *SPINK1, Spink3,* autophagy, EGF, EGFR

Chronic pancreatitis (CP) is a common disease characterized by progressive, destructive, and inflammatory process of multifactorial etiology that leads to irreversible obliteration of the exocrine and endocrine pancreatic tissues and to its replacement by fibrous tissue, which ultimately results in the clinical manifestations typical of an "end-stage" disorder of pancreatic function (Steer et al., 1995; Mergener and Baillie, 1997; Braganza et al., 2011). Furthermore, CP is a well-described risk factor for pancreatic adenocarcinoma (Whitcomb, 2004; Lowenfels and Maisonneuve, 2005), especially in cases of hereditary pancreatitis (HP; Lowenfels et al., 1997). In the Western countries, alcohol is generally considered as an important risk factor for the development of CP (Gullo et al., 1988). In addition, other metabolic, anatomical, obstructive, and autoimmune etiological factors have also been recognized (Steer et al., 1995; Etemad and Whitcomb, 2001). Furthermore, in recent years, several genetic risk factors for CP have been identified. HP is a very rare form of early onset CP. With the exception of the young age at diagnosis and a slower progression, the clinical course, morphological features, and laboratory findings of HP do not differ from those of patients with alcoholic CP. Gene mutations of cationic trypsinogen (protease serine 1; *PRSS1*), anionic trypsinogen (protease serine 2; *PRSS2*), pancreatic secretory trypsin inhibitor (PSTI; serine protease inhibitor Kazal type 1; *SPINK1*), cystic fibrosis transmembrane conductance regulator (*CFTR*), chymotrypsinogen C (*CTRC*), and calcium-sensing receptor (*CASR*) have been shown to be associated with HP (Whitcomb, 2010). Although the pathogenesis of CP, including HP, is not completely understood, the necrosis–fibrosis concept is supported by both clinical and experimental data. Necrosis–fibrosis concept is that repeated attacks of acute pancreatitis (AP) induce CP. Animal models of CP have been developed by inducing repeated episodes of AP in the pancreas using an administration of cerulein, an analog of cholecystokinin (Neuschwander-Tetri et al., 2000), or choline-deficient ethionine-supplemented diet (Ida et al., 2010).

The main mechanism in the onset of AP is believed to be the autodigestion of pancreatic structural cells by various proteases that are activated in response to the ectopic (intrapancreatic) activation of trypsinogen (trypsin production). A relationship between the trypsinogen gene mutations and the onset of pancreatitis was initially reported in 1996 (Whitcomb et al., 1996). The effect of mutations in *SPINK1* gene on the onset of pancreatitis was reported in 2000 (Witt et al., 2000). Mutations in *PRSS1* gene, encoding cationic trypsinogen, play a causative role in HP (Whitcomb et al., 1996). It has been shown that *PRSS1* mutations increase autocatalytic conversion of trypsinogen to active trypsin, and thus probably cause premature, intrapancreatic trypsinogen activation disturbing the intrapancreatic balance of proteases and their inhibitors (Whitcomb et al., 1996).

CATIONIC TRYPSINOGEN (*PRSS1*) AND HP

Whitcomb et al. (1996) determined the sequence of five exons of the *PRSS1* and *PRSS2* genes using genomic DNA from patients with HP. To explain why the R122H mutation might cause pancreatitis, Whitcomb proposed that the Arg122-Val123 autolytic peptide bond in trypsin plays an important role in the degradation of prematurely activated trypsin in the pancreas (Whitcomb et al., 1996). Destruction of this "failsafe mechanism" by the R122H mutation would increase intrapancreatic trypsin activity and disturb the protease–antiprotease equilibrium and eventually precipitate pancreatitis. Although, in the following years, further mutations of *PRSS1* gene were discovered in patients with hereditary or idiopathic CP (Teich et al., 2006), the R122H, and the N29I mutations are the most common *PRSS1* mutations worldwide. They have been frequently reported from Europe, North America, and Asia (Nishimori et al., 1999).

Several mechanisms have been proposed to explain how mutations in the cationic trypsinogen gene can lead to increase of trypsin activity. At first, R122H mutation prevents inactivation (autolysis) of activated trypsin (Whitcomb et al., 1996) and also leads to an increase in the autoactivation of trypsinogen (Sahin-Toth and Toth, 2000). N29I mutation was found to have no effect on trypsin activity and trypsinogen stability by biochemical analysis using recombinant trypsinogen. N29I mutation is hypothesized to change the higher-order structure of trypsin, resulting in decreased SPINK1 binding and increased autoactivation. As it has been shown that changes in the sequence of the cationic trypsinogen N-terminal peptide increased its rate of degradation, mutations A16V, D22G, and K23R (all of which change the signal peptide cleavage site of trypsin) may lead to increased autoactivation of trypsinogen to trypsin. 228delTCC has been hypothesized to enhance cationic trypsinogen transcription, thereby increasing activity. On the basis of these mechanisms, any of these gene mutations can increase trypsin activity in the pancreas.

To examine the link between *PRSS1* mutations and the initiation and progression of HP, Archer et al. (2006) generated a transgenic mouse that carries a missense mutation (R122H mutation) in the *PRSS1* gene. The pancreas from these transgenic mice displayed early onset acinar cell injury and inflammatory cell infiltration. With progressing age, the transgenic mice developed pancreatic fibrosis and display acinar cell dedifferentiation. Moreover, the expression of mutated *PRSS1* transgene is associated with enhanced response to cerulein-induced pancreatitis.

ANIONIC TRYPSINOGEN (*PRSS2*) AND HP

Although increased proteolytic activity caused by the *PRSS1* gene mutations enhances the risk for CP, it was thought that mutations in the *PRSS2* gene may predispose to disease (Witt et al., 2006). *In vitro* studies showed that recombinant mutated PRSS2 protein (G191R) showed a loss of trypsin activity due to a novel tryptic cleavage site that renders the enzyme hypersensitive to autocatalytic proteolysis. Thus, it appears that the PRSS2 variant (G191R) mitigates intrapancreatic trypsin activity, thereby playing a protective role against CP. Although the overall contribution of G191R to disease pathogenesis is low, the functional characterization of G191R provides the first example in pancreatitis for a disease-protective genetic variant.

INTRACELLULAR TRYPSINOGEN ACTIVATION AND CP

Biological consequences of intracellular trypsinogen activation have not been directly examined. Gaiser et al. (2011) generated mice, which were engineered to conditionally express an endogenously activated trypsinogen within pancreatic acinar cells. The mice can express trypsin activity conditionally in the pancreatic acinar cells. Although initiation of AP was observed with high levels of active trypsin expression, chronic inflammation, or fibrosis did not develop in this mice model, suggesting that intra-acinar activation of trypsinogen is sufficient to initiate AP, but not to CP. Because CP is sometimes observed without evidence of associated necrosis, mutated trypsinogen may have other functions, and cause HP.

SPINK1 AND HP

SPINK1 molecule was found by two independent groups. At first, Kazal et al. extracted SPINK1 from bovine pancreas as a pancreatic secretory trypsin inhibitor (PSTI) in 1948 (Kazal et al., 1948). The animal pancreas usually has two types of trypsin inhibitors, basic pancreatic trypsin inhibitor (BPTI) and PSTI. Docking site of SPINK1 and PRSS1 which is predicted by using biological information, ZDOCK, was very similar to that of BPTI and PRSS1 (**Figure 1**). However, human pancreas does not have BPTI. The gene encoding human PSTI was named as serine protease inhibitor, Kazal type 1 (*SPINK1*). Mouse gene homologous to human *SPINK1* is *Spink3*. Hydra gene homologous to human *SPINK1* is *Kazal1*. The human *SPINK1* gene encodes mRNA of 237 bp, which is translated to a 79 amino acid peptide including a 23 amino acid signal peptide with three intramolecular disulfide bridges (Cys9–Cys33, Cys16–Cys35, and Cys24–Cys56; Bartelt et al., 1977; Horii et al., 1987), and the molecular weight is estimated to be 6240 based on the amino acid composition. SPINK1 (PSTI) is secreted by the acinar cells of exocrine pancreas into the pancreatic duct. It binds rapidly to trypsin and inhibits its activity both intracellularly and extracellularly. Hence, it is an important protective factor in the onset of pancreatitis (Hirota et al., 2006). The liver also secretes SPINK1 protein in the systemic circulation as one of acute phase proteins to inhibit trypsin activity in tissues such as the pancreas. Although it is known that SPINK1 molecule is widely expressed in extrapancreatic tissues, especially in the gastrointestinal and urinary tract (Marchbank et al., 1998), its roles in these tissues are not known.

At second discovery, SPINK1 was isolated by Stenman et al. (1982) from urine of ovarian cancer patients and was reported as tumor-associated trypsin inhibitor (TATI). TATI was later shown to be identical to SPINK1 (Huhtala et al., 1982). Increased expression of SPINK1 protein was reported in various cancers, such as lung cancer (Tomita et al., 1987; Higashiyama et al., 1992), colon cancer (Higashiyama et al., 1990; Tomita et al., 1990), liver cancer (Ohmachi et al., 1993), breast cancer (Ogawa et al., 1987), prostate cancer (Tomlins et al., 2008), and pancreas cancer (Ogawa et al., 1987; Ozaki et al., 2009). Clinically, measurement of SPINK1 protein is most useful for monitoring of patients with mucinous ovarian cancer. Increased serum concentration ofSPINK1 protein may occur in most types of cancer (Paju and Stenman, 2006). And, increased expression of SPINK1 protein in tumor tissues has been reported to be associated with the poor survival in various

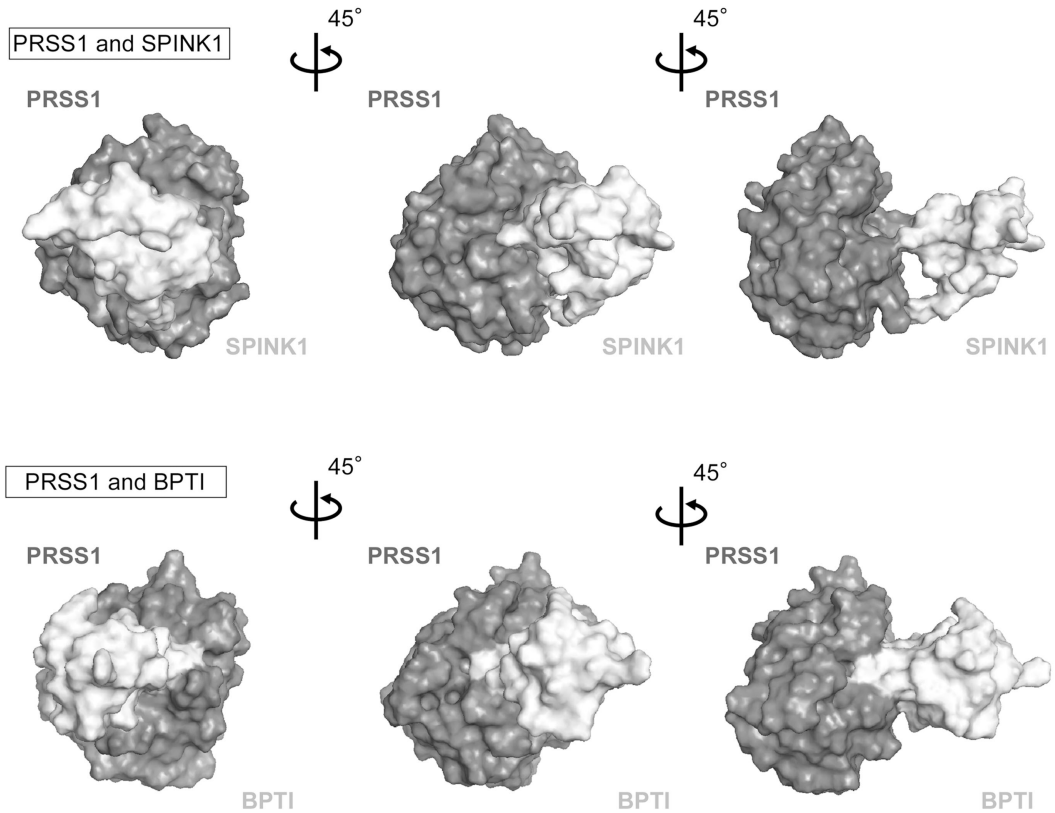

FIGURE 1 | PRSS1 and trypsin inhibitor binding. The 3-D structures of SPINK1, BPTI, and PRSS1 were downloaded from Protein Data Bank (accession number: SPINK1, 1cgj; BPTI, 2ptc, and PRSS1, 2ra3). Hydrogen atoms were added to these structures by using PyMOL software. Molecular docking simulations for PRSS1 and SPINK1 were the carried out by using ZDOCK.

cancers. Although this appears to be explained by the coexpression of SPINK1 molecule and tumor-associated trypsin, which is thought to participate in tumor-associated protease cascades mediating tumor invasion (Stenman et al., 1991), but it has not been established yet. Tomlins et al. (2008) reported that SPINK1 outlier expression is exclusively in a subset of ETS rearrangement-negative cancer (∼10% of total cases). ETS rearrangement is that the translocation of an ETS (E26 transformation specific) transcription factor (ERG or ETV1) to the TMPRSS2 promoter region, which contains androgen responsive elements, and it results in the aberrant androgen-regulated expression of ERG (Tomlins et al., 2005). They found that SPINK1 outlier expression is an independent predictor of biochemical recurrence after resection, and observed that SPINK1 outlier expression is an independent predictor of biochemical recurrence after resection. Tonouchi et al. (2006) reported that by the DNA microarray analysis and quantitative RT-PCR reaction, SPINK1 molecule is a candidate suggesting early recurrence of intrahepatic cholangiocarcinoma after resection. They described that the patients with higher levels of SPINK1 mRNA expression had significantly shorter recurrence-free survival. Gouyer et al. (2008) reported that from the conditioned medium of HT-29 5M21 human colon cancer cells, which are expressing a spontaneous invasive phenotype, SPINK1 molecule was identified and characterized as the major proinvasive secreted factor. SPINK1, which has a signal peptide, is secreted from not only pancreatic acinar cells, but also, colon cancer cells. SPINK1 protein may work as an autocrine and/or paracrine transforming factor, which is potentially involved in cancer progression, including local invasion of the primary tumor and its metastatic spread.

Interestingly, there are some structural similarities between SPINK1 and epidermal growth factor (EGF); both have similar numbers of amino acid residues (56 and 53, respectively), molecular weights (about 6 kD), and three intra-chain disulfide bridges (Fukuoka et al., 1987; Marchbank et al., 1998). There is 50% gene sequence homology between SPINK1 and EGF (Hunt et al., 1974; Scheving, 1983; Yamamoto et al., 1985). Ogawa et al. (1985) reported that SPINK1 protein was mitogenic for human fibroblasts. Some studies support the concept that SPINK1 protein binds to the EGF receptor (EGFR). Rat monitor peptide (rat homolog of human *SPINK1*) has been reported to compete with mouse EGF for binding to EGFR of Swiss 3T3 fibroblasts (Fukuoka et al., 1987) and an EGFR-blocking antibody removed the promigratory effects of SPINK1 on human HT-29 cells (Marchbank et al., 1996).

Recently we elucidated several functions of SPINK1 molecule. These include trypsin inhibition *in vivo*, autophagy regulation, and growth stimulation.

SPINK1 AS TRYPSIN INHIBITOR

Pancreatic digestive enzymes are stored as inactivated precursors in pancreatic acinar cells. Under normal conditions, digestive enzyme activation is strictly controlled to prevent autodigestion of the pancreas, which is called pancreatitis. However, in certain circumstances, excessive amounts of pancreatic trypsinogen are activated to trypsin intracellularly, resulting in activation of other zymogens and autodigestion of the pancreas. SPINK1 molecule is synthesized in acinar cells of the pancreas and is thought to inhibit the trypsin activity in the pancreas.

Intrapancreatic balance between trypsin and SPINK1 activities is important for pancreatitis development. It is hypothesized that mutations in *SPINK1* gene that affect SPINK1 binding with trypsin will contribute to the onset of pancreatitis. There have been many reports of mutations in *SPINK1* genes in patients with pancreatitis, and several hypothetical roles of these mutant proteins in pancreatitis (Pfutzer et al., 2000; Witt et al., 2000; Chen et al., 2001; Kaneko et al., 2001; Kuwata et al., 2001, 2003).

To analyze the importance of trypsinogen activation (trypsin production) and its regulation by SPINK1 molecule in the onset of pancreatitis, we generated *Spink3* gene knockout mice by gene targeting and analyzed their phenotypes (Ohmuraya et al., 2005). The pancreatic acinar cells in knockout mouse showed excessive autophagy (**Figure 2**) and enhanced tryptic activity was detected in pancreatic acini prepared at 1 day after birth (Ohmuraya et al., 2006). All acinar cells disappeared completely after birth, indicating that Spink3 is not only a trypsin inhibitor within pancreatic acinar cells, but also important in maintaining the integrity of these cells.

The general mechanism to deliver cytoplasmic components to the lysosomes is called autophagy (**Figure 3**). The best understood role of autophagy is cellular housekeeping, a function that extends beyond the simple removal of damaged or unwanted products (Seglen and Bohley, 1992; Kim and Klionsky, 2000; Meijer, 2003). In fact, along with other proteolytic systems, lysosome participates in the continuous turnover of intracellular constituents. Not only soluble cytosolic proteins but also organelles, such as mitochondria and peroxisomes, can be removed by autophagy (Lemasters et al., 2002; Bellu and Kiel, 2003; Roberts et al., 2003). In addition to maintaining cellular homeostasis, there is growing evidence for the participation of autophagy in processes such as cellular differentiation, tissue remodeling, growth control, cell defense, and adaptation to adverse environments (Hennig and Neufeld, 2002; Jacinto and Hall, 2003; Melendez et al., 2003; Otto et al., 2003).

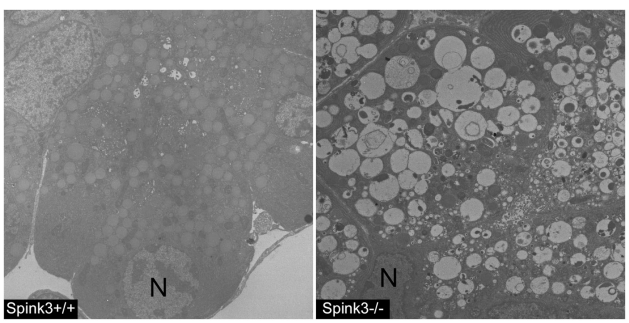

FIGURE 2 | The pancreatic acinar cells of Spink3 deficient mice. Many vacuoles, autophagosomes, are appeared in Spink3 deficient pancreatic acinar cells (Right panel). N, nucleus of the acinar cell.

SPINK1 AS AN AUTOPHAGY REGULATOR

The vacuoles were observed by histological examination correspond to autophagosomes using autophagosome specific probe, microtubule-associated protein 1 light chain 3 (LC3-II), which are the hallmark of autophagy (Hashimoto et al., 2008). We reported that conversion of trypsinogen to trypsin within the pancreatic acinar cell was greatly suppressed in autophagy-related gene 5 (*Atg5*) deficient acinar cells, suggesting involvement of autophagy in trypsinogen activation by lysosomal hydrolase such as cathepsin B (**Figure 4**; Hashimoto et al., 2008; Ohmuraya and Yamamura, 2008). In previous study, the results indicate that all known mutations of *SPINK1* gene are functionally innocuous for the trypsin inhibitory activity (Kuwata et al., 2002; Kiraly et al., 2007; Ohmuraya et al., 2009). Hence, it is possible that HP caused by mutation of *SPINK1* gene is due to autophagy induction, but not to loss of binding to trypsin.

Interestingly, Chera et al. (2006) reported that similar phenotypes parallel in the endodermal epithelial cells observed upon silencing of *Kazal1* gene, which is a homolog of *SPINK1* gene in hydra. In hydra, the endodermal epithelial cells carry out the digestive function together with the gland cells that produce zymogens and express the evolutionarily conserved gene *Kazal1*. A progressive *Kazal1* silencing induced excessive autophagy in the cytoplasm of digestive cells, and dramatic disorganization followed by a massive death of these cells. These data suggests that SPINK1 activity is required to prevent excessive autophagy in food digestive systems.

In mammalian cells, autophagy continuously occurs at basal level, but can be induced in response to environmental signals including nutrients and hormones (Mizushima, 2005; Mizushima et al., 2008). The molecular machinery of autophagy is regulated by a class I PI3K and mammalian target of rapamycin (mTOR), which act to inhibit autophagy (Klionsky, 2005; Mizushima et al., 2008). It is interesting to note that SPINK1 and EGF have structural similarities, including the number of amino acid residues and the presence of three intrachain disulfide bridges (Scheving, 1983). Can SPINK1 bind to EGFR and stimulate its, as a growth factor?

SPINK1 AS A GROWTH FACTOR

Spink3 deficient pancreas shows no sign of regeneration of acinar cells (Ohmuraya et al., 2005), and *Kazal1* silencing decreases budding rate in hydra (Chera et al., 2006). SPINK1 protein was previously shown to stimulate the growth of non-neoplastic (Ogawa et al., 1985; McKeehan et al., 1986) and neoplastic cells (Freeman et al., 1990; Niinobu et al., 1990).

Marchbank et al. (1996) reported that SPINK1 molecule involved in the regenerative process at the ulcer edge. The cell migration induced by SPINK1 protein was inhibited by adding an EGFR-blocking antibody, which suggests that SPINK1 protein mediated this effect through binding to the EGFR. This idea is supported by a previous report that rat PSTI competed with mouse EGF for binding to mouse Swiss 3T3 cells (Fukuoka et al., 1987),

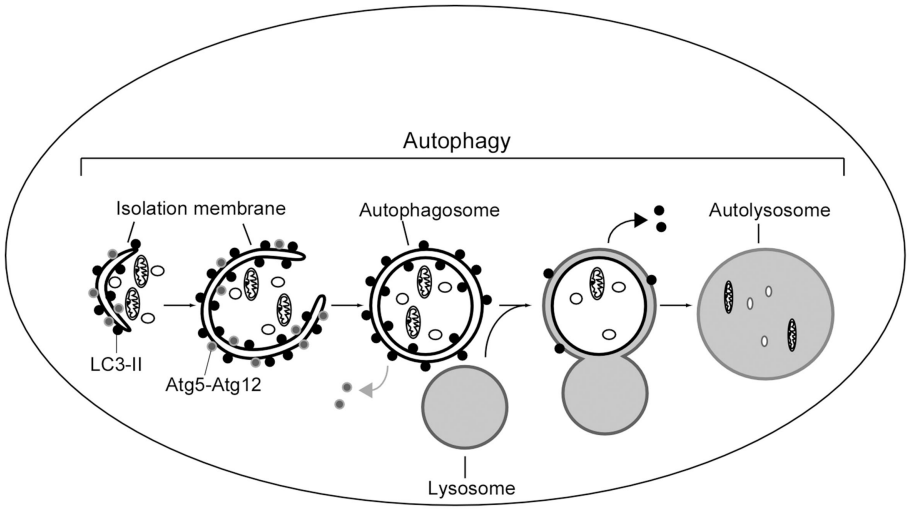

FIGURE 3 | Scheme of autophagy in mammalian cells. A portion of cytoplasm is enclosed by isolation membrane to form an autophagosome. Autophagosome fuses with lysosome to degrade the inside materials. The Atg5–Atg12 conjugate localizes to the isolation membrane throughout its elongation process. LC3 is recruited to the membrane in the Atg5-dependent manner. Atg5–Atg12 dissociate from the membrane upon completion of autophagosome formation, while LC3 (-II) remains on the autophagosome membrane. Atg5 is required for elongation of the isolation membrane.

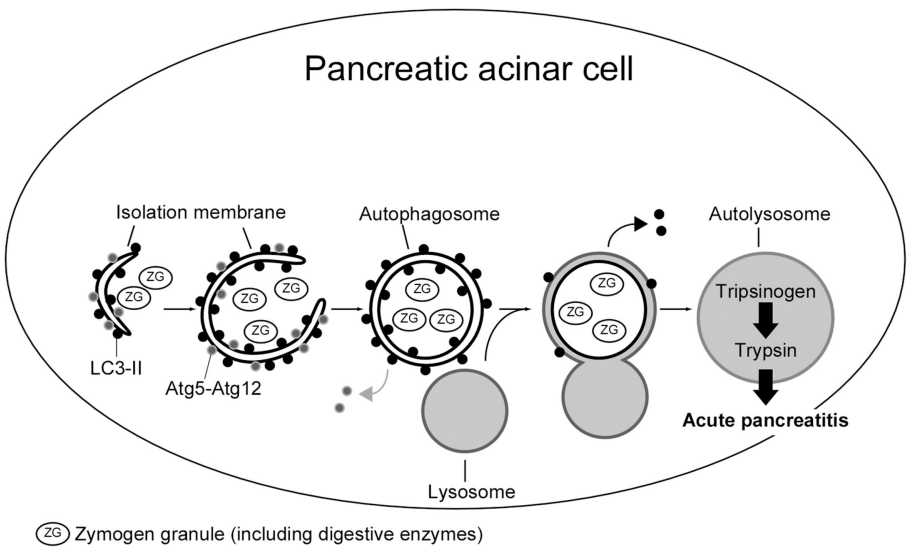

FIGURE 4 | Schematic representation of autophagy within a pancreatic acinar cell in acute pancreatitis. After autophagic vacuole containing zymogen granules fuses lysosome, the trypsinogen is activated by lysosomal hydrolases in autophagic process, and release of activated digestive enzymes inside the cell.

although Niinobu et al. (1990) reported that the binding of human ^{125}I-labeled SPINK1 protein to the same cells was displaced by cold SPINK1 protein but not EGF, suggesting a separate receptor.

Recently, we showed that SPINK1 protein binds to EGFR to activate its downstream signaling; resulting in proliferation of pancreatic and breast cancer cells (Ozaki et al., 2009). We showed that SPINK1 protein coprecipitated with EGFR in an immunoprecipitation experiment and that the binding affinity of SPINK1 to EGFR is about half of that of EGF using quartz-crystal microbalance technique. In addition, we performed molecular docking simulations using ZDOCK for the following pairs: EGF and EGFR; SPINK1 and EGFR. In 2000 docking runs, each binding pairs bound 795 and 287 times at the same position, respectively. These data indicate that SPINK1 binds to EGFR at the same position of EGF–EGFR binding site, but binding affinity is about half compared to EGF–EGFR affinity.

Although binding affinity of SPINK1–EGFR is lower than that of EGF–EGFR, EGFR, and its downstream molecules, signal transducer and activator of transcription 3 (STAT3), v-Akt murine thymoma viral oncogene homolog (AKT), and extracellular

signal-regulated kinase 1/2 (ERK1/2), were phosphorylated by SPINK1 as well as EGF. These results suggest that SPINK1 protein stimulates the proliferation of pancreatic cancer cells through EGFR (Ozaki et al., 2009). Autophagy is regulated by PI3K–AKT–mTOR pathway (Klionsky, 2005; Mizushima et al., 2008). Secreted SPINK1 protein may activate this pathway via EGFR to prevent insufficient or excessive autophagy, which induces cell death.

CONCLUSION

In summary, SPINK1 is a multifunctional fascinating molecule. It is important not only in pancreatitis pathogenesis, but also in cellular growth and death. Although generally favoring a central role for trypsin activity during pancreatitis, none of the hereditary variants provides direct evidence that intracellular trypsin activity alone is sufficient to initiate the CP. We proved new roles of SPINK1 molecule, namely autophagy regulation and growth stimulation via EGFR. These new functions of SPINK1 molecule may be related to the development of CP. It is possible that HP caused by mutation of the *SPINK1* gene is due to autophagy induction, but not to loss of binding to trypsin (Ohmuraya and Yamamura, 2008). As next steps, human *SPINK1* replacement models in *Spink3* knockout mice are in progress.

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Molecular mechanisms of pancreatic stone formation in chronic pancreatitis

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Chronic pancreatitis (CP) is a progressive inflammatory disease in which the pancreatic secretory parenchyma is destroyed and replaced by fibrosis. The presence of intraductal pancreatic stone(s) is important for the diagnosis of CP; however, the precise molecular mechanisms of pancreatic stone formation in CP were left largely unknown. Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel expressed in the apical plasma membrane of pancreatic duct cells and plays a central role in HCO_3^- secretion. In previous studies, we have found that CFTR is largely mislocalized to the cytoplasm of pancreatic duct cells in all forms of CP and corticosteroids normalizes the localization of CFTR to the proper apical membrane at least in autoimmune pancreatitis. From these observations, we could conclude that the mislocalization of CFTR is a cause of protein plug formation in CP, subsequently resulting in pancreatic stone formation. Considering our observation that the mislocalization of CFTR also occurs in alcoholic or idiopathic CP, it is very likely that these pathological conditions can also be treated by corticosteroids, thereby preventing pancreatic stone formation in these patients. Further studies are definitely required to clarify these fundamental issues.

Keywords: chronic pancreatitis, pancreatic stone formation, bicarbonate secretion, CFTR, cytoplasmic mislocalization

INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammatory disease of the pancreas, and is characterized by pancreatic exocrine and endocrine dysfunction resulting from tissue damage caused by inflammation. The pancreatic exocrine gland is composed of two types of cells, duct cells and acinar cells. Duct cells secrete fluid and HCO_3^- to neutralize gastric acid from the stomach. Acinar cells secrete digestive enzymes essential for the digestion of food. Regardless of the cause of pancreatitis, HCO_3^- and digestive enzyme secretion are more or less compromised in all forms of chronic pancreatitis.

For a diagnosis of CP (Homma et al., 1997), it is essential to show the inflammation and destruction of the gland; however, it is often difficult to obtain pancreatic tissues due to the anatomy of the gland, except in cases of a pancreatic resection for malignant tumors. Another way to diagnose CP is to show exocrine pancreatic dysfunction. Pancreatic ductal dysfunction, especially a low HCO_3^- concentration in the pancreatic juice, is the most important finding for the diagnosis of CP with mild or moderate exocrine dysfunction, since an impairment in ductal HCO_3^- secretion is one of the earliest defects in CP (Freedman, 1998). For evaluating exocrine function, the secretin test was the only reliable test which can directly measure pancreatic ductal function (Ko et al., 2010) and acinar cell function separately; however, it became impossible to diagnose mild or moderate CP Functionally because the secretin test is no longer available in Japan due to the lack of supply of the clinical grade secretin. For that reason, the presence of pancreatic ductal stones became the most reliable diagnostic criterion for chronic pancreatitis.

Pancreatic stones are thought to be formed at first as protein plugs in pancreatic ducts in CP (Freedman, 1998). However, detailed molecular mechanisms of how pancreatic stones are formed in pancreatic ducts of CP still remain elusive. In previous studies, while elucidating the molecular mechanisms of aberrant HCO_3^- transport in pancreatic ducts in chronic pancreatitis, we have found that the Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, which plays a central role in HCO_3^- transport in pancreatic ducts, is largely mislocalized in the cytoplasm of pancreatic duct cells in autoimmune pancreatitis (Ko et al., 2010). As the cytoplasmic mislocalization of CFTR has been observed in all other forms of CP (i.e., alcoholic, idiopathic, or obstructive), we concluded that the mislocalization of CFTR is a cause of pancreatic ductal dysfunction and subsequent pancreatic stone formation. This notion was further supported by the observation that corticosteroids, a potent anti-inflammatory drug, normalize the CFTR localization from the cytoplasm of pancreatic duct cells to the proper apical plasma membrane, and subsequently restored aberrant pancreatic HCO_3^- secretion.

In this article, we propose that the cytoplasmic mislocalization of the CFTR in pancreatic duct cells is a cause of pancreatic

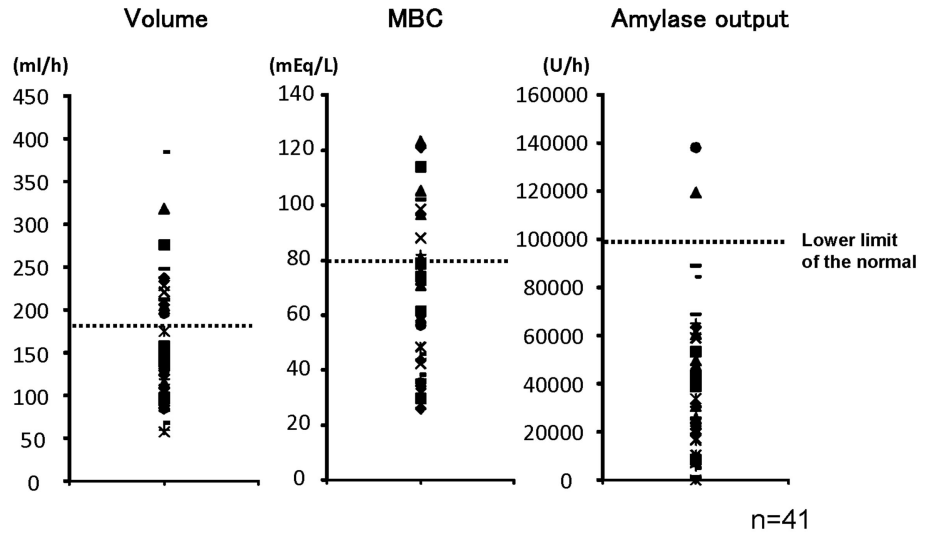

FIGURE 1 | Pancreatic exocrine functions are compromised in chronic pancreatitis. Pancreatic exocrine functions of patients with alcoholic chronic pancreatitis were evaluated by a canonical secretin-stimulated exocrine function test. Pancreatic fluid volume (lower limit of normal, 183 ml/h), maximum bicarbonate (HCO_3^-) concentration in pancreatic juice (MBC: lower limit of normal, 80 mEq/l), and total amylase output (AO: lower limit of normal, 99,000 U/h), were all impaired in most of the patients with chronic pancreatitis (Ko et al., 2010).

stone formation in chronic pancreatitis. Currently, steroid treatment for autoimmune pancreatitis is the only established therapy to restore impaired pancreatic ductal function. It might be possible that pancreatic ductal dysfunction seen in alcoholic or idiopathic pancreatitis can also be cured by the administration of corticosteroid treatment. Further studies are definitely required to clarify these important issues in the treatment of chronic pancreatitis.

HCO_3^- SECRETION FROM PANCREATIC DUCT CELLS IS COMPROMISED IN CHRONIC PANCREATITIS

The normal pancreas secretes the most alkaline fluid among exocrine organs in humans (maximum HCO_3^- concentration in pancreatic juice around 140 mM, pH ∼8.5) to neutralize acid from the stomach (Steward et al., 2005). In pancreatic ducts in chronic pancreatitis, it is well known that the alkalinization of pancreatic juice is impaired, and results in a low pH in secreted fluid. However, the precise molecular mechanisms of pancreatic ductal dysfunction remained elusive.

Figure 1 shows pancreatic exocrine functions in patients with alcoholic chronic pancreatitis. Pancreatic fluid volume (V: lower limit of normal, 183 ml/h), maximum bicarbonate concentration (MBC) in pancreatic juice (lower limit of normal, 80 mEq/l), and total amylase output (AO: lower limit of normal, 99,000 U/h), were all impaired in alcoholic chronic pancreatitis. In a severe form of chronic pancreatitis, all three factors are impaired. In some cases with mild or moderate forms of chronic pancreatitis, fluid volume, and AO may stay within normal range; however, HCO_3^- secretion is frequently impaired even in a mild form of the disease. Therefore direct measurement of maximum HCO_3^- concentration in pancreatic juice was very valuable to diagnose mild or moderate forms of CP when the secretin test was available.

MOLECULAR MECHANISM OF PANCREATIC HCO_3^- SECRETION FROM THE NORMAL DUCT CELLS

The pancreatic duct epithelium is capable of secreting HCO_3^- at a concentration of around 140 mM (Steward et al., 2005). Molecular mechanisms of how the pancreatic duct epithelium secretes such a high concentration of HCO_3^- have long been examined (**Figure 2**). A primary fluid rich in digestive enzymes secreted from pancreatic acinar cells contains around 24 mM HCO_3^-. The digestive hormone secretin was secreted from the endocrine cells in the duodenum when these cells were stimulated with gastric acid from the stomach. Secretin binds to its receptor on the basolateral membrane of pancreatic duct cells. An increase of intracellular cyclic AMP levels stimulates the CFTR chloride channel which is located at the apical plasma membrane of small pancreatic duct cells. Cl^- ions pass through the CFTR chloride channel into the luminal space of pancreatic ducts. HCO_3^- was secreted from pancreatic duct cells in exchange for Cl^- absorption by an electrogenic anion exchanger, SLC26 transporters, expressed also at the apical plasma membrane of pancreatic ducts (Ko et al., 2002, 2004; Song et al., 2012). When Cl^- concentration in the juice becomes quite low, HCO_3^- was secreted to the luminal space of pancreatic ducts through CFTR chloride channels (Ishiguro et al., 2009).

CFTR CHLORIDE CHANNEL IS MISLOCALIZED TO THE CYTOPLASM OF PANCREATIC DUCTS IN CHRONIC PANCREATITIS

It has been well known that the HCO_3^- concentration in pancreatic juice is reduced in CP (Braganza et al., 2011). In previous studies, however, it was unclear why HCO_3^- concentration in pancreatic juice in CP is low. It has been shown that the CFTR plays a most pivotal role in HCO_3^- secretion in pancreatic duct cells. Thus, it is reasonable to consider whether or not the expression of CFTR is

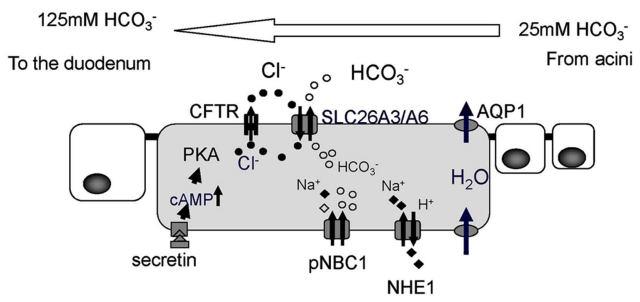

FIGURE 2 | Cellular mechanism proposed for ion and fluid secretion by the pancreatic duct epithelium. The primary fluid secreted by acinar cells contains a large amount of digestive enzymes and 24 mM HCO_3^-. Intracellular HCO_3^- leaves the cell in exchange for Cl^-, mediated by SLC26 anion exchangers. Cl^- is supplied to the lumen by a secretin-stimulated Cl^- channel, the Cystic fibrosis transmembrane conductance regulator (CFTR). For fluid movement across the epithelium, aquaporin (AQP) water channels are present at both the apical and basolateral membranes of smaller ducts in the rat (Furuya et al., 2002) and human pancreas (Burghardt et al., 2003). PKA, protein kinase A; pNBC1, pancreatic sodium bicarbonate cotransporter1; NHE1, sodium proton exchanger1.

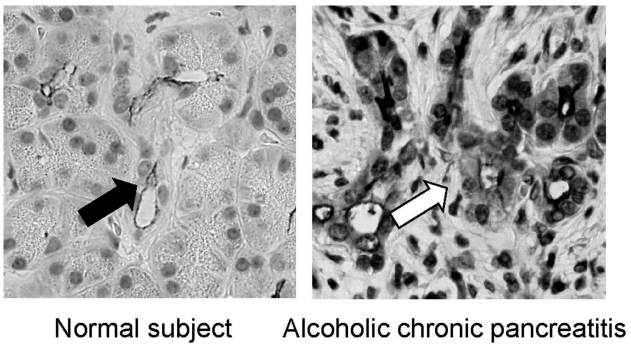

FIGURE 3 | Immnolocalization of CFTR in the pancreas of normal and chronic pancreatitis. In the normal subjects, the CFTR chloride channel is exclusively localized in the apical plasma membrane of small pancreatic ducts (left panel). In contrast, the CFTR chloride channel is largely retained at the cytoplasm of pancreatic ducts and is not transported to the proper apical plasma membrane domain in alcoholic chronic pancreatitis (right panel; modified from Freedman, 1998).

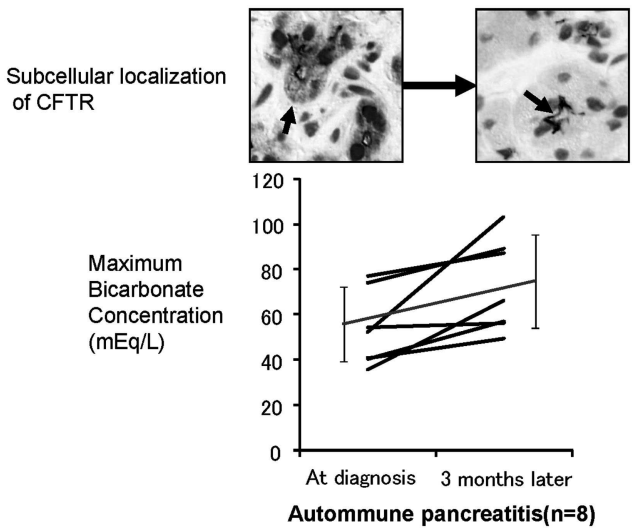

FIGURE 4 | Effects of corticosteroids on the localization of CFTR and pancreatic ductal dysfunction in autoimmune pancreatitis. The lower panel shows the changes in HCO_3^- concentrations in the pancreatic juice of autoimmune pancreatitis treated by corticosteroids. The HCO_3^- concentration in pancreatic juice is quite low prior to treatment. However, 3 months of corticosteroid treatment significantly increased HCO_3^- concentration in the pancreatic juice in autoimmune pancreatitis. The upper panel shows the changes of immunolocalization of the CFTR in human pancreatic ducts (modified from Freedman, 1998). Much of the CFTR proteins were retained at the cytoplasm of small pancreatic ducts and were not transported to the proper apical plasma membrane domain (left). Three months of corticosteroid treatment completely corrected the localization of the CFTR from the cytoplasm of pancreatic ducts to the apical membrane (right).

compromised in pancreatic duct cells in patients with chronic pancreatitis. To this end, we have examined the immunolocalization of the CFTR in these patients. In the normal pancreas the CFTR is expressed exclusively at the plasma membrane of small pancreatic ducts (**Figure 3**). However, in chronic pancreatitis, trafficking of the CFTR is largely compromised and the protein is largely retained at the cytoplasm of pancreatic ducts (**Figure 3**). CFTR plays a central role in HCO_3^- secretion from pancreatic duct cells; therefore, the reduced CFTR expression at the apical membrane of pancreatic ducts should result in the low HCO_3^- concentration seen in CP (Ko et al., 2011).

STEROID THERAPY NORMALIZES CYTOPLASMIC MISLOCALIZATION OF CFTR TO THE APICAL PLASMA MEMBRANE OF PANCREATIC DUCT CELLS IN AUTOIMMUNE PANCREATITIS AND RECOVERS ABERRANT HCO_3^- SECRETION IN CHRONIC PANCREATITIS

Autoimmune pancreatitis is one form of chronic pancreatitis. Autoimmunity is suspected to be its pathogenesis (Yoshida et al., 1995). Severe exocrine insufficiency has been reported in most of the cases (Ito et al., 2007; Frulloni et al., 2010; Ko et al., 2010). Steroid therapy improves the swelling of the gland and narrowing of the main pancreatic ducts, and reduces serum gamma globulin and immunoglobulin G subtype 4 (IgG4) levels.

To further elucidate the role of CFTR mislocalization in the aberrant ductal HCO_3^- secretion in chronic pancreatitis, we have examined histology (Mizuno et al., 2009) and exocrine functions of patients with autoimmune pancreatitis at the diagnosis and 3 months after maintenance steroid treatment (Ko et al., 2010). In autoimmune pancreatitis, HCO_3^- concentration in pancreatic juice is remarkably reduced prior to treatment as well as in other forms of CP (**Figure 4**), whereas 3 months of steroid therapy restored the mislocalization of the CFTR to the proper apical plasma membrane and significantly improved the HCO_3^- concentration in pancreatic juice (**Figure 4**). These data indicate that the mislocalization of the CFTR is a direct cause of a low HCO_3^- concentration in pancreatic juice and that steroid

treatment restores both the localization of CFTR and HCO_3^- concentration in pancreatic juice in autoimmune pancreatitis.

PANCREATIC DUCTAL DYSFUNCTION IS NOT RECOVERED SPONTANEOUSLY IN CHRONIC ALCOHOLIC PANCREATITIS

We have shown that the mislocalization of the CFTR in the cytoplasm of pancreatic ducts is a possible cause of the low HCO_3^- concentration found in pancreatic juice in chronic pancreatitis. If this proposed molecular mechanism is correct, one would speculate that the retargeting of the CFTR at the apical plasma membrane by the specific anti-inflammatory treatment will restore pancreatic HCO_3^- secretion in chronic pancreatitis, and prevent pancreatic stone formation in chronic pancreatitis. Thus, we next examined HCO_3^- concentrations in the juice of 18 cases of chronic alcoholic pancreatitis to see if pancreatic ductal dysfunction spontaneously recovers (**Figure 5**). As shown in **Figure 5**, a 1-year observation period did not affect the HCO_3^- concentrations of pancreatic juice in chronic alcoholic pancreatitis without an active anti-inflammatory regimen, indicating that pancreatic ductal dysfunction does not improve spontaneously in chronic pancreatitis.

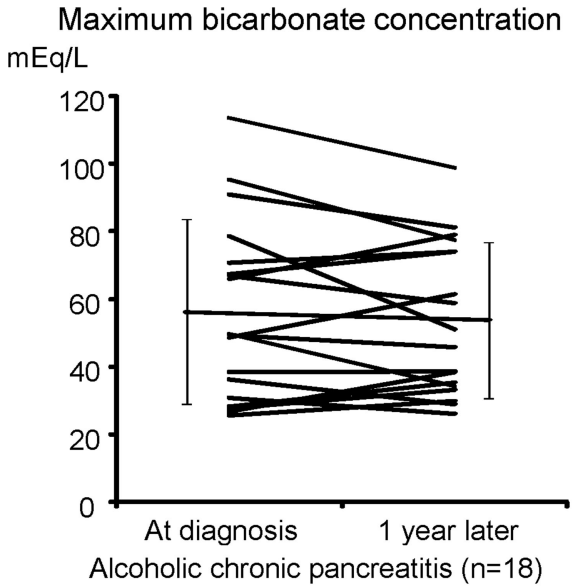

FIGURE 5 | HCO_3^- secretory defect is not spontaneously recovered in alcoholic pancreatitis. The secretin-stimulated pancreatic exocrine test shows that a 1-year interval does not affect the HCO_3^- secretory defect in alcoholic chronic pancreatitis.

PROPOSED MOLECULAR MECHANISM OF PANCREATIC STONE FORMATION IN CHRONIC PANCREATITIS

Figure 6 shows the possible steps/mechanisms for pancreatic stone formation in chronic pancreatitis.

Step 1: Chronic inflammation occurred in the pancreas by drinking, gallstone, or autoimmune mechanisms.
Step 2: Trafficking of membrane proteins such as the CFTR is compromised and the proteins are mislocalized to the cytoplasm of pancreatic ducts.
Step 3: Decrease of the CFTR expression at the plasma membrane results in a low HCO_3^- concentration in pancreatic juice.
Step 4: Decrease of HCO_3^- secretion further decreases the pH and volume of the fluid secreted by pancreatic ducts.
Step 5: Low fluid volume can result in the precipitation of digestive enzymes in pancreatic fluid and protein plugs are formed in pancreatic ducts in chronic pancreatitis.

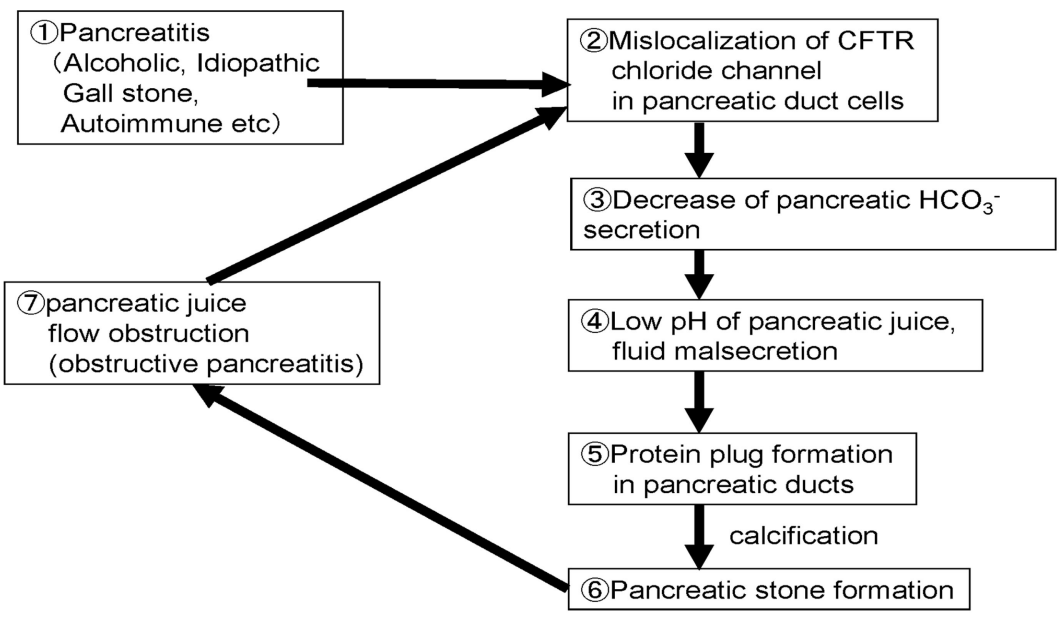

FIGURE 6 | Proposed cellular mechanisms of pancreatic stone formation in chronic pancreatitis.

Step 6: Protein plugs formed in pancreatic ducts disturb pancreatic juice outflow and protein plugs are calcified.
Step 7: Protein plugs and pancreatic stones obstruct pancreatic juice flow further and cause obstructive/upstream pancreatitis. Obstructive pancreatitis caused by protein plugs and stones in pancreatic ducts exacerbate the cytoplasmic mislocalization of CFTR, and then compromise pancreatic ductal dysfunction.

CONCLUSIONS

Extensive research has revealed the molecular mechanisms of ion and fluid secretion in the physiological and pathological status (chronic pancreatitis) of the pancreas. Cytoplasmic mislocalization of the CFTR chloride channel results in aberrant HCO_3^- secretion from the pancreatic ducts in chronic pancreatitis. Nonetheless, there is no cure for pancreatic ductal dysfunction in CP such as alcoholic or idiopathic pancreatitis. However, we have found for the first time that pancreatic ductal dysfunction of patients with autoimmune pancreatitis was partially reversed by the corticosteroid treatment. Further investigation to examine the effects of an anti-inflammatory regimen on pancreatic ductal dysfunction in other forms of Cp should eventually lead to the establishment of the treatment for chronic pancreatitis.

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Pancreatic stellate cells: a starring role in normal and diseased pancreas

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While the morphology and function of cells of the exocrine and endocrine pancreas have been studied over several centuries, one important cell type in the gland, the pancreatic stellate cell (PSC), had remained undiscovered until as recently as 20 years ago. Even after its first description in 1982, it was to be another 16 years before its biology could begin to be studied, because it was only in 1998 that methods were developed to isolate and culture PSCs from rodent and human pancreas. PSCs are now known to play a critical role in pancreatic fibrosis, a consistent histological feature of two major diseases of the pancreas—chronic pancreatitis and pancreatic cancer. In health, PSCs maintain normal tissue architecture via regulation of the synthesis and degradation of extracellular matrix (ECM) proteins. Recent studies have also implied other functions for PSCs as progenitor cells, immune cells or intermediaries in exocrine pancreatic secretion in humans. During pancreatic injury, PSCs transform from their quiescent phase into an activated, myofibroblast-like phenotype that secretes excessive amounts of ECM proteins leading to the fibrosis of chronic pancreatitis and pancreatic cancer. An ever increasing number of factors that stimulate and/or inhibit PSC activation via paracrine and autocrine pathways are being identified and characterized. It is also now established that PSCs interact closely with pancreatic cancer cells to facilitate cancer progression. Based on these findings, several therapeutic strategies have been examined in experimental models of chronic pancreatitis as well as pancreatic cancer, in a bid to inhibit/retard PSC activation and thereby alleviate chronic pancreatitis or reduce tumor growth in pancreatic cancer. The challenge that remains is to translate these pre-clinical developments into clinically applicable treatments for patients with chronic pancreatitis and pancreatic cancer.

Keywords: pancreatic fibrosis, stellate cells, chronic pancreatic, desmoplastic reaction, pancreatic cancer, review

INTRODUCTION

"I will love the light for it shows me the way, yet I will endure the darkness because it shows me the stars." Og Mandino (American Essayist and Psychologist, 1923–1996).

Although both exocrine and endocrine functions of the pancreas and the cell types relevant to these functions (acinar cells, ductal cells, and cells of the islets of Langerhans) have been extensively studied since the pancreas was first described by Herophelus (335–280 BC) (Howard and Hess, 2002), one major cell type remained in the dark until as recently as 20 years ago. This cell type, the pancreatic stellate cell (PSC, so called because of its star-like appearance *in situ*) was first reported by Watari (Watari et al., 1982) in 1982 using electron microscopy of rodent and human pancreas. Watari likened these cells to hepatic stellate cells, which are well established as the key effector cells in liver fibrosis. However, there was little further effort in the field to characterize PSCs or to determine whether they played a similar fibrogenic role in the pancreas as their hepatic counterparts. This possibly reflected a general disinterest at the time in the mechanisms of pancreatic fibrosis which, despite being a predominant histological feature of two major pancreatic diseases (chronic pancreatitis and pancreatic cancer), was mostly considered to be an epiphenomenon of chronic injury.

It was to be another 16 years since Watari's initial report before methods were developed to isolate and culture PSCs, which finally provided researchers with a tool to study the mechanisms responsible for pancreatic fibrosis. In general, fibrosis is defined as the excessive accumulation of extracellular matrix (ECM) proteins (particularly fibrillar collagens) as a result of a loss of the normal balance between the deposition and the degradation of ECM. The concept of fibrosis as an inert, reactive tissue has changed significantly in recent times. Indeed, it is now well recognized that fibrogenesis in the pancreas is an active, dynamic process that may be reversible, at least in the early stages. Importantly, there is unequivocal evidence to indicate that PSCs play a central role in pancreatic fibrogenesis (Apte et al., 2011).

Abbreviations: PSCs, pancreatic stellate cells; PDGF, platelet derived growth factor; TGFb, transforming growth factor beta; HGF, hepatocyte growth factor; VEGF: vascular endothelial growth factor; IL, interleukin; NFkB, nuclear factor kappa B; AP-1: activator protein 1; αSMA, alpha smooth muscle actin; TLR, toll like receptor.

THE HISTORY OF STELLATE CELLS

In contrast to the pancreas, where the fibrogenic process has only received attention in recent years, the mechanisms of fibrosis in the liver have been well studied over several decades. Stellate cells

were identified in the liver more than 130 years ago by the famous pathologist Karl Wilhelm von Kupffer. In a letter written in 1876 to his colleague Heinrich von Waldeyer, Kupffer described star shaped cells ("sternzellen") stained with gold chloride in perisinusoidal spaces in the liver. However, Kupffer was unsure at the time as to whether these cells were different from resident liver macrophages, and this resulted in considerable confusion in the field for several decades. Almost 75 years after Kupffer's first description of "sternzellen," Ito (Ito, 1951) reported the presence of lipid-containing cells in a perisinusoidal location in the liver which he termed as Ito cells. Finally in 1971, Wake and colleagues (Wake et al., 1987), used multiple techniques (gold chloride staining, lipid staining and electron microscopy) which clearly demonstrated that vitamin A storing hepatic stellate cells were the same as the Ito cells and also the same as the sternzellen initially reported by Kupffer, but were distinctly different from liver macrophages (also known as Kupffer cells). Rapid progress was made in the field following this clarification, with detailed characterization of the biology and functions of hepatic stellate cells, from the first report of the possible role of HSCs in collagen synthesis by Kent and colleagues (Kent et al., 1976) in 1976, to the current time where HSCs are established as not only central to the production of ECM proteins but also as serving several other functions including roles in liver development and regeneration, retinoid metabolism and immunomodulation (Lee and Friedman, 2011).

As noted earlier, the pancreatic counterparts of HSCs were first described by Watari in 1982, (more than a hundred years after Kupffer's initial reports in the liver). Watari examined the pancreas of vitamin A loaded mice by fluorescence microscopy and electron microscopy and described the presence of cells exhibiting a rapidly fading blue-green fluorescence characteristic of vitamin A in the periacinar areas of the pancreas (Watari et al., 1982). In 1990, a similar (albeit sparsely distributed) vitamin A autofluorescence was reported in normal pancreatic sections from rats and humans by Ikejiri (Ikejiri, 1990). A few years later, in 1998, two seminal papers were published describing the isolation and culture of PSCs from rat and human pancreas (Apte et al., 1998; Bachem et al., 1998). These methods proved to be a major breakthrough because they finally provided an invaluable *in vitro* tool for researchers to characterize the biology of PSCs in health and disease.

PANCREATIC STELLATE CELLS

PSCs are located adjacent to the basolateral aspects of pancreatic acinar cells and have also been identified around small pancreatic ducts and blood vessels (**Figure 1**) (Watari et al., 1982; Ikejiri, 1990; Apte et al., 1998). They comprise approximately 4–7% of the total cell mass in the gland (Apte et al., 1998; Bachem et al., 1998). In the healthy pancreas, PSCs exhibit abundant, vitamin A containing lipid droplets in their cytoplasm and are in their quiescent (non-activated) state. They can be differentiated from fibroblasts due to their expression of selective markers such as desmin, glial fibrillary acidic protein (GFAP), vimentin and nestin (intermediate filament proteins) and neuroectodermal markers such as nerve growth factor (NGF) and neural cell adhesion molecule (NCAM). On electron microscopic examination, PSCs reveal a prominent rough endoplasmic reticulum, collagen fibrils and vacuoles (lipid droplets) surrounding a central nucleus.

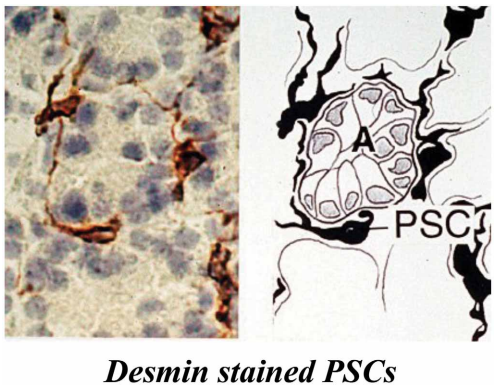


FIGURE 1 | Pancreatic stellate cells in rat pancreas stained for the selective marker desmin. The left panel shows a representative photomicrograph of normal rat pancreas immunostained for desmin. The right panel depicts the corresponding line diagram. Desmin positive PSCs with long cytoplasmic projections are located at the basolateral aspect of acinar cells (A). *Reprinted with permission from BMJ Group.*

CHARACTERISTICS OF QUIESCENT (NON-ACTIVATED) PSCs

These have been essentially determined using PSCs isolated from normal rat and human pancreas. Taking their cues from the method used to isolate HSCs, Apte et al. (1998) developed a technique to isolate PSCs based on the knowledge that in the normal pancreas, PSCs contain abundant lipid droplets in their cytoplasm which decreases cell density. Consequently, when a suspension of pancreatic cells is centrifuged through a density gradient, PSCs can be readily separated from other pancreatic cells (Apte et al., 1998). When placed in plastic culture wells, quiescent PSCs exhibit a flattened polygonal shape with prominent lipid droplets in the cytoplasm surrounding the central nucleus (**Figure 2**). Exposure of the cells to UV light at 328 nm elicits a transient blue-green fluorescence typical of vitamin A. After being in culture for a period of about 48 h, these quiescent PSCs become activated, a process that is inevitably associated with a loss of the cytoplasmic vitamin A droplets and a transformation of cell shape to a myofibroblast like phenotype that now expresses the cytoskeletal protein α smooth muscle actin (αSMA).

Although the presence of vitamin A containing lipid droplets in the cytoplasm is a specific marker of quiescent stellate cells, little is known about the mechanisms mediating their accumulation. Two recent studies by the same group of researchers have endeavoured to shed some light on this process. Kim and colleagues (Kim et al., 2009, 2010) have postulated that albumin (a protein that is endogenously expressed in PSCs and is co-localized with vitamin A in the lipid droplets) may play a role in lipid droplets formation. When the authors transfected activated PSCs (which had lost their lipid droplets) with expression plasmids for albumin, the cells exhibited a re-accumulation of lipid droplets that contained vitamin A (as confirmed by UV exposure). This was associated with increased resistance of the cells to the activating effects of the well known profibrogenic factor transforming

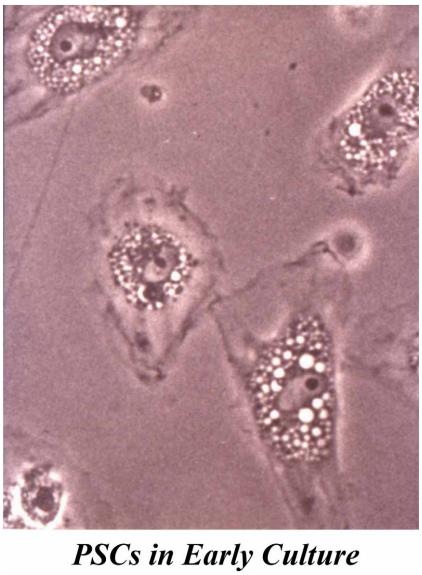


FIGURE 2 | PSCs in early culture. The cells exhibit a flattened polygonal shape with abundant lipid droplets (containing vitamin A) in the cytoplasm, surrounding the central nucleus. *Reprinted with permission from BMJ Group.*

growth factor beta (TGFβ). The authors have further shown that albumin is a downstream effector of the nuclear receptor peroxisome proliferator activated receptor γ (PPARγ) which is known to inhibit PSC activation (vide infra). While these studies provide interesting insights into the formation of vitamin A lipid droplets in PSCs, the mechanisms mediating their loss during PSC activation remain to be elucidated.

PSCs have the capacity to proliferate, to migrate and to synthesize and secrete proteins ECM proteins. Each of these functions is significantly stimulated during the activation process (see below). In addition to the production of ECM proteins, PSCs also produce matrix degrading enzymes (matrix metalloproteinases, MMPs) and their inhibitors (TIMPs, tissue inhibitor of metalloproteinases) (Phillips et al., 2003), suggesting that in health the cells may be responsible for the maintenance of normal ECM turnover in the pancreas. However, during pancreatic injury, when PSCs are activated, the balance between ECM production and ECM degradation is severely disturbed, leading to excessive ECM synthesis and eventually to the development of pathological fibrosis. With the availability of improved techniques for proteomic analyses based on mass spectrometry, three recent studies have assessed the differences in the proteomes of non-activated versus activated mouse (Paulo et al., 2011a), rat (Paulo et al., 2011b) and human (Wehr et al., 2011) PSCs. A detailed description of all differences is beyond the scope of this article, but it is interesting to note that numerous proteins were found to be differentially expressed in the two states, with proteins in the activated states being those related to the cell cytoskeleton, cell metabolism, motility, growth and invasion. (**Table 1** summarizes the different characteristics of quiescent and activated PSCs).

Table 1 | Characteristics of quiescent and activated PSC phenotypes.

	Quiescent PSCs	Activated PSCs
Vitamin A lipid droplets	Present	Absent
α Smooth muscle actin	Absent	Present
Proliferation	Limited	Increased
Migration	Limited	Increased
Extracellular matrix production	Limited	Increased
Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix proteinases (TIMPs)	Complement of MMPs and TIMPs to maintain normal ECM turnover	Change in types of MMPs and TIMPs to facilitate ECM deposition
Production of cytokines	Limited	Increased (PDGF, TGFβ, CTGF, IL1, IL6, IL15)
Capacity for phagocytosis	Absent	Present
Proteomic analyses	Basal protein expression	Differential expression of proteins related to the cell cytoskeleton, cell metabolism, motility, growth and invasion

While the initial focus of PSC related research was on understanding the role of activated PSCs in fibrosis (discussed in detail later), more recent efforts have been directed toward other non-fibrogenic functions of quiescent cells. Accumulating evidence suggests that PSCs may function as (1) progenitor cells; (2) immune cells or (3) intermediary cells in cholecystokinin (CCK)-induced pancreatic digestive enzyme (exocrine) secretion. With regard to their possible progenitor function, Mato et al. (2009) isolated and expanded pancreatic cells from lactating rats using mitoxantrone (a drug that acts through multidrug transporter systems) selection. They have reported that the surviving, mitoxantrone-resistant cells showed a PSC-like morphology (fibroblast-like with vitamin A lipid droplets), expressed the stem cell marker ABCG2 transporter (ATP binding cassette G2 transporter) and were able to secrete insulin after cell differentiation. However, whether such a selected "drug resistant" population is representative of normal PSCs remains to be examined. In order to determine whether the cells are true progenitor cells, additional work is needed to assess whether PSCs express other stem cell markers and can transform (under physiological conditions) into other cell types.

In terms of an immune function, Shimizu et al. (2005) were the first to observe that PSCs could internalize necrotic acinar cells and apoptotic neutrophils, but this was associated with necrotic cell death of the PSCs themselves. These *in vitro* observations were supported by the authors' *in vivo* work using a mouse model

of bile-duct ligation induced acute pancreatitis and a model of spontaneous chronic pancreatitis (WBN/Kob rats) in which they found that PSCs engulfed damaged parenchymal cells. Thus, the authors speculated that PSCs may exhibit a locally protective "innate" immune function to inhibit disease progression in early pancreatic injury. The role of PSCs in innate immunity is supported by the fact that the cells express Toll like receptors (TLR2, 3, 4, 5 and 9) which recognize foreign pathogen-associated molecular patterns (PAMPs) (Vonlaufen et al., 2007b; Masamune et al., 2008a; Nakamura et al., 2011). More recently, Shimizu and colleagues (2012) investigated whether PSCs may also have an "acquired" immune function by acting as antigen presenting cells. However, they found that rat PSCs did not express any antigen presenting cell markers such as MHC class II molecules or HLA-DR molecules. This finding differs from reports with HSCs which have been shown to process lipid antigens and present them to natural killer cells via CD1d. HSCs have also been shown to process protein antigens and present them to CD4 and CD8 positive T cells (Unanue, 2007; Winau et al., 2007). It is possible that the antigen-presenting capacity in HSCs develops because of the consistent exposure of the liver to foreign antigens from the gastrointestinal tract via the portal vein, whereas the pancreas (and PSCs) in comparison would be less likely to be exposed to the same load of exogenous antigens.

The question as to whether PSCs may play an intermediary role in CCK-induced digestive enzyme secretion arose from the known proximity of the PSCs to acinar cells *in situ* and the debate in the literature about the presence of functional CCK receptors on human acinar cells (in contrast to rat acinar cells where CCK receptors have been well identified). Two recent studies have convincingly demonstrated the presence of CCK receptors 1 and 2 on human PSCs (Berna et al., 2010; Phillips et al., 2010). Furthermore, the study by Phillips et al. (2010) has shown that PSCs respond to CCK by producing the neurotransmitter acetylcholine which can act on muscarinic receptors on acinar cells. Using a co-culture system of PSCs and acinar cells, the authors have also demonstrated an increase in amylase output by acinar cells in the presence of PSCs, which could be inhibited by the muscarinic receptor blocker atropine. These findings indicate that in humans, PSCs may play a significant intermediary role in regulating CCK-induced exocrine pancreatic secretion.

CENTRAL ROLE OF ACTIVATED PSCs IN PANCREATIC FIBROSIS

In vitro and *in vivo* studies over the past 14 years (ever since the first descriptions of methods to isolate PSCs) have now convincingly demonstrated that when activated during pancreatic injury, PSCs play a critical role in the pathogenesis of pancreatic fibrosis.

ACTIVATION OF PSCs – *In vitro* STUDIES

As noted earlier, transformation of PSCs from their quiescent to an activated state is a key event in fibrogenesis, resulting in excessive synthesis and deposition of ECM proteins. Specific molecules/factors and cellular pathways that mediate PSC activation were initially identified mostly by using cultured PSCs *in vitro*. The selection of putative activating factors for examination was based upon the knowledge that during the process of tissue injury, PSCs are likely to be exposed to factors such as: (1) alcohol and its metabolites acetaldehyde and fatty acid ethyl esters (FAEEs) [in view of the well known role of alcohol in pancreatitis (Apte et al., 2011)]; (2) endotoxin [given the known association of alcohol abuse and endotoxinaemia (Parlesak, 2005) and the correlation of circulating endotoxin levels with severity of pancreatitis (Windsor et al., 1993; Ammori et al., 1999)]; (3) growth factors and cytokines - transforming growth factor β (TGFβ), platelet derived growth factor (PDGF), tumour necrosis factor α (TNFα) and interleukins (IL), all of which are upregulated during pancreatic damage (Vonlaufen et al., 2007b); (4) oxidant stress (known to occur during both acute and chronic pancreatitis) (Uden et al., 1990; Casini et al., 2000); and (5) increased pancreatic pressure due to the "compartment syndrome" of chronic pancreatitis (Jalleh et al., 1991). To this list of activating factors have been added several others in recent years, on the basis of their overexpression in and/or association with chronic pancreatitis. These include hyperglycaemia [given that diabetes is a known complication of chronic pancreatitis (Nomiyama et al., 2007)], the endothelial cell derived vasoconstrictor endothelin-1 (Jonitz et al., 2009), cyclooxygenase 2 (COX-2, the inducible form of the rate limiting enzyme that converts arachidonic acid to prostaglandin) (Aoki et al., 2007), galectin-1 (a beta-galactoside binding lectin) (Masamune et al., 2006a) and the haemostatic protein fibrinogen (Masamune et al., 2009).

PSC activation in response to the above factors has generally been assessed using one or more of a number of "activation" parameters such as cell proliferation, αSMA expression, ECM protein synthesis, matrix degradation via the production of matrix metalloproteinases, loss of vitamin A stores, cell migration, cytokine release and contractility. Alcohol (ethanol) itself directly activates PSCs most likely due to the oxidative metabolism of ethanol to acetaldehyde via the enzyme alcohol dehydrogenase (ADH, known to be active in PSCs), and the subsequent generation of oxidant stress within the cell (Apte et al., 2000). Interestingly, ethanol upregulates PDGF-induced NADPH oxidase activity within PSCs (Hu et al., 2007), supporting the concept that reactive oxygen species (ROS) generated within PSCs play a role in PSC activation. It is noteworthy that ethanol can activate PSCs from their quiescent state and does not require the cells to be pre-activated to exert its stimulatory effects (Apte et al., 2000), suggesting that *in vivo*, PSC activation may occur early during chronic alcohol intake even in the absence of necroinflammation. This activation may then be perpetuated further during ethanol-induced necroinflammatory episodes leading to the development of fibrosis. Ethanol also inhibits PSC apoptosis (as assessed by the standard apoptosis indices Annexin V staining, TUNEL staining and caspase 3 and 9 activities) thereby facilitating cell survival (Vonlaufen et al., 2011). Furthermore, ethanol enhances the inhibitory effect of endotoxin lipopolysaccharide (LPS) on PSC apoptosis, suggesting that these two factors may exert synergistic effects on PSCs which promote cell activation and survival, thereby promoting pancreatic fibrosis. In contrast to the effects of the oxidative ethanol metabolite acetaldehyde on PSCs, the non-oxidative ethanol metabolites (FAEEs) have not

yet been reported to activate PSCs. However, one of the FAEEs, palmitic acid ethyl ester (PAEE), has been shown to stimulate specific signaling molecules within PSCs (see below) (Masamune et al., 2004).

With regard to cytokines, it is now well established that (a) PDGF is a potent proliferative and chemotactic factor for PSCs; (b) TGFβ and its downstream effector connective tissue growth factor (CTGF) stimulate the synthesis and secretion of ECM proteins (collagen, fibronectin, and laminin) by PSCs; TGFβ also induces matrix metalloproteinase 2 (MMP2) production by the cells [it is postulated that degradation of normal basement membrane collagen by MMP2, facilitates the deposition of abnormal (fibrillar) collagen, thereby promoting fibrosis]; (c) the proinflammatory cytokines TNFα, monocyte chemotactic protein (MCP-1) and IL1, IL6 and IL13 stimulate proliferation, αSMA expression and/or collagen synthesis in PSCs (Apte et al., 1999; Schneider et al., 2001; Mews et al., 2002; Michalski et al., 2007).

It is important to note that, in addition to responding to exogenous cytokines via paracrine pathways, PSCs themselves produce inflammatory mediators including TGFβ, CTGF, MCP-1, IL1, IL8, IL15 and RANTES (Regulated on Activation Normal T Cell Expressed and Secreted), all of which are capable of activating the cells via autocrine pathways (Andoh et al., 2000; Shek et al., 2002). The production of these endogenous cytokines can be stimulated by exogenous compounds such as ethanol, acetaldehyde, TGFβ and CTGF (Mews et al., 2002; Karger et al., 2008) and also by autocrine loops between certain cytokines in PSCs. For example, Aoki et al. (2006) have shown that IL-1β and IL6 produced by PSCs can each stimulate the autocrine secretion of TGFβ by the cells, and vice versa. In contrast IL13 (a Th2 lymphokine) suppresses TGFβ secretion by rat PSCs, although it induces PSC proliferation (Shinozaki et al., 2010). However, the relevance to human pancreatic fibrosis of the IL13 induced suppression of TGFβ secretion by rat PSCs is difficult to assess since the IL13 receptor system has not been detected in human pancreatitis specimens. Nonetheless, the ability of PSCs to be activated via autocrine pathways suggests that once activated, PSCs are capable of being in a perpetually activated state even in the absence of the initial trigger factors (**Figure 3**). This phenomenon may represent one of the mechanisms responsible for progression of chronic pancreatitis despite the cessation of the initial insult, for example alcohol and/or acute flare.

Oxidant stress (produced by exposure of cells to a pro-oxidant complex such as iron sulphate/ascorbic acid or hydrogen peroxide) activates PSCs and this activation is prevented by the antioxidant α-tocopherol (vitamin E) (Apte et al., 2000; Kikuta et al., 2004, 2006). PSCs have also been shown to generate ROS within the cell (Apte et al., 2000). Interestingly, (Masamune et al., 2008b) have demonstrated that PSCs express NADPH oxidase (an enzyme that is primarily found in phagocytic cells such as neutrophils and macrophages) to generate intracellular ROS, which, in turn, mediate activation of PSCs.

As noted earlier, pancreatic tissue pressure is elevated in chronic pancreatitis compared to normal pancreas. Asaumi et al. (2007) have reported activation of PSCs upon exposure to high pressure conditions (80 mmHg) produced using helium gas and a sealed pressure loading apparatus into which culture flasks bearing PSCs were placed. This activating effect was prevented by anti-oxidants such as N-acetyl cysteine (NAC) and epigallocatechin gallate (a green tea polyphenol), suggesting that it was mediated by intracellular oxidant stress.

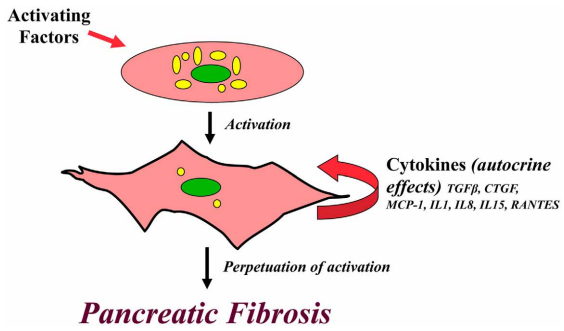


FIGURE 3 | Perpetuation of PSC activation. A diagrammatic representation of the postulated pathway for a perpetually activated state for PSCs. Pancreatic stellate cells are activated via paracrine pathways by exogenous factors such as cytokines, oxidant stress, ethanol and its metabolites. Activated PSCs synthesize and secrete endogenous cytokines which influence PSC function via autocrine pathways. It is possible that this autocrine loop in activated PSCs perpetuates the activated state of the cell, even in the absence if the initial trigger factors, leading to excessive ECM production and eventually causing pancreatic fibrosis.

Other factors that have recently been reported to induce PSC activation (as assessed by proliferation, migration, collagen production, αSMA expression or cytokine expression) include hyperglycaemia, endothelin 1, COX-2, galectin 1 and fibrinogen.

SIGNALING PATHWAYS IN PSCs

Having established the functional responses of PSCs to exogenous and endogenous factors, the logical next step for researchers in the field was to identify the intracellular signaling pathways mediating these responses, with the ultimate aim of developing approaches to target specific signaling molecules so as to interrupt PSC activation and inhibit abnormal fibrogenesis.

The activating effects of ethanol, acetaldehyde and oxidant stress on PSCs are mediated by activation of the mitogen activated protein kinase (MAPK) pathway (extracellular signal regulated kinase (ERK1/2), p38 kinase and c-jun amino terminal kinase (JNK), as well as the nuclear transcription factor AP-1 (Gukovskaya et al., 2002; McCarroll et al., 2003a,b). The non-oxidative metabolite of ethanol, PAEE also activates the same pathways in human PSCs (Masamune et al., 2004). Ethanol and acetaldehyde also activate two signaling molecules upstream of the MAPK cascade, phosphatidylinositol 3 kinase (PI3K) and protein kinase C (PKC) (McCarroll et al., 2003a,b). Given the synergistic effects of ethanol and endotoxin on PSCs *in vitro* (noted above), the LPS signaling pathway has recently been examined in PSCs. The cells express the LPS receptor TLR4 (toll like receptor 4) as well as the adapter molecules CD14 and MD2 (Vonlaufen et al., 2007b). Interestingly, TLR4 expression is upregulated in PSCs upon exposure to LPS (Vonlaufen et al., 2007b). PSCs are also known express other toll-like receptors, TLR2, 3 and 5 and

exposure of the cells to relevant TLR ligands activates the transcription factor NFκB (Masamune et al., 2008a). This finding is of interest because NFκB can induce anti-apoptotic proteins such as IAPs (inhibitor of apoptosis proteins) (Bhanot and Moller, 2009) and may provide an explanation for the LPS-induced inhibition of PSC apoptosis observed *in vitro*.

PDGF-induced PSC proliferation is mediated by ERK and JAK/STAT (Janus activated kinases/Signal induced activation of transcription) (Jaster et al., 2002; Masamune and Shimosegawa, 2009), while PDGF-induced migration is regulated by the PI3K pathway (McCarroll et al., 2004). There is significant cross-talk between PI3K and ERK in PSCs, so that modulation of one pathway is often associated with a change in the function of the other (McCarroll et al., 2004). Another signaling molecule which influences PSC migration is Indian hedgehog (IHH) (Shinozaki et al., 2008), a peptide belonging to the hedgehog protein family that is active in pancreas development, patterning and differentiation. PSCs express smoothened (Smo) and patched-1 (Ptch1) proteins which are essential components of the hedgehog receptor system. IHH – receptor binding leads to relocation of the transcriptional factor Gli-1 to the nucleus and results in chemotactic as well as chemokinetic migration of PSCs. This is associated with localization of membrane type I – matrix metalloproteinase (MT1-MMP) to the surface of PSCs, where it is thought to aid basement membrane degradation so as to facilitate cell movement. The profibrogenic growth factor TGFβ exerts its effect on PSCs via the intracellular signaling mediators SMAD2 and 3 (Ohnishi et al., 2004). TGFβ also exerts autocrine effect on PSCs whereby it induces its own mRNA expression; this process is regulated by the ERK pathway (Ohnishi et al., 2004).

The well-established association of PSC activation with the expression of the cytoskeletal protein αSMA, has prompted studies on the regulation of the actin cytoskeleton and PSC morphology. The small GTP protein Rho and its downstream effector Rho kinase regulate the actin cytoskeleton, stress fibre formation and alteration of cell shape during the PSC activation process (Masamune and Shimosegawa, 2009).

Intracellular calcium signaling, which is closely linked to the pathways mentioned above is modulated in PSCs in response to the binding of growth factors and cytokines to relevant receptors on the cell surface (Masamune and Shimosegawa, 2009). PSCs also respond to the extracellular nucleotides purines and pyrimidines (known to be involved in cell-cell communication of inflammatory signals after cell injury) via P2X and P2Y receptors (Hennigs et al., 2011). Activation of P2 receptors elicits robust intracellular Ca signaling known to mediate the fibrogenic function of activated PSCs (Hennigs et al., 2011).

Most recently, attention has turned toward microRNAs, the small non-coding RNAs that are implicated in many biological processes including cell differentiation, proliferation, apoptosis and tumorigenesis. Shen et al. (2012) have reported that miR-15b and miR-16 modulate rat PSC apoptosis by targeting the anti-apoptotic factor Bcl-2.

While the above discussion focuses on PSC activation pathways, factors and signaling molecules that inhibit PSC activation have also been examined in recent times. In this regard, it is now known that inhibition of MAPK (ERK, JNK and p38 kinase) signaling mediates the induction of quiescence of PSCs in response to retinol and its metabolites ATRA and 9-cisRA (McCarroll et al., 2003a,b). Curcumin, a polyphenol compound found in turmeric, decreases PDGF-induced PSC proliferation via inhibition of the ERK pathway; it also inhibits cytokine-induced PSC activation by inhibiting the MAPK pathway and by preventing activation of the transcription factor AP-1 (Masamune et al., 2006b). The peroxisome proliferator-activated receptor γ (PPAR γ, a ligand-activated transcription factor which controls cellular growth and differentiation) mediates the inhibitory effect of its ligand troglitazone on PDGF-induced and culture-induced activation of PSCs (Masamune et al., 2002; Shimizu et al., 2004).

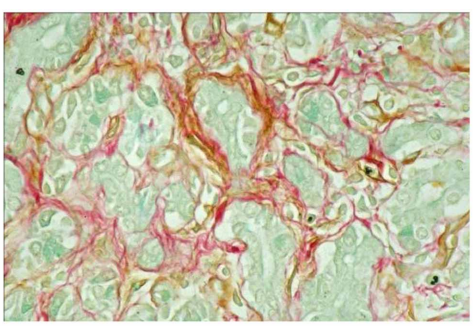
PSCs IN CHRONIC PANCREATITIS

The role of PSCs in chronic pancreatitis has been assessed predominantly via *ex vivo* studies using pancreatic sections from patients with chronic pancreatitis and *in vivo* studies using animal models of chronic pancreatitis.

HUMAN STUDIES

Human chronic pancreatitis sections have mainly been examined using standard histological stains (H and E, Masson's trichrome for connective tissue and Sirius Red for collagen) and using immunohistochemistry for the presence of specific proteins. These studies have clearly established that in the fibrotic pancreas, areas that stain positive for collagen also stain for alpha smooth muscle actin indicating the presence of activated PSCs (**Figure 4**). Moreover, using dual immunostaining for αSMA, it has been established that it is predominantly the activated PSCs that produce the collagen in the fibrotic areas (Haber et al., 1999).

The expression of growth factors known to activate PSCs is also increased in chronic pancreatitis. Pancreatic acinar cells adjacent to areas of fibrosis exhibit strong staining for TGFβ, while such staining is absent in acinar cells remote from bands of fibrosis (Haber et al., 1999), suggesting that TGFβ secreted by pancreatic



Collagen – red; α smooth muscle actin - brown

FIGURE 4 | Dual staining of a human chronic pancreatitis section immunostained for the PSC activation marker α smooth muscle actin (αSMA) and for collagen using Sirius Red. The brown staining for αSMA is co-localized with the red staining for collagen indicating the presence of activated PSCs in fibrotic areas of the pancreas. *Reprinted with permission from Elsevier.*

acinar cells may have a paracrine effect on PSCs, leading to increased collagen synthesis by the cells. TGFβ staining is also evident in spindle shaped cells in the fibrotic bands. The expression of NGF, one of the stellate cell selective markers) is also increased in human chronic pancreatitis (Friess et al., 1999). Since NGF is expressed by neuronal cells as well as PSCs, it is possible that proliferating PSCs in fibrotic areas may contribute to the observed increase in NGF staining in this disease. Expression of the receptor for PDGF is also increased (at both mRNA and protein levels) in areas of fibrosis in chronic pancreatitis (Haber et al., 1999). Given that PDGF is a potent mitogenic and chemotactic factor for PSCs, increased PDGF receptor expression on the cells may be one of the mechanisms responsible for the increased numbers of PSCs observed in fibrotic areas.

Interestingly, there is evidence of increased oxidant stress in fibrotic areas of chronic pancreatitis as indicated by positive staining for 4-hydroxynonenal (4HNE, a lipid peroxidation product) (Casini et al., 2000). This finding is highly relevant because PSCs are known to be activated in response to oxidant stress (as noted earlier).

ANIMAL MODELS

The human studies described above were important in terms of confirming the association of activated PSCs with pancreatic fibrosis in chronic pancreatitis. However, being cross-sectional studies, they did not allow examination of chronological events in the development of pancreatic fibrosis. This limitation was overcome by studies with animal models (predominantly rodent models) of pancreatic fibrosis. Fibrosis has been produced in rodents via various approaches. Rat models described in the literature include: (1) trinitrobenzene sulfonic acid (TNBS) injection into the pancreatic duct (Haber et al., 1999); (2) intravenous injection of an organotin compound dubutyltin chloride (DBTC) (Emmrich et al., 2000); (3) spontaneous chronic pancreatitis in WBN/Kob rats (Ohashi et al., 1990); (4) severe hyperstimulation obstructive pancreatitis (SHOP), involving intraperitoneal (IP) injections of supramaximal doses of caerulein (a synthetic analogue of CCK, a major pancreatic secretagogue) + bile-pancreatic duct ligation (Murayama et al., 1999); (5) repeated IP injections of a superoxide dismutase inhibitor (Matsumura et al., 2001); (6) intragastric high dose alcohol administration + repeated caerulein injections (Tsukamoto et al., 1988; Uesugi et al., 2004); (7) chronic alcohol administration (liquid diet) with repeated cyclosporin and caerulein injections (Gukovsky et al., 2008) and (8) chronic alcohol administration with repeated endotoxin LPS, injections (Vonlaufen et al., 2007b). Mouse models of pancreatic fibrosis include: (i) transgenic mice overexpressing TGFβ or the EGF receptor ligand heparin binding epidermal growth factor-like growth factor (HB-EGF) (Blaine et al., 2009); (ii) repetitive pancreatic injury induced by repeated injections of supramaximal caerulein (Neuschwander-Tetri et al., 2000); (iii) transgenic mice overexpressing IL-1β (Marrache et al., 2008b).

The overall results from animal studies to date support the concept that PSCs are activated early in the course of the injury, most likely due to paracrine effects of factors (cytokines and ROS) produced by injured acinar cells and/or inflammatory cells during the acute phase of the injury. Activated PSCs are the major source of collagen in fibrotic areas (confirming findings from in human studies). As with human chronic pancreatitis, expression of factors known to activate PSCs are all reported to be upregulated in experimental pancreatic fibrosis including PDGF and its receptor, TGFβ and two TGFβ regulated genes SM22α and Cygb/STAP, and oxidant stress.

Although the above models have provided useful data, caution needs to be exercised in assessing their direct clinical relevance, since most have involved relatively non-physiological methods (e.g., injections of toxin into the pancreatic duct, administration of supraphysiological levels of caerulein or interventions such as bile duct ligation), to produce pancreatic damage. However, there is one rat model produced by chronic alcohol administration and repeated endotoxin exposure (Vonlaufen et al., 2007a,b) that is based on a well recognized clinical phenomenon, namely endotoxinaemia (secondary to increased gut mucosal permeability) in alcoholics (Bode et al., 1993; Parlesak, 2005). Thus, the alcohol feeding, LPS challenge model possibly represents the most physiologically relevant model of chronic alcoholic pancreatitis described to date.

SOURCE OF ACTIVATED PSCs IN THE FIBROTIC PANCREAS – EVIDENCE FROM ANIMAL MODELS

The significant increase in PSC numbers observed during pancreatic injury has raised the question as to whether these increased numbers are made up largely of resident "pancreatic" PSCs or whether migratory cells homing to the pancreas from extra-pancreatic sources such as the bone marrow (in response to chemotactic signals from the injured organ) also contribute to the PSC population. Two recent studies have used a gender mismatch and chimeric approach whereby green fluorescent protein (GFP) labeled bone marrow derived cells (BMDC) obtained from GFP transgenic male donor mice were transplanted into lethally irradiated wild type female rodents (Marrache et al., 2008a,b; Sparmann et al., 2010). Pancreatic injury was then induced in recipient mice either by repeated injections of caerulein or using the chemical toxin dibutyltin chloride (DBTC). Both studies showed that a small proportion (5–18%) of the proliferative PSCs in the pancreas could be bone marrow derived, but additional studies with different models of pancreatic fibrosis need to be performed to fully characterize the contribution of bone marrow derived PSCs to progression (or repair) of pancreatic injury.

FATE OF ACTIVATED PSCs AND REVERSAL OF PANCREATIC FIBROSIS

As the processes of PSC activation are becoming increasingly clear, the fate of activated PSCs is also attracting increasing attention. Three possibilities that have been considered include: (1) reversion to quiescence; (2) apoptosis; and (3) senescence. Partial reversion to quiescence has been described *in vitro* upon exposure to retinol and its metabolites, albumin or culture on matrigel (a basement membrane like matrix) (McCarroll et al., 2003b; Kim et al., 2009), however, there is no *in vivo* evidence yet to support these findings. On the other hand, apoptosis of PSCs has been well described *in vitro* and also recently *in vivo* using the alcohol-fed endotoxin challenged model of chronic

pancreatitis. Vonlaufen et al. (2011) have demonstrated increased apoptosis of activated PSCs *in vivo* upon withdrawal of alcohol in this model. In terms of cell senescence, a very recent paper by Fitzner and colleagues (2012) has reported that PSCs in long-term culture (6 weeks) express the senescence marker senescence associated b-galactosidase (SA-βGal) and are highly susceptible to immune cell-mediated cytotoxicity. Furthermore, the authors report that in a rat model of DBTC-induced pancreatitis, PSCs not only express activation markers, but also senescence markers leading them to speculate that inflammation, PSC activation and senescence are timely coupled processes in the injured pancreas. However, this study did not assess PSC apoptosis. Indeed, the relative contributions of the processes of apoptosis versus senescence versus reversion to quiescence in the removal of activated PSCs after pancreatic injury remain to be clarified.

Regardless of the eventual fate of activated PSCs, advances in our knowledge of the processes of PSC activation have helped underpin evidence-based rationales for the development of potentially useful anti-fibrotic therapies *in vivo* (albeit only in experimental models so far). Several treatments/approaches have been reported to prevent/retard fibrosis in animal models, including: (i) Antioxidants - vitamin E (the subclass tocotrienol has been shown to induce PSC death via apoptosis and autophagy) (Gomez et al., 2004; Vaquero et al., 2007), oxypurinol and allopurinol, both xanthine oxidase inhibitors (Pereda et al., 2004; Tasci et al., 2007), ellagic acid, a plant derived polyphenol with antioxidant, anti-inflammatory and anti-fibrosis activities (Suzuki et al., 2009), and salvianolic acid, a herbal medicine with free radical scavenging properties (Lu et al., 2009); (ii) TGFβ suppression - using TGFβ neutralizing antibodies (Menke et al., 1997), a herbal medicine Saiko-keishi-to (Su et al., 2001) or a plant alkaloid halofuginone which inhibits downstream Smad3 phosphorylation (Zion et al., 2009); (iii) TNFα inhibition - using a TNFα antibody (Hughes et al., 1996), soluble TNFα receptors or an inhibitor of TNFα production pentoxifylline (Pereda et al., 2004); (iv) anti-inflammatory agents - protease inhibitors such as camostat mesilate which inhibit proinflammatory cytokine production by monocytes (Gibo et al., 2005) and the synthetic carboxamide derivative IS-741 which suppresses macrophage infiltration into the pancreas, with a consequent decrease in *in vivo* PSC activation (Kaku et al., 2007); and (v) modulation of signaling molecules using the PPARγ ligand troglitazone (Shimizu et al., 2004).

In terms of alcoholic pancreatic fibrosis, withdrawal of alcohol from the diet after established early pancreatitis, has been shown to result in complete reversal of pancreatic fibrosis (Vonlaufen et al., 2011). The authors have attributed this effect to the fact that the alcohol-induced inhibition of PSC apoptosis (described earlier) is removed in the absence of alcohol, thereby enabling the loss of activated PSCs through cell death and interrupting the fibrogenic process. These observations provide a strong experimental basis for the advocacy of abstinence in patients with alcoholic pancreatitis in a bid to prevent disease progression.

PSCs IN PANCREATIC CANCER

Pancreatic ductal adenocarcinomas are characterized by an abundant stromal/desmoplastic reaction which, up until recently had received little attention in terms of its possible role in the pathogenesis of the disease. It was the emerging evidence of the central role of PSCs in the fibrosis of chronic pancreatitis that stimulated researchers to investigate whether the same cells were responsible for the production of the stroma of pancreatic cancer, and if so, whether PSCs interacted with cancer cells to influence disease progression (Apte and Wilson, 2012). Of particular relevance in this regard were the known increased risk of pancreatic cancer in patients with chronic pancreatitis (Raimondi et al., 2010) and the commonalities in gene expression between the stromal compartments of chronic pancreatitis and pancreatic cancer (Binkley et al., 2004).

Histological and immunohistochemical studies of human pancreatic cancer sections have shown that activated PSCs are present in the desmoplastic areas of pancreatic cancer (Apte et al., 2004). Furthermore, dual staining for activated PSCs (αSMA) and for collagen mRNA (*in situ* hybridisation) has established that the predominant cells responsible for producing the fibrosis in pancreatic cancer are PSCs (**Figure 5**) (Apte et al., 2004).

The possibility of a close interaction between PSCs and pancreatic cancer cells has been examined *in vitro* (using co-cultures of PSCs and pancreatic cancer cell lines and/or exposure of one cell type to conditioned medium from the other) as well as *in vivo* (using subcutaneous, orthotopic and transgenic mouse models of pancreatic cancer) (Apte and Wilson, 2012).

In vitro STUDIES

Exposure of PSCs to cancer cells (either directly or via conditioned media) results in activation of PSCs (increased proliferation, ECM synthesis, and migration) (Apte and Wilson, 2012). In turn, PSCs stimulate cancer cell proliferation but inhibit cancer cell apoptosis thereby effectively enhancing the survival of cancer cells and induce cancer cell migration. The PSC-induced cancer cell migration is associated with epithelial-mesenchymal transition in cancer cells as indicated by decreased expression of epithelial markers such as E-cadherin and increased expression of mesenchymal markers such as vimentin and Snail in cancer cells (Kikuta et al., 2010). It is possible that this PSC-induced epithelial-mesenchymal transition in cancer cells facilitates the migration of these cells. Recently, a study by Ikenaga et al (Ikenaga et al., 2010) has reported that a subset of PSCs that overexpress CD10 (a cell membrane associated MMP) induce cancer cell invasion and proliferation significantly more than CD10 negative PSCs, suggesting that functional heterogeneity of PSCs may influence their effects on tumour progression. Overall, the above observations suggest that pancreatic cancer cells recruit host PSCs to their immediate vicinity and that PSCs reciprocate by facilitating cancer cell growth as well as local invasion (**Figure 6**). Most recently, it has been reported that PSCs increase the stem cell phenotype of cancer cells, as assessed by increased expression of stem cell markers such as nestin, ABCG2, and LIN28 in cancer cells upon co-culture with PSCs (Hamada et al., 2012). These findings have implications for the possible resistance to treatment of a cancer stem cell niche which then facilitates recurrence of tumour.

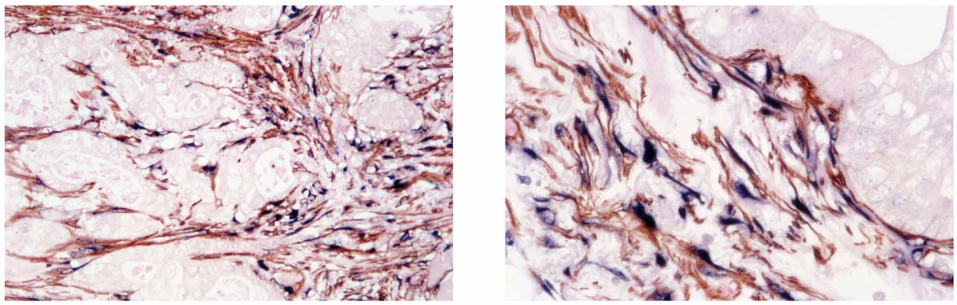


FIGURE 5 | Dual staining of a human pancreatic cancer section for α smooth muscle actin (αSMA) and mRNA for collagen. The Figure depicts low and high power views of a pancreatic cancer section immunostained for αSMA and for collagen mRNA using *in situ* hybridisation. The brown staining for αSMA is co-localized with the blue staining for collagen mRNA. Importantly, both stains are restricted to the stromal areas of the section, with no staining of the tumour elements. The findings indicate that activated PSCs are the predominant source of collagen in the stroma of pancreatic cancer. *Reprinted with permission from Wolters Kluwer Health.*

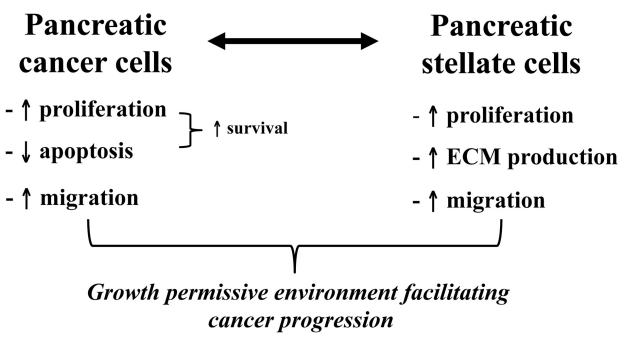


FIGURE 6 | Bidirectional interactions between pancreatic cancer cells and PSCs. The diagram outlines the effects of pancreatic cancer cells on PSCs and vice versa. The observed interaction between these two cell types facilitates local tumour growth as well as regional and distant metastasis of pancreatic cancer.

The cancer cell induced increase in ECM synthesis by PSCs is thought to be mediated by TGFβ1 and fibroblast growth factor 2 (FGF2), while PSC proliferation is likely mediated by PDGF. Recent studies have also implicated cycloxygenase 2 (COX-2, the inducible form of cycloxygenases, enzymes involved in conversion of arachidonic acid to prostaglandin) (Yoshida et al., 2005) and trefoil factor 1 (TFF1) (Arumugam et al., 2011) (a stable secretory protein that is upregulated in pancreatic cancer but not expressed in the normal pancreas) in PSC proliferation in response to cancer cell secretions. COX-2 is upregulated in PSCs exposed to the pancreatic cancer cell line PANC1 and inhibition of COX-2 prevents PANC1 induced PSC proliferation. ERK1/2 has been identified as the signaling pathway regulating cancer cell-induced PSC proliferation (Yoshida et al., 2004).

The possible factors mediating the effects of PSCs on cancer cells remain to be characterized. However, PSC-induced proliferation of cancer cells is thought to be mediated, at least in part, by PDGF (Xu et al., 2010). Other candidate factors in PSC secretions that require further study as possible mediators include the growth factors insulin-like growth factor (IGF), EGF, hepatocyte growth factor (HGF), TGFßand other proinflammatory cytokines.

In vivo STUDIES

In order to obtain *in vivo* evidence to support the stromal-tumour interactions observed *in vitro*, scientists have turned to murine xenograft or transgenic models. Using an immunocompromised mouse model of pancreatic cancer produced by subcutaneous injection of either a suspension of pancreatic cancer cell cells alone or an admixture of cancer cells and PSCs, Bachem et al. (2005) have shown a significantly increased rate of tumour growth in the latter group. The larger tumours in the mice injected with cancer cells + PSCs were due not only to the expected PSC-mediated fibrosis but also to proliferation of tumour cells themselves, suggesting that the presence of PSCs stimulated cancer cell growth.

One of the drawbacks of subcutaneous xenografts is the absence of the natural tumour microenvironment. Therefore, orthotopic models which involve injection/implantation of cancer cells directly into the organ of interest are a preferred option. In these models, tumours develop in a relevant anatomical location, so that the implanted cancer cells are exposed to the same microenvironment as may be expected in human cancer. In addition, orthotopic tumours have the capacity to metastasise thus allowing studies of tumour progression. Early studies by Lohr et al. (2001) reported that orthotopic injections of pancreatic cancer cells (PANC-1 cell line) transfected with TGFβ1 cDNA resulted in the induction of an extensive stromal reaction around the pancreatic tumour. Although not specifically studied at the time, this stromal reaction was most likely via the TGFβ-induced activation of stromal cells/fibroblasts/ in the host (mouse) pancreas.

More recently, orthotopic models of pancreatic cancer have been described wherein human pancreatic cancer cells (MiaPaCa-2, AsPC-1) with or without human pancreatic stellate cells (hPSCs) were injected directly into the mouse pancreas (Vonlaufen et al., 2008; Xu et al., 2010). In the presence of hPSCs, local tumour growth, and importantly, regional and distant metastasis were significantly enhanced. Tumours produced by the mixture of cancer cells and PSCs exhibited bands of fibrosis (resembling desmoplasia) and the presence of αSMA positive activated PSCs as well as increased proliferation and decreased apoptosis of cancer cells. These data concur well with the interactions between PSCs and cancer cells observed *in vitro* and strongly support an active role for PSCs in cancer progression (increased local growth and distant metastasis).

Neo-angiogenesis is a well recognized event in malignant tumours and is thought to be a major factor influencing cancer metastasis. PSCs significantly enhance tumour angiogenesis as indicated by upregulation of the endothelial cell marker CD31 in orthotopic tumours produced by cancer cell + PSCs compared to tumours produced by injection of cancer cells alone (Xu et al., 2010). These *in vivo* findings are supported by *in vitro* observations that PSCs stimulate tube formation of human microvascular endothelial cells, an effect that is mediated by vascular endothelial growth factor (VEGF) secreted by PSCs (Xu et al., 2010).

The process of angiogenesis in human pancreatic cancers may be somewhat more complex than that in mouse models. Studies with human pancreatic cancer sections indicate that neoangiogenesis is limited to the invading front of the tumour while the central areas of the tumour have few patent blood vessels and are relatively hypoxic (Erkan et al., 2009). In an attempt to address this issue, Erkan et al. (2009) assessed the effects of hypoxia on the interactions of PSCs with endothelial cells. Using co-cultures of the two cell types, they found that the VEGF-mediated proliferative effect of PSCs on endothelial cells observed under normoxic conditions were dampened under hypoxic conditions. At the same time however, hypoxia significantly increased PSC activation and ECM synthesis. Further studies are needed to clarify the relative importance of new blood vessel formation versus tumour hypoxia in terms of the influence of PSCs on cancer behavior.

One of the intriguing features of PSC biology reported recently, is the ability of the cells migrate through an endothelial layer *in vitro*, suggesting that PSCs have the capacity to intravasate/extravasate to and from blood vessels *in vivo* (Xu et al., 2010). In the presence of cancer cells transendothelial migration of PSCs is further stimulated, an effect which may be mediated by PDGF in cancer cell secretions (Xu et al., 2010). More interestingly, it has now been shown, using a gender mismatch approach, that PSCs from the primary tumour (produced by implantation of female pancreatic cancer cells + male PSCs into the pancreas of female mice) can be detected in distant metastatic sites as y chromosome positive cells using fluorescent *in situ* hybridization. These findings suggest that PSCs can travel to distant metastatic sites (possibly with cancer cells) where they likely facilitate the seeding, survival, and growth of cancer cells. Indeed, similar observations have now been reported in a model of lung cancer (Duda et al., 2010). These findings challenge the long held concept that metastasis is the sole preserve of cancer cells.

The above studies provide convincing evidence of an active role of PSCs in pancreatic cancer progression. It is now also acknowledged that PSCs (via the production of dense stroma) may play a role in the well documented resistance of pancreatic cancer to chemotherapy and radiotherapy (Hanahan and Weinberg, 2011). In this regard, Olive et al. (2009) have shown in an orthotopic model of pancreatic cancer, that gemcitabine (a widely used chemotherapeutic agent for pancreatic cancer) is sequestered in the stromal area of pancreatic cancer, thereby limiting the availability of drug to cancer cells and providing a possible explanation for the chemoresistance of the disease. In addition, Mantoni and colleagues (2011) have reported that PSCs protect cancer cells from radiation via a ß1-integrin dependent pathway.

Given the accumulating evidence of the influence of PSCs on pancreatic cancer behavior, it is logical that the stroma is now seen as an important alternative therapeutic target to improve the outcome of this disease. Several recent studies have reported encouraging findings with such approaches in pre-clinical models. In a transgenic mouse model of pancreatic cancer, Olive et al. (2009) have shown that inhibition of the Sonic hedgehog pathway in PSCs (achieved by using vitamin A containing liposomes to specifically target stellate cells) transiently decreased PSC activation resulting in stromal depletion and increased accumulation of gemcitabine in cancer cells leading to cancer cell destruction. Von Hoff et al. (2011) have observed stromal depletion upon treatment of subcutaneous xenografts in mice with nanoparticle albumin bound paclitaxel, while Froeling and colleagues (2011) have reported decreased tumour growth in a transgenic mouse model of pancreatic cancer treated with the PSC inhibitor all trans retinoic acid (ATRA). It is anticipated that ongoing research will also target the signaling pathways/molecules that mediate PSC-cancer cell interactions so as to inhibit the facilitatory effects of PSCs on cancer progression.

To conclude, over the past two decades there has been a steep rise in our understanding of pancreatic fibrogenesis and the central role of PSCs in this process. It is also clear that PSCs have functions over and beyond the regulation of pathologic fibrosis in the pancreas, with the cells likely playing important roles in health as immune and/or progenitor cells and as intermediary cells in digestive enzyme secretion (at least in humans). Improved understanding of PSC biology will underpin the development of novel therapies in the future for the treatment of chronic pancreatitis and pancreatic cancer.

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Commensal flora, is it an unwelcomed companion as a triggering factor of autoimmune pancreatitis?

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The etiopathogenesis of many autoimmune disorders has not been identified. The aim of this paper is to focus on the involvement of bacterial exposure, as an environmental factor, in the pathogenesis of autoimmune pancreatitis (AIP), which is broadly categorized as autoimmune disorders involving pancreatic lesions. Avirulent and/or commensal bacteria, which may have an important role(s) as initiating/progressing factors in the pathogenesis of autoimmune disorder AIP, will be emphasized.

Keywords: autoimmune pancreatitis, bacteria, innate immunity, autoimmune disease, commensal flora

AUTOIMMUNE DISEASES ASSOCIATED WITH MICROBIAL INFECTION

Autoimmune diseases arise from an overactive immune response of the body against tissues normally present in the body. Organ-specific autoimmune disease can develop through a combination of hereditary and environmental factors that regulate adaptive immune responses to self antigens (Mills, 2011). There are well-established links between infection and autoimmune diseases. Several studies have implicated microorganisms in the environmental etiology of autoimmune disorders based on observations such as, infection with *Chlamydia pneumoniae*, human herpesvirus 6, and Epstein–Barr virus (EBV) are triggered or exacerbated by multiple sclerosis (Mills, 2011). The regression of autoimmune thrombocytopenia is seen after the eradication of *Helicobacter pylori* (Gasbarrini et al., 1998). In Guillain–Barré syndrome (GBS), amino acid similarities exist between the gangliosides of the nerve system and the lipopolysaccharides (LPSs) of *Campylobacter jejuni*, suggesting that sensitization by microbes may be based on autoimmunity from molecular mimicry between bacteria and the targeted system of the host (Yuki et al., 2004; Houliston et al., 2011). The link has been attributed to either molecular mimicry between pathogen-derived antigens and self antigens or non-specific activation of innate immunity leading to a breakdown in immunological tolerance and the development of self antigen-specific T cell and antibody response (Mills, 2011). A common recent theory of the cause of autoimmune diseases is that an infectious agent triggers a cycle of events, which leads to the upregulation of the host immune response to self antigens (Aoki, 1999; Tlaskalová-Hogenova et al., 2004).

AUTOIMMUNE PANCREATITIS AND IgG4-RELATED DISEASES

Autoimmune pancreatitis (AIP) is a putative autoimmune disease and is a chronically progressing inflammatory disease of the pancreas (Park et al., 2009; Okazaki et al., 2011a). The morphological characteristics of AIP include diffuse or localized enlargement of the pancreas and irregular narrowing of the main pancreatic duct. Histologically, the disease is also associated with progressive lymphoplasmacytic infiltration, predominantly localized to the ductal structures, and varying degrees of parenchymal and acinar destruction (Okazaki et al., 2008).

There are two types of AIP that differ in their clinical features, such as the gender ratio, mean age, and associated immune-related diseases. Type 1 AIP is associated with the histological finding of lymphoplasmacytic sclerosing pancreatitis (LPSP). Its serological hallmark is an elevation in the serum levels of the IgG4 subclass of IgG (Okazaki et al., 2011a). Type 1 AIP appears to be the pancreatic manifestation of a systemic disease called IgG4-associated systemic disease (ISD) or IgG4-related sclerosing disease, affecting not only the pancreas, but also other organs including the bile duct, retroperitoneum, kidney, lymph nodes, and salivary glands (Kamisaw and Okamoto, 2006; Okazaki et al., 2011b). Type 2 AIP is a form of idiopathic chronic pancreatitis (ICP), histologically associated with granulocyte-epithelial lesions (Shimosegawa

Abbreviations: AIP, autoimmune pancreatitis; ANAs, antinuclear antibodies; CA-II, carbonic anhydrase II; CPS, capsular polysaccharide; CTLA-4, cytotoxic T-lymphocyte antigen-4; DAMPs, damage-associated molecular patterns; FCRL3, Fc receptor-like gene 3; GBS, Guillain–Barré syndrome; GEL, granulocyte-epithelial lesions; HLA, human leukocyte antigen; HSP, heat shock protein; IDCP, idiopathic duct-centric pancreatitis; LF, lactoferrin; LPS, lipopolysaccharide; LPSP, lymphoplasmacytic sclerosing pancreatitis; MAMPs, microorganism-associated molecular patterns; PAMP, pathogen-associated molecular patterns; PBC, primary biliary cirrhosis; PBP, plasminogen-binding protein; poly I:C, polyinosinic:polycytidylic acid; PRR, pattern-recognition receptor; T_{reg}, regulatory T cell; TGF, transforming growth factor; TLR, toll-like receptor; UBR2, ubiquitin-protein ligase E3 component N-recognin 2.

and Kanno, 2009). The pathogenesis of AIP remains unknown. Genetic associations between susceptibility to the disease and the human leukocyte antigen (HLA) DRB1∗0405-DQB1∗0401 haplotype (Kawa et al., 2002; Muraki et al., 2006; Ota et al., 2007), Fc receptor-like gene 3 (FCRL3; Kojima et al., 2007), and the CTLA-4 gene (Umemura et al., 2008) have been suggested.

An outstanding finding in type 1 AIP is hypergammaglobulinemia and the existence of a high serum concentration for IgG4, which has been documented in 90% of patients (Hamano et al., 2001). This occurs in parallel to an abundant IgG4 positive plasma cell infiltration in the pancreatic tissue (Aoki et al., 2005). The fibroinflammatory process characterizing AIP occurs at the pancreatic basement membranes where IgG4/IgG/complement–immune complexes are deposited (Detlefsen et al., 2010). IgG1 is believed to be an opsonizing antibody and activates the complement classical pathway, namely opsonization of a bacterium by activated complement and antibodies. Combined opsonization by both complement and antibodies considerably enhances the uptake of the bacterium by the phagocyte (Wilson et al., 2011). AIP is occasionally associated with elevated circulating immune complex levels, which are significantly linked to increased serum IgG1 and complement activation via the classical pathway (Muraki et al., 2006). IgG4 is unable to activate the classical pathway of complements, but binds IgG1, 2, and 3 and forms an immune complex by Fc–Fc interaction in patients with AIP (Kawa et al., 2008). Although the role of IgG4 in the immune response and autoimmunity has not yet been fully elucidated, it may be hypothesized that IgG4 blocks the Fc-mediated effector functions of IgG1 and dampens the inflammatory response to an as yet unidentified primary trigger of the inflammatory process in AIP (Ito et al., 2010).

IS THERE ANY LINK BETWEEN BACTERIA AND THE PATHOGENESIS OF AIP?

To which antigens the immunoglobulins in the deposits along the basal membranes of the pancreatic ducts and acini react remains unclear and definitive disease-specific antibodies have not been identified. However, several candidates for AIP-specific autoantibodies have been reported, such as anti-lactoferrin (LF; Okazaki et al., 2000), anti-carbonic anhydrase (CA)-II (Okazaki et al., 2000), anti-CA IV (Nishimori et al., 2005), anti-pancreatic secretory trypsin inhibitor (PSTI; Asada et al., 2006), anti-amylase-alpha (Endo et al., 2009), anti-heat shock protein (HSP) 10 (Takizawa et al., 2009), and anti-plasminogen-binding protein (PBP) peptide autoantibodies (Frulloni et al., 2009). These putative AIP-specific autoantibodies give us some hints toward considering the possibility that microorganisms are involved in the pathogenesis of AIP.

In order to survive in a host, a pathogenic microbe has to follow these steps; (i) attach to the host cells for colonization, (ii) evade the host's innate and adaptive immune defense and persist in the host, (iii) obtain iron and other nutrients, (iv) disseminate or spread within a host, and (v) produce symptoms of disease in the host (although production of symptoms is not necessary; Wilson et al., 2011). Following the above mentioned processes, it might be valid to hypothesize that microbes are the pathogenic factors of AIP, based upon the possible roles of AIP-specific antibodies.

(i) *LF*: Considering the fact that LF is distributed in the ductal cells of several exocrine organs, including the pancreas, salivary gland, biliary duct, lungs, and renal tubules (Okazaki et al., 2011b), an anti-LF antibody might be induced as a consequence of the tissue damage caused by microbial infection and anti-LF might exert direct effects on these ductal cells of exocrine organs.

Iron is essential for the growth of bacteria. Most pathogenic bacteria require iron and thus there is a strong correlation between iron availability and virulence (Wilson et al., 2011). However bacteria have to cope with the fact that iron concentrations in nature are quite low. The concentration of free iron is particularly low in the host body due to the actions of host proteins, such as lactoferrin, transferring, ferritin, and heme, that bind most of the available iron. To survive in the body, bacteria must have some mechanism for acquiring this sequestered iron (Wilson et al., 2011).

LF is a glycoprotein, and a member of a transferrin family, thus belonging to those proteins capable of binding and transferring Fe^{3+} ions (Wilson et al., 2011). LF binds Fe^{3+} with an affinity and stability much higher than that of transferrin in the serum (Valenti and Antonini, 2005). LF is secreted from the exocrine glands and in specific granules of neutrophils, which are the main source of lactoferrin in blood plasma after degranulation. Neutrophils are at the front line of the innate immunity process (Wilson et al., 2011). LF binds iron released from transferrin, which prevents its further usage for bacterial proliferation. Besides iron, LF is capable of binding a large amount of other compounds and substances such as LPS, heparin, glycosaminoglycans, or other metal ions and certain DNA and RNA viruses. The concentration of LF in the blood increases during infection and inflammation (Adlerova et al., 2008). Since many bacteria synthesize and secrete small iron-chelating molecules (siderophores) that can compete with LF for insoluble Fe^{3+} ions, the growth-inhibiting activity of the protein is thus reversed. Iron sequestration by apo-LF, which is an iron-free form of LF, can effectively inhibit the growth of many bacterial species (Valenti and Antonini, 2005). LF has been related to the decreased survival of microorganisms in the host. LF combines with the bacterial surface through electrostatic and hydrophobic interactions, resulting in a perturbation of bacterial membranes. The molecular mechanisms of this bactericidal activity of LF, which is not related to iron withholding, appears to be similar for either Gram-negative or Gram-positive bacteria. Taking these characteristics of LF into consideration, blocking the LF function by an anti-LF antibody might be beneficial for microbes to get the essential growth element iron to survive and prolong persistent infection in the body of AIP patients. Assessing whether or not the anti-LF antibody obtained from AIP patients actually possesses the blocking function of the binding of $Fe3^{+}$ ion to LF would be of interest.

(ii) *HSP*: Mammalian HSP10 and HSP60, also known as chaperonins 10 and 60, are mitochondrial proteins involved in protein folding. HSPs exported to the plasma membrane

or released from dying cells are believed to be a source of "danger" signals, informing the innate and adaptive immune systems of tissue damage induced by various insults including infection, toxins, and cellular stress (Johnson et al., 2005; Shields et al., 2011).

It has not been clarified whether anti-HSP10 production is the cause or the consequence of AIP progression, but HSP10 consistently inhibits LPS-induced TLR4 signaling by interacting with HSP60 in the extracellular milieu and inhibits LPS-induced secretion of the pro-inflammatory cytokines, TNF-α, IL-6, and the pro-inflammatory chemokine regulated upon activation, normal T cell expressed and secreted (RANTES) and anti-inflammatory cytokine IL-10 (Johnson et al., 2005). From this point of view, it might be possible that the production of anti-HSP10 antibodies indicates the implication of bacteria in the pathogenesis of AIP.

(iii) *Molecular mimicry*: Homology between alpha-CA of *H. pylori* and human CA-II has been also reported. Guarneri et al. showed a significant homology between human CA-II and α-CA of *H. pylori*. Moreover, the homologous segments contained the binding motif of DRB1∗0405 (Guarneri et al., 2005). Notably, the possession of the HLA-DRB1∗0405-DQB1∗0401 genotype confers a risk for AIP development (Ota et al., 2007). The anti-PBP peptide antibody was newly identified in patients with AIP. Frulloni et al. (2009) reported that 94% of AIP patients, but only 5% of pancreatic cancer patients, exhibit IgG antibodies to a PBP that is homologous to the human protein ubiquitin-protein ligase E3 component n-recognin 2 (UBR2), which is expressed in pancreatic acinar cells and is also homologous to the PBP of *H. pylori*. These data suggest that *H. pylori* infection may trigger AIP in genetically predisposed subjects through autoimmune responses triggered by molecular mimicry.

(iv) *CAs*: Immunization with CA-II or LF has been reported to induce systemic lesions such as pancreatitis, sialadenitis, cholangitis, and interstitial nephritis (Nishimori et al., 1995; Ueno et al., 1998). The distribution of CA-II and IV are both in the ductal cells of several exocrine organs, including the pancreas, salivary gland, biliary duct, lungs and renal tubules (Okazaki et al., 2011b). Nishimori et al. (2005) reported that serum antibodies to CAs I and II might be detected in patients with Sjögren's syndrome and ICP perhaps as a consequence of the cross-reactivity of an antibody against another unknown antigen that mimics CAs I and II. Expression of CA IV is more restricted in tissues and cell types in the inflamed lesions that have been observed in patients with AIP and related diseases. It has been suggested that CA IV is more likely to function as the target antigen (Nishimori et al., 2005). CAs on the luminal surface of the ductal cells would eliminate excess acid from the ductal lumen, inhibiting spontaneous autoactivation of trypsin and other proteases (Nishimori et al., 1999), and therefore may contribute to attrition of bacterial killing by these enzymes in the pancreas.

(v) *PSTI*: PSTI, a 56-amino-acid peptide, is synthesized in pancreatic acinar cells, distributed in the ductal cells of several exocrine organs, including the pancreas, salivary gland, biliary duct, lungs, and renal tubules, and colocalizes with trypsinogen in zymogen granules. In addition to its protective role in acinar cells, PSTI inhibits activation of trypsinogen in the pancreatic duct (Asada et al., 2006). It might be beneficial for preventing degradation of bacterial pathogen(s). Additionally, it would be of interest to study whether or not the anti-PSTI antibody inhibits the function of PSTI.

Besides putative AIP-specific autoantibodies, other factors such as channel(s), transporters, and some of AIP associated genes also give us hints to consider the possible involvement of bacteria in the pathogenesis of AIP.

(vi) *AQP1*: Aquaporin (AQP) water channels are intrinsic membrane proteins expressed most of the cell types which have high osmotic water permeability. Among them AQP1 (Aquaporin 1) is a predominant water channel expressed in the plasma membranes of human pancreatic ducts (Ko et al., 2009). Altered tissue water homeostasis may contribute to edema formation during various stress including bacterial infection (Schweitzer et al., 2007). AQP1 indeed contributes to maintain water homeostasis even in cells with plasma membrane damage following insult with bacterial infection (Schweitzer et al., 2007). Interestingly, AQP1 expression was significantly increased in plasma membranes of pancreatic ducts in AIP (Ko et al., 2009). Upregulation of AQP1 expression seen in pancreatic ducts of patients with AIP is speculated to be caused by the reduced fluid secretion from the pancreas as compensation (Ko et al., 2009).

Membranes serve as a barrier to prevent pathogenic bacteria from entering the nutrient-rich host cytosol (Radtke and O'Riordan, 2008). Aquaporins may be an integral part of infection and pathology caused by some microbial pathogens, so regulation of aquaporin expression and function is a key aspect of host–pathogen interaction (Radtke and O'Riordan, 2008). In addition, AQP1 regulates swelling of secretory vesicles, associated with pathogen-containing vacuoles (PCV). AQP1-dependent modulation of PCV was triggered by bacterial induced membrane damage and ion flux (Radtke and O'Riordan, 2008). As described above, in the pancreas of AIP patients, expression of AQP1 was increased markedly, not only to on the plasma membrane but also in the cytoplasm of pancreatic duct cells. To clarify the association between the upregulation of AQP1 in the cytoplasm and PCV would be of interest.

(vii) CFTR: It was reported that aberrant cystic fibrosis transmembrane conductance regulator (CTFR) localization in the pancreatic ducts was observed in AIP patients. The CTFR regulates overall pancreatic ductal fluid/overall ion transport across most epithelia and plays a central role in pancreatic ductal HCO_3^- secretion (Ko et al., 2010). Altered acidity of the pancreatic fluid may affect growth of the normal bacterial inhabitant and immunity of the upper intestinal environment.

(viii) *ABCF1*: The *ABCF1* (ATP-binding cassette, sub-family F) gene proximal to *C3-2-11* microsatellite in HLA class I regions, is thought to be one of the HLA-linked

susceptibility regions for AIP (Ota et al., 2007). At this point, biological function of the putative ABCF1 protein is mostly speculative, however it is thought to be regulated by TNF-α, a prime cytokine in inflammatory reactions (Ota et al., 2007). From this point, it could be possible to suspect that bacteria would lie in the pathogenesis of AIP.

ANIMAL MODELS OF AIP

Several experimental models of AIP have been described (**Table 1**). As for specific induction models, CA-II, LF, and amylase-specific T cells could be listed (Nishimori et al., 1995; Uchida et al., 2002; Davidson et al., 2005). As for the microorganisms related induction group, virus-induced AIP models, such as C57BL/6 mice infected with the murine leukemia retrovirus LP-BM5, developed histological findings similar to human AIP (Suzuki et al., 1993; Watanabe et al., 2003). The spontaneous development of pancreatitis via an autoimmune mechanism in MRL/Mp mice is accelerated by the administration of polyinosinic:polycytidylic acid (poly I:C), a synthetic double-stranded RNA and TLR 3 ligand (Qu et al., 2002; Soga et al., 2009; Asada et al., 2010; Nishio et al., 2011). Sensitization occurs with not only viral components, such as double-stranded RNA poly I:C, but also bacterial LPS in interleukin (IL)-10-deficient mice (Nishio et al., 2011). TLRs play important roles in innate immunity by initiating intracellular signaling to macrophages and dendritic cells after stimulation with various antigens. The majority of known TLRs mediate the development of Th1 cell-inducing dendritic cells (Li et al., 2009). Thus, pattern-recognition receptors (PRRs) that bind

Table 1 | Experimental animal models of autoimmune pancreatitis.

Animal	Organs with lesions	Induction	Effector cells	Reference
MICROORGANISMS RELATED INDUCTION				
SMA mice	Pancreas	*K. pneumoniae*, pancreatic extract	?	Yamaki et al. (1980)
MRL/Mp, MRL/lpr mice	Pancreas	poly (I:C)	$CD4^+$ T cells	Qu et al. (2002)
MRL/Mp mice	Pancreas	poly (I:C)	?	Soga et al. (2009) Asada et al. (2010) Nishio et al. (2011)
IL-10KO mice	Pancreas	poly (I:C), LPS	?	Nishio et al. (2011)
C57BL/6 mice	Pancreas, salivary gland, bile duct, kidney, lung	LP-BM5	$CD4^+$ T cells	Suzuki et al. (1993) Watanabe et al. (2003)
C57BL/6 mice	Pancreas, salivary gland	*E. coli*	T cells	Haruta et al. (2010a)
SPECIFIC INDUCTION (EXCEPT MICROORGANISMS)				
DA(RP) rats	?	Amylase-specific T cell	$CD4^+$ and $CD8^+$ T cells	Davidson et al. (2005)
Lewis rats	Pancreas			
PL/J mice (H-2u), (H-2s)	Pancreas, salivary gland	CA-II	?	Nishimori et al. (1995)
nTx-BALBc mice and nude mice	Pancreas, salivary gland, bile duct	CA-II, LF	$CD4^+$ Th1 cells	Uchida et al. (2002)
SPONTANEOUS INDUCTION				
MRL/Mp mice	Pancreas, salivary gland	Spontaneous	T cells	Kanno et al. (1992) Hosaka et al. (1996)
aly/aly mice	Pancreas	Spontaneous	$CD4^+$ T cells	Tsubata et al. (1996) Nakamura et al. (2007) Wang et al. (2010)
WBN/Kob rats	Pancreas, salivary gland, thyroid, bile duct, kidney	Spontaneous	$CD8^+$ T cells	Sakaguchi et al. (2008)
MHC-II $^{-/-}$ mice	Pancreas	Spontaneous	$CD8^+$ T cells	Vallance et al. (1998)
T-cell+ HLA-DR*0405Ab0 NOD mice	Pancreas	Spontaneous	?	Freitag et al. (2010)
$Tgfbr2^{(fspKO)}$ mice	Pancreas	Spontaneous	$CD4^+$ T cells	Boomershine et al. (2009)
NOD.CD28KO mice	Pancreas	Spontaneous	$CD4^+$ T cells	Meagher et al. (2008)

CA-II, carbonic anhydrase II, LF, lactoferrin; PSTI, pancreatic secretary trypsin inhibitor; poly I:C, polyinosinic:polycytidylic acid; nTx, neonatal thymectomy. Modified from references Frulloni et al. (2009), Takizawa et al. (2009), and Yanagisawa et al. (2011).

pathogen-associated molecular patterns (PAMPs) may trigger an autoimmune response.

As for specific induction, thymectomized mice immunized with CA-II or LF develop a pathology that closely resembles AIP under a regulatory T (T_{reg}) cell-depleted background (Uchida et al., 2002). However, T cells specific for CA-II and LF were unable to induce pancreatitis in the adoptive transfer of an amylase-specific rat model (Davidson et al., 2005), suggesting that autoantibodies against these enzymes in AIP represent a late consequence of tissue destruction.

As for the spontaneous induction group, recently, the spontaneous development of AIP in $CD4^+$ T cell-competent HLA-DR*0405 transgenic Ab0 NOD mice (Freitag et al., 2010), T_{reg}-deficient backgrounds in neonatally thymectomized mice (Uchida et al., 2002), NOD.CD28 knockout mice (Meagher et al., 2008), WBN/Kob rats (Sakaguchi et al., 2008), and $Tgfbr2^{fsp}$ knockout mice (Boomershine et al., 2009) have demonstrated genetic polymorphisms of the effector cells in the etiologies of AIP. However, because these mice are genetically engineered they may not completely reflect the onset of human diseases.

COMMENSAL BACTERIA-INDUCED MOUSE MODEL OF AIP

Instances of where microbial antigens might underlie the pathogenesis of IgG4-related disease have been reported (Akitake et al., 2010; Watanabe et al., 2012). We previously reported that when C57BL/6 mice were inoculated intraperitoneally (i.p.) with heat-killed *E. coli* weekly for 8 weeks, marked cellular infiltration with fibrosis was observed in the exocrine pancreas accompanied by a high serum gamma globulin level and the production of autoantibodies against CA-II and LF. Bacterial infection apparently triggered AIP-like pathological alterations in mice that strikingly resembled AIP in humans (Haruta et al., 2010a).

C57BL/6 mice inoculated weekly with *E. coli* for 8 weeks were utilized as donors, and the spleens were intravenously transferred to $RAG2^{-/-}$ mice. The pancreas in the recipient $RAG2^{-/-}$ mice showed cellular infiltration in the exocrine pancreas, especially around the pancreatic ducts, indicating that the *E. coli*-inoculated mouse spleen cells possess the ability to reproduce pathological alterations in the pancreas of naïve mice. Similarly, when the spleen cells of donor *S. intermedius*-inoculated mice were transferred to $RAG2^{-/-}$ mice, primary biliary cirrhosis (PBC)-like cholangitis

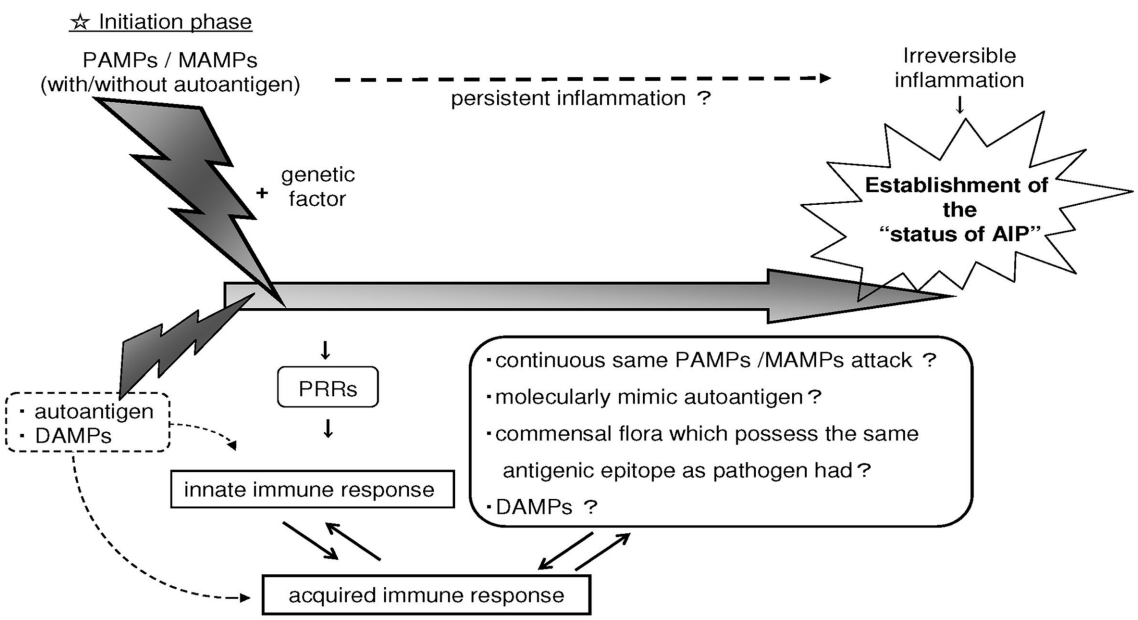

FIGURE 1 | Hypothetical pathogenesis of AIP. Under normal condition, commensal bacteria are not pathogenic and may exist in the host from early life. However, additional factor(s) such as genetic factors would be synchronized with the existence of microorganisms, and the progression switch to generate the status of AIP would be turned on. During the initiation phase, PAMPs and/or non-pathogenic MAMPs trigger and upregulate the innate immune system. These respective PAMPs/MAMPs and DAMPs induce inflammatory response PRRs. Second, the progressive phase features the persistence of this PAMPs/MAMPs attack or stimulation by a molecular-mimicking antigen, perhaps released as DAMPs and/or exposure to or stimulation from commensal flora possessing the same antigenic epitope that the initial pathogen possessed, thereby upregulating the host immune response to the target antigen. These slowly progressive steps eventually lead to the development of autoimmune diseases. Modified from previous reports (Haruta et al., 2010a; Yanagisawa et al., 2011).

in the liver was induced, similar to that seen in the donor (Haruta et al., 2010b). The AIP-like inflammatory region in the pancreases of recipient mice with spleen cells transferred from *E. coli*-inoculated mice (Haruta et al., 2010a), showed that most of the cellular infiltrates in the target organs were CD3-positive, indicating that these cells in both models originated from the donor mice.

The criteria for determining whether a condition may be considered to be autoimmune, according to Witebsky's postulates with modern revision by Rose and Bona (1993), include (i) indirect evidence based on the reproduction of the autoimmune disease in experimental animals, (ii) direct evidence of the transfer of pathogenic antibodies, or (iii) pathogenic T cells and indirect evidence of the isolation of autoantibodies or autoreactive T cells. Several lines of evidence have suggested that repeated *E. coli*-inoculated AIP-like inflammation in the C57BL/6 mice pancreas is possibly of autoimmune origin.

It is worth mentioning that long before the concept of AIP was proposed as a definite disease entity, Yamaki et al. (1980) had established inflammation of the exocrine pancreas using syngeneic pancreatic antigens. Repeated injection of pancreatic extract from syngeneic mice mixed with capsular polysaccharide (CPS) of the *Klebsiella pneumoniae* type 1 Kasuya strain (CPS-K), as an adjuvant, caused pathological alteration in the pancreas, consistent with infiltration by lymphocytes, plasma cells and mononuclear cells, degradation and lysis of the acinar cells, and destruction of the lobular architecture, replacement of fatty tissue and fibrous connective tissue and anti-pancreas antibody production. No histological changes were observed in tissues other than the pancreas and either CPS-K or the pancreatic extract alone were insufficient to induce inflammation in the pancreas. *K. pneumoniae* is one of the commonly found bacteria in Enterobacteriaceae (Yamaki et al., 1980), the natural habitat of the intestinal tract, and it is an opportunistic bacterium. The CPS is a voluminous outer layer and is involved in protection against complement deposition that occurs mainly in the inhibition of macrophage phagocytosis (Evrard et al., 2010). It is of great interest that inflammation had been induced only when the syngeneic pancreatic antigen and bacterial component both existed, which suggest the possibility that the bacterial component may exhibit an adjuvant effect to the antigen.

CONCLUSION

It is currently believed that the microbiota is essential for the development of a functional immune system and the gut microbiota has a far-reaching influence on the immune system, beyond the gastrointestinal tract, i.e., within and outside the gut. In other words, gut microbiota has a greater influence on the systemic immune system than previously anticipated (Kranich et al., 2011). From the aspect of gut microbiota and autoimmunity, several animal models have been reported. For instance, it was reported that the relationship between certain harmful species of gut bacteria affect T cell populations in the periphery and thereby control the development of autoimmune arthritis. Segmented filamentous bacteria could promote experimental autoimmune encephalomyelitis (Kranich et al., 2011).

We propose a hypothetical pathogenesis of bacteria-induced AIP. Under normal conditions, commensal bacteria are not pathogenic and may exist in the host from early life. However, additional factor(s) such as genetic factors including the haplotype of class II antigen of the major histocompatibility complex (MHC) and so on would be synchronized with the existence of microorganisms, and the progression switch to generate the status of AIP would be turned on. During the initiation phase, infection with microorganisms possessing PAMPs and/or non-pathogenic microorganisms associated molecular patterns (MAMPs) trigger and upregulate the innate immune system. In this step, we are especially focusing on the commensal bacteria. Inflammatory T cells are induced by molecules derived from pathogens or commensal microorganisms, as well as by endogenous stress-induced self molecules. These respective MAMPs and damage-associated molecular patterns (DAMPs) induce inflammatory T cells either indirectly, through the induction of pro-inflammatory cytokine production by binding to pattern recognition receptors (PRRs) on innate immune cells, or directly, by binding to pathogen-derived peptide specific receptors on T cells (Mills, 2011).

Second, the progressive phase features the persistence of this PAMPs/MAMPs attack, stimulation by molecular-mimicking antigen, perhaps released as DAMPs, exposure to or stimulation from commensal flora possessing the same antigenic epitope, and/or non-specific activation of innate immunity. These slowly progressive steps lead to a breakdown in immunological tolerance and the development of self antigen-specific T cell and antibody responses (Mills, 2011) and eventually establish the autoimmune diseases. Throughout these processes, TLRs might participate as the key member of PRRs involved in driving autoimmune inflammation (Mills, 2011; **Figure 1**). Taking these considerations together, the pancreas is the field of the specific inflammation which would be triggered by the interaction with microorganisms, and a provider of the autoantigen to induce the production of autoantibodies. These sequential processes would be orchestrated to constitute the status of AIP.

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Recent advances in autoimmune pancreatitis

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It is now clear that are two histological types (Type-1 and Type-2) of autoimmune pancreatitis (AIP). The histological pattern of Type-1 AIP, or traditional AIP, is called lymphoplasmacytic sclerosing pancreatitis (LPSP). The histological pattern of Type-2 AIP is characterized by neutrophilic infiltration in the epithelium of the pancreatic duct. In general, Type-2 AIP patients are younger, may not have a male preponderance, and rarely show elevation of serum IgG4 compared with Type-1 AIP patients. Unlike Type-1 AIP patients, Type-2 AIP patients rarely have associated sclerosing diseases, but they are more likely to have acute pancreatitis and ulcerative colitis. Although Type-2 AIP is sometimes observed in the USA and Europe, most AIP cases in Japan and Korea are Type-1. The international consensus diagnostic criteria for AIP comprise 5 cardinal features, and combinations of one or more of these features provide the basis for diagnoses of both Type-1 and Type-2 AIP. Due to the fact that steroid therapy is clinically, morphologically, and serologically effective in AIP patients, it is the standard therapy for AIP. The indications for steroid therapy in AIP include symptoms such as obstructive jaundice and the presence of symptomatic extrapancreatic lesions. Oral prednisolone (0.6 mg/kg/day) is administered for 2–4 weeks and gradually tapered to a maintenance dose of 2.5–5 mg/day over a period of 2–3 months. Maintenance therapy by low-dose prednisolone is usually performed for 1–3 years to prevent relapse of AIP.

Keywords: autoimmune pancreatitis, IgG4, international consensus diagnostic criteria, steroid

INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare disease that has recently emerged as a peculiar type of pancreatitis with a presumed autoimmune etiology. In 1991, Kawaguchi et al. reported two cases of an unusual inflammatory disease involving the pancreas and biliary tract that were resected on suspicion of pancreatic cancer and, based on the peculiar findings of dense infiltration of lymphocytes and plasma cells with marked fibrosis, described the condition as lymphoplasmacytic sclerosing pancreatitis (LPSP) (Kawaguchi et al., 1991). In 1995, Yoshida et al. first proposed the concept of AIP, and they summarized the clinical features as follows: increased serum γ-globulin or IgG levels and the presence of autoantibodies; diffuse irregular narrowing of the main pancreatic duct and enlargement of the pancreas; occasional association with stenosis of the lower bile duct and other autoimmune diseases; mild symptoms, usually without acute attacks of pancreatitis; effectiveness of steroid therapy; and histological finding of LPSP (Yoshida et al., 1995). In 2001, serum IgG4 levels were found to be frequently elevated in AIP patients (Hamano et al., 2001). Since, AIP is frequently associated with various extrapancreatic sclerosing lesions with the same peculiar histological findings as seen in the pancreas, AIP is currently considered to represent a pancreatic lesion of an IgG4-related systemic disease (Kamisawa et al., 2003a,b, 2010c).

Using retrospective, histological examination of pancreases resected on suspicion of pancreatic cancer from patients with mass-forming chronic pancreatitis, American and European pathologists have described another unique histological pattern, called idiopathic duct-centric pancreatitis (IDCP) (Notohara et al., 2003), or AIP with granulocytic epithelial lesion (GEL) (Zamboni et al., 2004). Recently, LPSP has been called Type-1 AIP, and IDCP has been called Type-2 AIP (Sah et al., 2010). This review focuses on the differences between Type-1 and Type-2 AIP, the recently proposed international consensus diagnostic criteria for AIP (Shimosegawa et al., 2011), and the Japanese standard therapeutic regimen for AIP.

TWO SUBTYPES OF AUTOIMMUNE PANCREATITIS: TYPE-1 AND TYPE-2

The pathological features of Type-1 and Type-2 AIP differ clearly. LPSP of Type-1 AIP consists of dense infiltration of lymphocytes and IgG4-positive plasma cells and fibrosis that involves pancreatic lobules, ducts and peripancreatic adipose tissue (Kawaguchi et al., 1991; Kamisawa et al., 2003a,b, 2010c). Storiform fibrosis, a swirling pattern of fibrosis mixed with inflammatory cells, and obliterative phlebitis are characteristic and diagnostic (**Figure 1**). Eosinophils are often present, but neutrophils are absent. Neutrophilic infiltration within the lumen and epithelium of interlobular ducts is a characteristic feature of IDCP of Type-2 AIP (Notohara et al., 2003; Zamboni et al., 2004). Infiltration of IgG4-positive plasma cells is rare (Kamisawa et al., 2010a; Shimosegawa et al., 2011).

Since no apparent serological markers have yet been found for Type-2 AIP, histological examination is necessary to diagnose

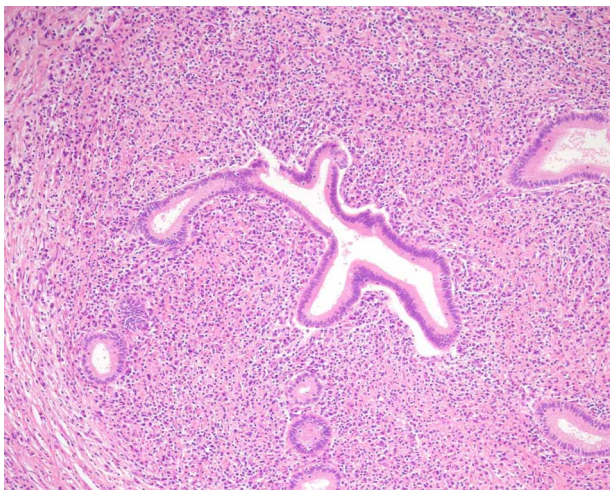

FIGURE 1 | Histological feature of the pancreas of Type-1 AIP.

Type-2 AIP, and thus its clinical diagnosis is difficult. Although the clinical features of Type-2 AIP are not as well-known as those of Type-1 AIP, Type-2 AIP patients show quite different clinical profiles from Type-1 AIP patients. Age at diagnosis of Type-2 AIP is more than a decade younger, and Type-2 AIP does not show a male preponderance. Type-2 AIP patients are less likely to show elevated serum IgG4 levels and IgG4-related extrapancreatic sclerosing lesions such as proximal sclerosing cholangitis, sclerosing sialadenitis, and retroperitoneal fibrosis. Type-2 AIP is more likely associated with acute pancreatitis and inflammatory bowel disease. Type-2 AIP tends to have focal features and is more commonly surgically resected due to the diagnostic difficulty. Although endocrine and exocrine functions of the pancreas are frequently impaired in Type-1 AIP, the functions in Type-2 AIP are unknown. Both Type-1 and Type-2 AIP respond well to steroids, but relapse of Type-2 AIP is rarely seen, whereas Type-1 sometimes relapses (Notohara et al., 2003; Zamboni et al., 2004; Kamisawa et al., 2010a; Sah et al., 2010; Shimosegawa et al., 2011). The prevalence of Type-1 and Type-2 AIP differ around the world. In Japan and Korea, most AIP cases are Type-1, and Type-2 AIP is quite rare (Kamisawa et al., 2011). However, Type-2 AIP may comprise about 20–40% of AIP cases in the United States of America and Europe (Notohara et al., 2003; Zamboni et al., 2004; Park et al., 2009). Whether these differences are induced by regional or racial elements or low recognition of the disease remains unclear.

INTERNATIONAL CONSENSUS DIAGNOSTIC CRITERIA

The international consensus diagnostic criteria for AIP (Shimosegawa et al., 2011) were developed to be applicable worldwide, diagnose AIP safely, avoid misdiagnosis of pancreatic cancer as AIP, and diagnose AIP in acute presentation. Criteria for Type-1 and Type-2 AIP were developed separately. The diagnosis of AIP is made by a combination of one or more of five cardinal features: (1) imaging features of the pancreatic parenchyma and pancreatic duct; (2) serology; (3) other organ involvement (OOI); (4) histology of the pancreas; and (5) an optional criterion of response to steroid therapy. Each feature has been categorized as level 1 and 2 findings depending on the diagnostic reliability. The diagnoses of Type-1 and Type-2 AIP can be definitive or probable, and in some cases, the distinction between the subtypes may not be possible (AIP-not otherwise specified). Diagnosis of sero-negative Type-1 AIP is sometimes difficult.

Cross-sectional pancreatic imaging on CT or MRI is considered as the first essential clue, and the findings have been divided into typical diffuse enlargement and indeterminate images of segmental or focal enlargement of the pancreas. For the criterion related to the pancreatic duct on endoscopic retrograde pancreatography (ERP), level 1 is long or multiple narrowings without marked upstream dilatation, and level 2 is segmental or focal narrowing without marked upstream dilatation. For the serological criterion for Type-1 AIP, level 1 is marked elevation of serum IgG4 levels (more than double upper limit of normal value), and level 2 is mild elevation of serum IgG4 levels. For the criterion of OOI for Type-1 AIP, level 1 is either histological findings in extrapancreatic organs (any three of the four features) or typical radiological evidence (proximal bile duct stricture or retroperitoneal fibrosis), and level 2 is histological findings or physical or radiological evidence (symmetrically enlarged salivary/lacrimal glands or renal involvement). For the histological criterion for Type-1 AIP, level 1 is LPSP with more than three features on core biopsy or resected specimens, and level 2 is any two features on core biopsy specimens. A diagnostic steroid trial is an optional criterion. Response to steroid therapy is defined as rapid (within 2 weeks), radiologically demonstrable resolution or marked improvement in pancreatic or extrapancreatic manifestations. However, a steroid trial should be used only after a negative work-up for cancer, including endoscopic ultrasound-guided fine needle aspiration (**Table 1**). For the criteria for Type-2 AIP, there is no serological criterion; the criterion of OOI is only level 2 (clinically diagnosed inflammatory bowel disease); and the histological criterion is granulocytic infiltration and absent or scant IgG4-positive cells.

Definitive Type-1 can be diagnosed only by histological examination of resected pancreas or core biopsy specimens showing LPSP. To diagnose definitive Type-1, in cases with diffuse imaging findings, any additional non-ductal cardinal criterion is necessary. In cases with segmental or focal imaging, two or more from any level 1 and ductal level 2 cardinal criteria are necessary. To make the diagnosis in association with response to steroid therapy, one non-ductal level 1 or ductal level 1 with any non-ductal level 2 cardinal criterion is necessary. Response to steroid with one non-ductal level 2 cardinal criterion is diagnosed as probable Type-1 AIP (**Table 2**).

To diagnose definite Type-2 AIP, histologically confirmed IDCP or clinical inflammatory bowel disease with level 2 histology and response to steroid is necessary.

JAPANESE STANDARD STEROID TREATMENT FOR AIP

Steroid therapy is clinically, morphologically, and serologically effective in AIP patients, and as a result, it has become the standard current therapy for AIP. From the international survey of AIP (Kamisawa et al., 2011), steroid therapy is standard for AIP

Table 1 | Level 1 and level 2 criteria for Type-1 AIP.

Criterion	Level 1	Level 2
Parenchymal imaging	Typical: diffuse enlargement with delayed enhancement (sometimes associated with rim-like enhancement)	Indeterminate (including atypical): segmental/focal enlargement with delayed enhancement
Ductal imaging (ERP)	Long (>1/3 length of the main pancreatic duct) or multiple strictures without marked upstream dilatation	Segmental/focal narrowing without marked upstream dilatation (duct size, <5 mm)
Serology	IgG4, >2x_ upper limit of normal value	IgG4, 1–2x_upper limit of normal value
Other organ involvement (OOI)	a or b a. Histology of extrapancreatic organs Any three of the following: (1) Marked lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration (2) Storiform fibrosis granulocytic infiltration (3) Obliterative phlebitis (4) Abundant (>10 cells/HPF) IgG4-positive cells b. Typical radiological evidence At least one of the following: (1) Segmental/multiple proximal (hilar/intrahepatic) or proximal and distal bile duct stricture (2) Retroperitoneal fibrosis	a or b a. Histology of extrapancreatic organs including endoscopic biopsy of bile duct Both of the following: (1) Marked lymphoplasmacytic infiltration with fibrosis without granulocytic infiltration (2) Abundant (>10 cells/HPF) IgG4-positive cells b. Physical or radiological evidence At least one of the following: (1) Symmetrically enlarged salivary/lacrimal glands (2) Radiological evidence of renal involvement described in association with AIP
Histology of the pancreas	LPSP (core biopsy/resection) At least 3 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (>10 cells/HPF) IgG4-positive cells	LPSP (core biopsy) Any 2 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosism (4) Abundant (>10 cells/HPF) IgG4-positive cells

Diagnostic steroid trial. Response to steroid (Rt) Rapid (e2 wk) radiologically demonstrable resolution or marked improvement in pancreatic/extrapancreatic manifestations.

Table 2 | Diagnosis of definitive and probable Type-1 AIP using international consensus diagnostic criteria.

Diagnosis	Primary basis for diagnosis	Imaging evidence	Collateral evidence
Definitive Type-1 AIP	Histology	Typical/indeterminate	Histologically confirmed LPSP (level 1 H)
	Imaging	Typical	Any non-D level 1/level 2
		Indeterminate	Two or more from level 1 (+level 2 D*)
	Response to steroid	Indeterminate	Level 1 S/OOI + Rt or level 1 D + level 2 S/OOI/H + Rt
Probable Type-1 AIP		Indeterminate	Level 2 S/OOI/H + Rt

** Level 2 D is counted as level 1 in this setting.*

in all countries. According to the Japanese consensus guideline for the management of AIP (Kamisawa et al., 2010b), the indications for steroid therapy in AIP include symptoms such as obstructive jaundice and the presence of symptomatic extrapancreatic lesions. Before steroid therapy, obstructive jaundice should be controlled by biliary drainage and blood glucose levels should be regulated, usually by administration of insulin in diabetes mellitus patients. The initial recommended dose of oral prednisolone for induction of remission is 0.6 mg/kg/day, administered for 2–4 weeks. Biochemical and serological blood tests, such as liver enzyme and IgG4 levels, as well as imaging tests, such as CT, MRCP, and ERCP, are performed periodically after the start of steroid therapy. Pancreatic size usually normalizes within a few weeks, and biliary drainage becomes unnecessary within about 1 month. Rapid response to steroids is reassuring and confirms the diagnosis of AIP. If steroid effectiveness is reduced, the patient should be re-evaluated on suspicion of pancreatic cancer. The dose is gradually tapered to a maintenance dose of 2.5–5 mg/day over a period of 2–3 months.

Remission is defined as, the disappearance of clinical symptoms and resolution of the pancreatic and/or extrapancreatic manifestations on imaging studies. In the Japanese multicenter survey of steroid therapy for AIP (Kamisawa et al., 2009), the remission rate of AIP patients treated with steroid

was 98% (451/459). At remission, the enlarged pancreas returned to near-normal size in 239 (80%) of 300 patients and became atrophic in 58 patients (20%). Elevated serum IgG4 levels decreased in all patients after the start of steroid therapy, but they failed to normalize in 115 (63%) of 182 patients. HbA1c and impaired pancreatic exocrine function improved and normalized in half of them.

Relapse of AIP is defined as reappearance of symptoms accompanied by the reappearance of pancreatic and/or extrapancreatic, including bile duct, salivary gland, and retroperitoneal abnormalities on imaging and/or elevation of serum IgG4 levels. In a multicenter survey (Kamisawa et al., 2009), the relapse rate of AIP patients treated with steroid was 24% (110/451). Relapse occurred in the pancreas ($n = 57$, 52%), bile duct ($n = 37$, 34%), and extrapancreatic lesions ($n = 19$). Maintenance steroid therapy was given after remission in 377 (82%) of 459 patients treated with steroid. The maintenance oral prednisolone dose was 10 mg/day ($n = 27$, 7%), 7.5 mg/day ($n = 13$, 3%), 5 mg/day ($n = 238$, 63%), 2.5 mg/day ($n = 78$, 21%), and others. The relapse rate with maintenance therapy was 23% (63/273), which was significantly lower than that of patients who stopped maintenance therapy (34%, 35/104; $p < 0.05$). In the USA and UK, where no maintenance therapy was given, relapse rates of patients treated with steroid were reportedly 38% (Sandanayake et al., 2009) and 53% (Ghazale et al., 2008). Whether maintenance therapy benefits AIP patients remains unconfirmed, but given these findings, maintenance therapy with low dose prednisolone (2.5–5 mg/day) was recommended to prevent relapse (Kamisawa et al., 2009, 2010b). In a multicenter study (Kamisawa et al., 2009), of the 377 patients who underwent maintenance therapy, maintenance therapy was stopped in 104 (28%) in whom complete radiological and serological improvement was achieved. As to the period from the start of steroid therapy to relapse, 32% (32/99) relapsed within 6 months, 56% (55/99) relapsed within 1 year, 76% (75/99) relapsed within 2 years, and 92% (91/99) relapsed within 3 years after starting medication. Maintenance therapy should be stopped within 1–3 years in cases with radiological and serological improvement to prevent steroid-related complications such as osteoporosis, diabetes, and infection (Kamisawa et al., 2009, 2010b) (**Figure 2**).

For relapsed AIP, re-administration or dose-up of steroid was effective. In the USA and UK, immunomodulatory drugs such as azathioprine were used in addition to re-administered steroid for relapsed patients, and remission was again achieved and maintained on long-term azathioprine (Ghazale et al., 2008; Sandanayake et al., 2009).

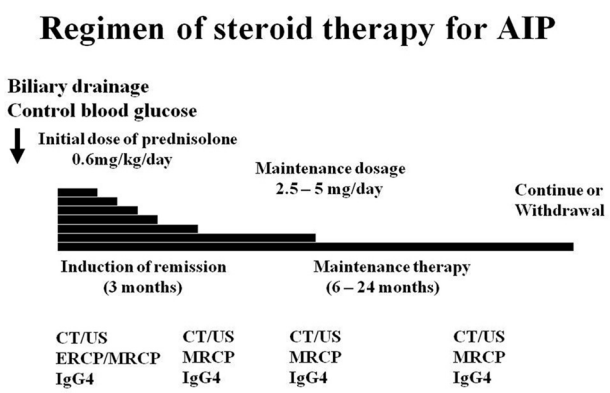

FIGURE 2 | Regimen of Japanese standard steroid treatment for AIP.

It has been reported that the predictors for relapse are the presence of proximal bile duct stenosis and elevated serum IgG4 levels (Ghazale et al., 2008; Kamisawa et al., 2009; Sah et al., 2010; Takuma et al., 2011). Pancreatic stones are formed in relapsing AIP patients, which might be induced by pancreatic juice stasis from intensified incomplete obstruction of the pancreatic duct system (Takuma et al., 2011; Maruyama et al., 2012). Since, AIP might transform into ordinary chronic pancreatitis after several relapses, relapses of AIP should be avoided as much as possible.

It is necessary to verify the validity of the Japanese regimen of steroid therapy for AIP: the necessity, drugs, and duration of maintenance therapy need to be clarified by prospective studies.

CONCLUSIONS

Type-1 and Type-2 AIP are clinicopathologically different entities. International consensus diagnostic criteria can be used to diagnose Type-1 and Type-2 AIP. Steroid therapy is standard therapy for AIP, but the regimen should be evaluated in prospective trials.

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Regulators of epithelial mesenchymal transition in pancreatic cancer

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Pancreatic cancer is a leading cause of cancer-related death due to its invasive nature. Despite the improvement of diagnostic strategy, early diagnosis of pancreatic cancer is still challenging. Surgical resection is the only curative therapy, while vast majority of patients are not eligible for this therapeutic option. Complex biological processes are involved in the establishment of invasion and metastasis of pancreatic cancer and epithelial-mesenchymal transition (EMT) has been reported to play crucial role. EMT is part of the normal developmental processes which mobilizes epithelial cells and yields mesenchymal phenotype. Deregulation of EMT inducing molecules in pancreatic cancer is reported, such as multiple cytokines, growth factors and downstream transcriptional factors. In addition to these molecules, non-coding RNA including miRNA also contributes to EMT. EMT of cancer cell also correlates with cancer stem cell (CSC) properties such as chemoresistance or tumorigenicity, therefore these upstream regulators of EMT could be attractive therapeutic targets and several candidates are examined for clinical application. This review summarizes recent advances in this field, focusing the regulatory molecules of EMT and their downstream targets. Further understanding and research advances will clarify the cryptic mechanism of cancer metastasis and delineate novel therapeutic targets.

Keywords: EMT, pancreatic cancer, BMP, MSX2, miR-126

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a normal cellular function which is indispensable during developmental processes such as gastrulation or neural crest cell migration (Pla et al., 2001; Nakaya and Sheng, 2008). However, pancreatic cancer cells misuse this machinery for their invasion toward surrounding tissue and dissemination into distant organs (Rhim et al., 2012). Since invasion and metastasis are the key step for untreatable disease, numerous approaches have been made to prevent EMT of pancreatic cancer cells. Unfortunately, these efforts have not yet conquered the EMT of pancreatic cancer cells due to the complex, cryptic mechanisms involved in this biological process.

Several cytokines and growth factors are reported to induce EMT in pancreatic cancer cells. These factors are derived from cancer cell itself (autocrine) or stromal cell (paracrine). For example, transforming growth factor β (TGFβ) or its family member bone morphogenetic protein (BMP) is reported to cause cellular morphological changes and altered expression of epithelial markers (Fensterer et al., 2004; Hamada et al., 2007). In addition, treatment of pancreatic cancer cells with vascular endothelial growth factor (VEGF) also promotes EMT in pancreatic cancer cells (Yang et al., 2006). These cytokines and growth factors utilize wide variety of receptors and downstream signaling molecules which synergistically or redundantly contribute to the tumor progression.

As a result of the cumulative gene mutations which amplify oncogenic signal, aberrant activation of several signaling pathways are observed in pancreatic cancer cells. Up to 95% of pancreatic cancer harbors constitutively active mutation of *K-ras* oncogene (Furukawa et al., 2006), which leads to the activation of downstream signals for unlimited cellular proliferation. On the other hand, specific gene deletion results in the defect of tumor suppressive signal such as Smad4 deletion, which is observed in 50% of pancreatic cancer (Maitra and Hruban, 2008). Alteration of these signaling pathways also contributes to the EMT induction in pancreatic cancer cells.

Tumor microenvironment also influences the biological behavior of pancreatic cancer. The characteristic feature of pancreatic cancer tissue is dense stroma surrounding tumor cells which is called desmoplastic reaction (Mahadevan and Von Hoff, 2007). The existence of desmoplastic reaction is reported to contribute to pancreatic cancer progression. Recent research identified a significant role of the tumor stromal cells in pancreatic cancer such as protection from chemotherapeutic agents (Muerkoster et al., 2004) or metastasis-promoting role (Xu et al., 2010). Based on these findings, tumor stromal cells are attracting interest as a novel therapeutic target of pancreatic cancer.

Recent advances in the cancer research field identified an additional regulatory molecule of cellular functions. MicroRNA is a member of non-coding RNA consists of 20–23 nucleotide which targets 3′UTR sequence of mRNA for translational repression and destabilization (Farazi et al., 2011). Several reports indicate that microRNA could orchestrate biological processes by targeting hundreds of target mRNAs, including cancer cell invasion and

metastasis (Sureban et al., 2011). There are several microRNAs which contribute to pancreatic cancer cell migration and invasion whose expression levels correlate with patients' prognosis (Ali et al., 2010a,b; Giovannetti et al., 2010).

These lines of evidences partially uncovered the complex machinery which keep invasive growth of pancreatic cancer cells. Dissecting the detailed mechanism of EMT in cancer cells will elucidate novel therapeutic targets against invasion and metastasis. Following sections describe current knowledge and future perspectives in this field.

EMT-INDUCING CYTOKINES AND GROWTH FACTORS

TGFβ is a characteristic cytokine which possesses distinct effects on cellular morphology and proliferation. TGFβ suppresses cellular proliferation accompanied by the induction of cell cycle regulator *p21/waf1*, whose induction requires intact Smad4 (Grau et al., 1997). On the other hand, TGFβ causes down-regulation of epithelial marker E-cadherin (Bardeesy et al., 2006) whose expression attenuates invasive growth of pancreatic cancer cells (Furuyama et al., 2000). Since TGFβ is enriched in plasma or other extracellular sources (Labelle et al., 2011), this cytokine is considered to be an important inducer of EMT during cancer progression. Similarly, a TGFβ family member BMP4 which phosphorylates different Smad (Smad1, 5 and 8) from TGFβ (Smad2 and 3) also induces EMT in pancreatic cancer cells via the induction of MSX2 (Hamada et al., 2007; Gordon et al., 2009). Interestingly, BMP4 also harbors growth inhibitory properties accompanied by the induction of cell cycle regulator *p21/waf1* (Kleeff et al., 1999; Hamada et al., 2009), suggesting redundant roles of these family members which affect pancreatic cancer progression.

Another growth factor is involved in the EMT induction of pancreatic cancer. VEGF is a potent angiogenic factor which stimulates endothelial cell proliferation and migration (Eilken and Adams, 2010). A characteristic tissue structure of pancreatic cancer, the desmoplastic reaction, hampers efficient blood perfusion which gives rise to increased hypoxia within the tumor. Hypoxic condition stabilizes hypoxia-inducible factor 1α (HIF1α) which promotes the transcription of VEGF mRNA (Dery et al., 2005). VEGF also affects cellular morphology of pancreatic cancer cells featured by the loss of polarity, loose cell to cell contact or decreased expression of the epithelial markers E-cadherin and plakoglobin which is in accordance with EMT induction (Yang et al., 2006).

Combination of multiple cytokines and growth factors depicts synergistic effects in EMT induction. Fibroblast growth factor 2 (FGF-2) contributes to the EMT induction in transformed epithelial cells as a downstream effector of *HOXB7*, a homeodomain protein which is overexpressed in breast cancer (Wu et al., 2006). Combined treatment of epithelial cells using recombinant FGF-2 and TGFβ further enhances the migratory phenotype (Shirakihara et al., 2011). This effect is mediated by the altered expression of FGF receptor subtype by TGFβ which sensitizes cells to FGF stimuli. Contribution of multiple cytokines and growth factors during EMT could overwhelm the effect of single-target therapy and enable redundant promotion of invasive growth.

EMT AND INTRACELLULAR SIGNALING

As mentioned in the previous section, pancreatic cancer cells are under the influence of various cytokines and growth factors. These extracellular stimuli activate intracellular signaling molecules which contribute to the invasive phenotype of cancer cells. Among these signaling molecules, the role of extracellular signal-regulated kinase (ERK) pathway is well characterized during EMT. Treatment of pancreatic cancer cell line Panc-1 by TGFβ leads to the increased phosphorylation of ERK which is indispensable for the EMT induction by TGFβ (Ellenrieder et al., 2001). Since ERK is activated by its upstream regulator Ras/Raf/MEK pathway (Lopez-Chavez et al., 2009), activating mutation of *Kras* could elevate the baseline activity of ERK pathway for further amplification of TGFβ signal.

Besides the Ras/Raf/MEK/ERK pathway, inflammatory signal-related pathway also plays important role for pancreatic carcinogenesis. Previous report suggested that addition of chronic inflammation by caerulein in genetically engineered mice model expressing pancreas-specific *Kras G12D* significantly enhanced the pancreatic cancer incidence (Guerra et al., 2007). Among those inflammatory signal-related molecules, nuclear factor kappa B (NFκB) plays aggravating role by inducing pro-inflammatory cytokines which sustains chronic inflammation within tumor (Ling et al., 2012). NFκB also promotes EMT in pancreatic cancer cells by inducing mesenchymal marker Vimentin and EMT-related transcriptional factor ZEB1 (Maier et al., 2010).

Aberrant activation of other signaling pathway is reported in pancreatic cancer. Notch is a cell surface receptor which regulates cell fate determination and differentiation during embryonic stage whose activation is also seen in pancreatic cancer (Kimura et al., 2007). Acquisition of gemcitabine-resistant phenotype of pancreatic cancer cells is accompanied by the elevated notch activity, whose silencing by siRNA attenuates the EMT phenotype such as vimentin, ZEB1, Slug, and Snail expression (Wang et al., 2009). Another signaling pathway, sonic hedgehog pathway is also involved in pancreatic carcinogenesis whose original role is an endodermal-mesodermal cross-talk during gut development (van den Brink, 2007). Inhibition of hedgehog pathway by several agents such as epigallocatechin-3-gallate or IPI-269609 abrogated EMT phenotype of pancreatic cancer cells (Feldmann et al., 2008; Tang et al., 2012). Targeting these signaling pathways could be an effective therapy for EMT inhibition, but complex cross-talk between multiple pathways could lead to the resistance against single-agent therapy.

EMT AND TUMOR MICROENVIRONMENT

The tumor microenvironment itself could exert EMT-promoting effects. Hypoxia is a characteristic feature in pancreatic cancer tissue, and besides the VEGF production, hypoxia alters intracellular signals by up-regulating HIF1α. Twist is a transcriptional factor involved in the EMT of pancreatic cancer cells (Satoh et al., 2008) and HIF1α induces its expression (Sun et al., 2009). This is a direct effect of hypoxia on cancer cells similar to the adaptation to the hypoxic conditions (Pasteur effect).

When considering the cellular component within pancreatic cancer stroma, the pancreatic stellate cells (PSC) should be

emphasized as a central regulator of fibrosis. (Masamune and Shimosegawa, 2009; Masamune et al., 2009; Erkan et al., 2012) PSCs contribute to the formation of desmoplastic reaction by producing extracellular matrix proteins such as fibronectin or collagen (Bachem et al., 2005). These matrix proteins yield growth stimulatory effects and EMT promotion (Kanno et al., 2008) on pancreatic cancer cells which could be recognized as preconditioning of cancer cells for metastasis.

Another report suggested that PSCs also contribute to establish metastatic site *in vivo* in collaboration with cancer cells (Xu et al., 2010). The detailed mechanism for this phenomenon remains elusive, but recent researches clarified the part of the picture. Indirect co-culture of PSCs promotes EMT phenotype in pancreatic cancer cells independently of TGFβ (Kikuta et al., 2010). This treatment also enhanced the cancer stem cell (CSC) related genes' expression and spheroid formation, a hallmark of CSC function (Hamada et al., 2012a), suggesting novel regulatory mechanism of PSCs in cancer metastasis. Since PSC itself is a non-transformed cell, inhibition of PSC function might be accomplished without acquiring therapy resistance. Targeting cancer supporting cells could be a promising therapeutic option. For the inhibition of PSC function, several signaling pathways are identified such as peroxisome proliferator-activated receptor-gamma, mitogen-activated protein kinases, and reactive oxygen species which could be modulated pharmaceutically (Masamune and Shimosegawa, 2009).

EMT-INDUCING microRNA

Recent advances in cancer research field identified novel regulatory molecule in EMT. MicroRNA is a member of non-coding RNA which targets hundreds of target mRNA, thereby orchestrating cellular functions including EMT. Until now, several microRNAs are reported to be involved in the regulation of pancreatic cancer cell motility and invasion. MiR-21 is highly expressed in pancreatic cancer compared with normal tissue, and introduction of miR-21 precursor results in increased cellular proliferation and invasion accompanied by the induction of matrix metalloproteinase-2 and -9 (Moriyama et al., 2009). Furthermore, miR-21 also contributes to the resistance against gemcitabine and correlates with patient's survival (Giovannetti et al., 2010). Expression levels of microRNAs are measurable in plasma samples, and their clinical application is expected. Serum miR-21 expression level is elevated in patient with pancreatic cancer and correlated with poor survival, which indicates miR-21 could be an efficient biomarker of pancreatic cancer (Ali et al., 2010a,b).

In contrast to the miR-21, EMT-inhibiting microRNA is also identified by comprehensive analysis. By comparing the microRNA expression profiles in invasive ductal adenocarcinoma

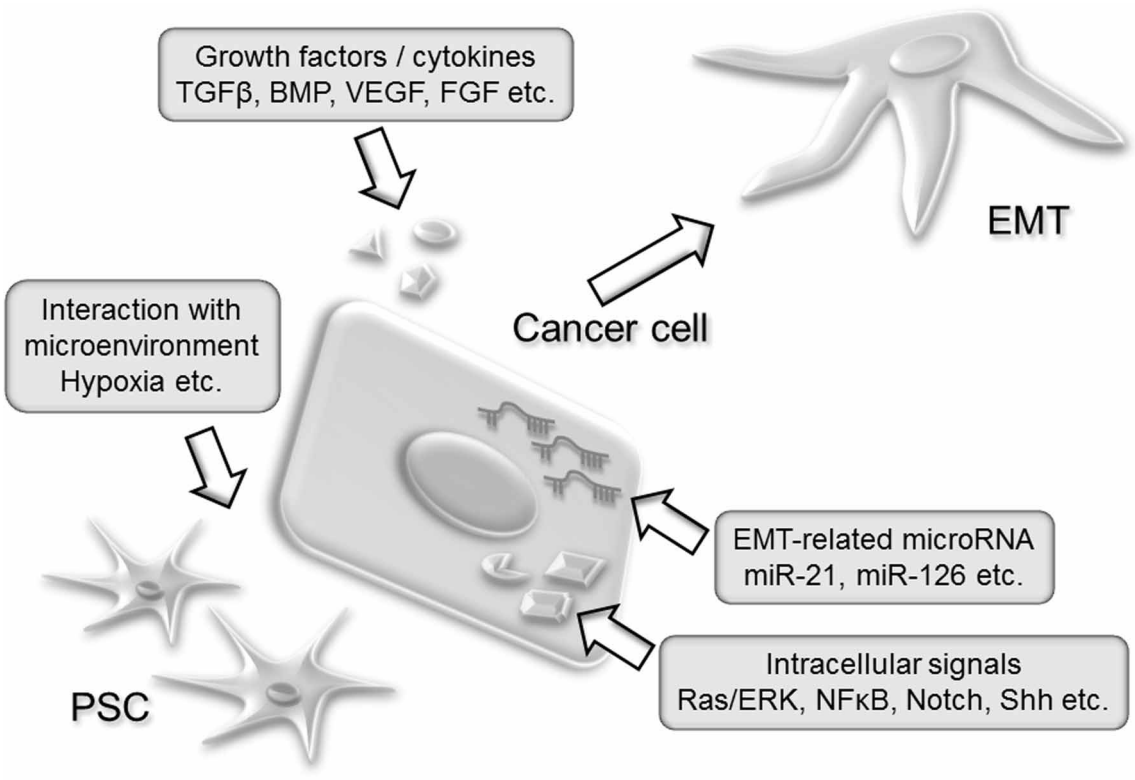

FIGURE 1 | A schematic view of EMT regulators in pancreatic cancer development. Secreted cytokines such as TGFβ or BMP activates intracellular signal which leads to the EMT induction. Activating mutation such as *Kras G12D* constitutively stimulates intracellular signal and amplifies extracellular signal. Endogenous alteration of microRNA expression modifies cancer cell function. Stromal cells including PSCs establish protective microenvironment for cancer cells such as desmoplasia.

with intraductal papillary mucinous neoplasm, the miR-126 was identified as a significantly down-regulated microRNA in invasive ductal adenocarcinoma (Hamada et al., 2012b). Database analysis identified that miR-126 potentially targets disintegrin and metalloproteinase domain-containing protein 9 (ADAM9), which is highly expressed in pancreatic cancer (Grutzmann et al., 2004). Expression of miR-126 and ADAM9 were mutually exclusive, and re-expression of miR-126 attenuated pancreatic cancer cell migration and invasion (Hamada et al., 2012b). These findings suggest the multimodal regulation of EMT during pancreatic cancer progression.

CONCLUSION

This review summarized the current knowledge about the regulatory mechanisms of EMT. The schematic view of these regulators of EMT is shown in **Figure 1**. Inhibition of specific cytokines, growth factors or signaling pathways met their limitations for clinical applications due to the redundant regulation of EMT in pancreatic cancer. Targeting normal cells such as endothelial cells, immune cells, or stromal cells which sustain cancer microenvironment would be novel therapeutic targets against cancer invasion and metastasis. In addition to this concept, a comprehensive regulator of cellular function, microRNA could be a powerful tool in regulating metastasis-promoting microenvironment. Further understanding about the EMT regulation will provide efficient therapy against pancreatic cancer.

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MSX2 in pancreatic tumor development and its clinical application for the diagnosis of pancreatic ductal adenocarcinoma

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MSX2, a member of the homeobox genes family, is demonstrated to be the downstream target for *ras* signaling pathway and is expressed in a variety of carcinoma cells, suggesting its relevance to the development of ductal pancreatic tumors since pancreatic ductal adenocarcinoma (PDAC) and intraductal papillary-mucinous neoplasia (IPMN) harbor frequent K-*ras* gene mutations. Recent studies revealed the roles of MSX2 in the development of carcinoma of various origins including pancreas. Among gastrointestinal tumors, PDAC is one of the most malignant. PDAC progresses rapidly to develop metastatic lesions, frequently by the time of diagnosis, and these tumors are usually resistant to conventional chemotherapy and radiation therapy. The molecular mechanisms regulating the aggressive behavior of PDAC still remain to be clarified. On the other hand, IPMN of the pancreas is distinct from PDAC because of its intraductal growth in the main pancreatic duct or secondary branches with rare invasion and metastasis to distant organs. However, recent evidence indicated that once IPMN showed stromal invasion, it progresses like PDAC. Therefore, it is important to determin how IPMN progresses to malignant phenotype. In this review, we focus on the involvement of MSX2 in the enhancement of malignant behavior in PDAC and IPMN, and further highlight the clinical approach to differentiate PDAC from chronic pancreatitis by evaluating MSX2 expression level.

Keywords: pancreatic ductal adenocarcinoma, intraductal papillary-mucinous neoplasm of the pancreas, cancer development, MSX2, homeobox gene

INTRODUCTION

Homeobox-containing genes regulate the morphological development of a variety of organs and their expression levels vary according to the development stages of organ (Wolgemuth et al., 1989; Morgan et al., 1992). The expression of MSX2, a member of the homeobox gene (Hox gene) family, is observed in a variety of sites, including premigratory cranial neural crest, tooth, and mammary gland, etc (Takahashi and Le Douarin, 1990; Davidson et al., 1991; Monaghan et al., 1991; Jowett et al., 1993; Davidson, 1995; Friedmann and Daniel, 1996; Phippard et al., 1996). The expression pattern of this gene in the development of organs suggests its pivotal role in epithelial–mesenchymal interactions (Satoh et al., 2004). On the other hand, accumulating evidence has revealed the active involvement of this gene in tumorigenesis and/or tumor development. MSX2 has been suggested to be a downstream target of the *Ras* signaling pathway because MSX2 was up-regulated in v-Ki-*ras* transfected NIH3T3 cells and antisense MSX2 cDNA and truncated MSX2 cDNA interfered with the transforming activities of both the v-K-*ras* and v-*raf* oncogene (Takahashi et al., 1996). In addition, the enhanced expression of MSX2 has been shown in a variety of carcinoma cell lines of epithelial origin compared to their corresponding normal tissues (Suzuki et al., 1993). In gastric cancer, MSX2 was identified as a cancer- specific hedgehog target and the down-regulation of this gene resulted in the inhibition of cancer cell growth *in vitro* (Ohta et al., 2009). Similarly, MSX2 has been shown to be a downstream target of WNT signal and has been correlated with the invasiveness of endometrioid adenocarcinoma (Zhai et al., 2011).

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant gastrointestinal tumors. Once PDAC is clinically evident, it progresses rapidly to develop metastatic lesions, frequently by the time of diagnosis. Moreover, this carcinoma usually shows resistance to conventional chemotherapy and radiation therapy. Although recent molecular analyses of precursor lesions revealed an association between gene alterations and carcinogenesis (Hong et al., 2011), the pathogenic mechanisms that

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary-mucinous neoplasia; MD-IPMN, main duct type intraductal papillary-mucinous neoplasia; BD-IPMN, branch duct type intraductal papillary-mucinous neoplasia; HPDE, human normal pancreatic duct epithelial cell line; PSC, pancreatic stellate cells; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; shRNA, small hairpin RNA; BrdU, 5-bromo-2-deoxyuridine; BMP4, Bone morphogenetic protein 4; EMT, epithelial to mesenchymal transition; QRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; CSC, cancer stem cell; SP, side population; CP, chronic pancreatitis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

regulate the aggressive behavior of this cancer still remain to be clarified. On the other hand, intraductal papillary-mucinous neoplasia (IPMN) of the pancreas is a unique neoplasm that is considered to be a precancerous lesion analogous to adenomatous polyps of the colon (Loftus et al., 1996). IPMNs are classified as main duct type (MD-IPMN) or branch duct type (BD-IPMN) based on the location of the main tumor, detected by imaging studies or histology. BD-IPMN is likely to have a less aggressive biological behavior than MD-IPMN (Kobari et al., 1999; Terris et al., 2000; Doi et al., 2002; Hara et al., 2002; Kitagawa et al., 2003; Matsumoto et al., 2003; Sugiyama et al., 2003). However, the activation of oncogenes such as K-*ras* (Satoh et al., 1996), sonic hedgehog (Satoh et al., 2008b), and c-*erb* B-2 (Satoh et al., 1993a), accumulation of *p53* (Satoh et al., 1996), or the expression of a member of inhibitor of apoptosis family, survivin, (Satoh et al., 2001) as well as loss of chromosome 18q (Fukushige et al., 1998) in BD-IPMN, indicate the malignant potential of this neoplasm. Furthermore, stromal infiltration and distal metastasis have been reported even in this type of tumor (Sugiyama et al., 1998; Yasuda et al., 1998).

Until recently there has been little information about the expression or function of MSX2 in pancreatic tumors, although both PDAC and IPMN harbor frequent K-*ras* gene mutations at codon 12 (Satoh et al., 1993b, 1996) and MSX2 was suggested to be a downstream target of the *ras* signal. In this review, we summarize the recently identified roles and functions of MSX2 in the development of pancreatic tumors (PDAC and IPMN). We also demonstrate the validity of measuring the MSX2 expression level in clinical samples for the diagnosis of PDAC.

EXPRESSION OF MSX2 IN PANCREATIC TISSUE

MSX2 was shown to be expressed in the expanding epithelia of the fetal murine pancreas, where PDX-1 was also detected, but not in the duct of the adult murine pancreas, suggesting that MSX2 might play a role in regulating the pancreatic developmental program (Kritzik et al., 1999). In cultured human cell lines, MSX2 expression was found in pancreatic cancer cell lines while it was not observed in human normal pancreatic duct epithelial cell line (HPDE) nor in pancreatic stellate cells (PSC) (**Table 1**) (Satoh et al., 2008a, 2010, 2011). Reverse transcription-polymerase chain reaction (RT-PCR) analysis of microdissected lesions also revealed that MSX2 expression occurred only in tumor lesions including carcinoma cells in PDAC tissues, and borderline to carcinoma cells in IPMN tissues, while no and faint expression of MSX2 transcripts was found in normal duct and adenoma cells of IPMN, respectively. The dominant MSX2 expression in cancer cells was also reported in other carcinomas such as bile duct (Ito et al., 2011), stomach (Ohta et al., 2009), and breast (Di Bari et al., 2009). Consistent with these findings, MSX2 expression is likely to be restricted to neoplastic duct cells in adult human pancreas.

The correlation between the up-regulation of MSX2 and clinicopathological factors was investigated in a number of carcinomas. The expression of this gene was associated with good prognosis in breast carcinoma (Lanigan et al., 2010) and malignant melanoma (Gremel et al., 2011), while this gene expression was increased significantly in tumors with metastasis compared to those without metastasis in prostate carcinoma (Chua et al., 2010). In human PDAC tissues, frequent MSX2 expression in cancer cells was observed by immunohistochemistry and a significant correlation was found between MSX2 expression and histological differentiation and vascular invasion, whereas there was no association between this gene expression and the tumor stage (**Table 2**) (Satoh et al., 2008a), suggesting that MSX2 expression may be associated with the aggressiveness of PDAC because poor differentiation of PDAC is correlated with reduced survival time (Cleary et al., 2004).

Table 1 | Relative expression of MSX2 in cultured pancreatic cells (Satoh et al., 2008a, 2010).

Cell	Relative MSX2 expression
Panc-1	1
AsPC-1	0.87
MIAPaca2	0.3
BxPC3	0.02
Pancreatic stellate cell	0.01
Human pancreatic epithelial cell (HPDE)	<0.001

THE EFFECT OF MSX2 ON GROWTH OF PANCREATIC CARCINOMA AND NORMAL EPITHELIAL DUCT CELLS

It has been suggested that MSX2 may induce the proliferation of osteoprogenitors (Dodig et al., 1999), as well as osteoblasts (Liu et al., 1999), and this was gene also related to the enhancement of branching morphogenesis in mouse mammary ducts (Satoh et al., 2007). In addition, knockdown of MSX2 by small interfering RNA (siRNA) or small hairpin RNA (shRNA) inhibited the cell growth of gastric (Ohta et al., 2009) and ovarian (Zhai et al., 2011) carcinoma, indicating that the function of MSX2 is likely to be relevant to the regulation of the proliferation of epithelial cells as well as

Table 2 | Correlation between clinicopathologic findings and MSX2 expression (Satoh et al., 2008a).

	MSX2<30%	≥30%	*P*-value*
Stage			0.957
I	1	2	
II	1	1	
III	4	4	
IV	8	11	
Histological classification			0.004
Well differentiated	8	2	
Moderately differentiated	6	9	
Poorly differentiated	0	7	
Vascular invasion			<0.0001
v0	0	3	
v1	6	1	
v2	8	1	
v3	0	12	

**Chi-square analysis.*

osteogenic cells. The involvement of MSX2 in normal pancreatic duct cell growth was examined using HPDE cells retrovirally transfected with MSX2 (Satoh et al., 2010). The effect of MSX2 on cell growth was analyzed by cell count every 48 h after seeding the cells. HPDE cells transfected with MSX2 demonstrated approximately 2.5-fold more cells compared to HPDE control cells at 4 days after seeding these cells. Similarly, forced expression of MSX2 in BxPC3 resulted in a significant induction of proliferation after 72 h of culture compared to control cells as determined by 5-bromo-2-deoxyuridine (BrdU) assay (Satoh et al., 2008a). In addition, MSX2 down-regulated Panc-1 cells by shRNA transfection significantly reduced the cellular growth rate. These findings clearly indicate that MSX2 facilitate the cellular growth of both benign and malignant pancreatic duct cells.

MSX2 AND INVASION OR METASTASIS OF PDAC

Bone morphogenetic protein 4 (BMP4) has been shown to induce epithelial to mesenchymal transition (EMT) in PDAC cells and that MSX2 is indispensable for this phenomenon (Hamada et al., 2007). This raised the question of whether MSX2 itself could cause the EMT of PDAC cells. The involvement of this gene in the EMT of PDAC was investigated using MSX2 up- and down-regulated PDAC cells in gain and loss of function manners, respectively. As endogenous MSX2 expression was low in BxPC3 and high in Panc-1 cells (**Table 1**), several clones of BxPC3 stably overexpressing MSX2 and Panc-1 stably expressing MSX2 shRNA were generated (Satoh et al., 2008a). A significant morphological difference was observed between MSX2-transfected and control cells (parental BxPC3 and empty vector transfected cells). MSX2-expressing cells showed loose cell junctions, scattered morphology, and a more fibroblast-like appearance compared to control cells. A similar morphological change was observed between MSX2 expressing and down-regulated Panc-1 cells. MSX2 expressing parental Panc-1 and empty-vector transfected cells showed loose cell junctions and scattered morphology, while the MSX2 down-regulated cell lines demonstrated a cobblestone-like phenotype. By immunofluorescent staining, BxPC3 cells transfected with MSX2 exhibited a weakly diffuse distribution of E-cadherin and β-catenin in the cytoplasm, while control cells showed dominant membrane-bound staining. These molecular changes in MSX2 expressing cells are consistent with EMT. Consistent with the morphological and molecular changes, MSX2-expressing pancreatic cancer cells showed enhanced cell migration by wound healing scratch assay and two-chamber assay, while down-regulation of MSX2 in Panc-1 is associated with the suppression of cell migration. This evidence clearly indicates that MSX2 itself plays a crucial role in the EMT of PDAC cells (Satoh et al., 2008a). The effect of MSX2 on EMT was also investigated in mammary and ovarian cells. MSX2 transfected NMuMG cell, a spontaneously immortalized normal mouse mammary epithelial cell line, showed morphological and molecular changes consistent with EMT (Di Bari et al., 2009). MSX2-expressing NMuMG cells appeared spindle-shaped or fibroblast-like in the monolayer culture and showed reduced expression of the epithelial marker E-cadherin concomitant with the increased expression of mesenchymal markers vimentin and N-cadherin. In addition, forced expression of MSX2 in NMuMG cells resulted in the promotion of invasiveness. On the other hand, Zhai et al. demonstrated that ectopic expression of MSX2 also enhanced the invasiveness of ovarian carcinoma cells *in vivo* (Zhai et al., 2011). Since the expression of MSX2 in selected ovarian carcinoma cells induced changes suggestive of EMT but MSX2 expression was not consistently correlated with EMT markers in primary tumor specimens, they speculated that the involvement of MSX2 in EMT was complex and context-dependent. Therefore, although the involvement of MSX2 in tumor invasion was consistently observed in several kinds of carcinoma, the role of MSX2 in EMT might depend on the tumor species.

MSX2 expression also promoted cell migration or metastasis formation in an orthotopic environment. Control cells, MSX2 expressing cells and shMSX2 cells were injected into the pancreas of nude mice. Tumors were observed in the pancreas of mice implanted with all MSX2-expressing or shRNA-transfected MSX2 cells and control cells. MSX2-expressing cells frequently showed metastases to the liver and peritoneal dissemination, while control cells demonstrated no liver metastasis or only one peritoneal invasion (**Table 3**). By contrast, the metastases to the liver and peritoneal dissemination were suppressed in the mice injected with MSX2-inactivated cells (**Table 3**). In this context, MSX2 is likely to facilitate PDAC metastasis through the induction of EMT.

The mechanisms underlying the induction of EMT by MSX2 in PDAC cells were assessed by cDNA microarray, which identified the differentially expressed genes between control and MSX2 expressing cells (National Center for Biotechnology Information Gene Expression Omnibus database, GSE6585). Among the genes significantly up-regulated by MSX2, Twist1 was one of the most strongly induced genes in MSX2-expressing cells compared to control cells. Twist 1 was initially identified as a crucial regulator of embryonic morphogenesis in *Drosophilia* (Yang et al., 2004). A recent study revealed that Twist 1 is involved in invasion and/or metastasis through the induction of EMT in various types of carcinoma cells (Mironchik et al., 2005). In PDAC, immunohistochemical analysis showed that Twist 1 expression was correlated with MSX2 expression and the colocalization of these proteins was confirmed by double staining of fluorescence immunohistochemistry. In addition, nuclear expression of Twist 1 disappeared when MSX2 was down-regulated in Panc-1 cells (Satoh et al., 2008a). These findings suggest that MSX2 is likely to function in leading the PDAC cells to the state of EMT through the up-regulation of Twist 1.

Table 3 | Summary of orthotopic implantation of MSX2-expressing or down-regulated cells in nude mice (Satoh et al., 2008a).

	Metastasis to liver	Peritoneal dissemination
BxPC3 control[#] ($n = 5$)	0	1
MSX2 expressing BxPC3 ($n = 5$)	3*	5*
Panc-1 control[#] ($n = 5$)	4*	3*
MSX2 down-regulated Panc-1 ($n = 5$)	0	0

[#]*Control cells were transfected with empty vector;* $^*P < 0.05$ *(Chi-square analysis).*

THE ROLE OF MSX2 IN THE DEVELOPMENT OF IPMN

IPMN is distinct from PDAC because it grows slowly and is rarely invasive, resulting in a better prognosis compared to PDAC (Loftus et al., 1996). However, recent evidence has indicated that once IPMN demonstrates stromal invasion, it progresses like PDAC (Sugiyama et al., 1998; Yasuda et al., 1998). Therefore, it is important to know how IPMN attains malignant phenotype. MSX2 is expressed in PDAC and its expression enhanced the aggressiveness of PDAC cells through the induction of EMT, as described above, and this gene is suggested to be a downstream target for *ras* signal (Takahashi et al., 1996). Since IPMN, like PDAC, harbors frequent K-*ras* mutations (Satoh et al., 1993b, 1996), the association of MSX2 expression with the tumor grade or clinicopathological features was examined in IPMN tissues to determine whether this gene could be involved in the process of benign-to-malignant progression in IPMN (Satoh et al., 2010). The expression levels of MSX2 mRNA in microdissected lesions from IPMNs were investigated by one-step quantitative real-time RT-PCR (QRT-PCR). The expression levels of MSX2 mRNA were increased in a stepwise manner from benign to malignant IPMN. Carcinoma lesions of IPMN expressed significantly higher levels of MSX2 mRNA than adenoma and borderline of IPMN cells did, while no significant difference was found between non-tumor lesions and adenoma-borderline IPMN cells. Consistent with the results of QRT-PCR, the immunoreactivity of MSX2 was frequently found in borderline IPMN (3/5, 60%), carcinoma of IPMN (12/19, 63.2%), and invasive carcinoma derived from IPMN (5/5, 100%), while its expression was seen in only one of 16 adenoma of IPMN tissues. When multivariate analysis among seven clinical parameters, including age, sex, branch duct size, nodule size, diameter of main pancreatic duct, serum CEA and CA19-9 levels, in addition to MSX2 expression, was done, MSX2 expression was identified as the only independent factor that predicted malignant BD-IPMN (**Table 4**).

A branch duct size cutoff of 30 mm has been widely accepted as a factor for predicting the malignancy of BD-IPMN (Tanaka et al., 2006). However, its low sensitivity for malignancy has also been reported (Pelaez-Luna et al., 2007). Thus, more specific predictive factors for malignant BD-IPMN were explored by various approaches. Clinical findings such as branch size, presence of nodule or dilatation of the main pancreatic duct have been described as signs of malignant BD-IPMN (Kitagawa et al., 2003; Sugiyama et al., 2003; Kobayashi et al., 2005; Pelaez-Luna et al., 2007). Similarly, molecular events including mutation of K-*ras* (Satoh et al., 1993b, 1996), inactivation of *p53* (Satoh et al., 1996) or *smad4* (Biankin et al., 2002) were demonstrated to be correlated with malignant BD-IPMN. However, it is known whether molecular markers would be better predictive factors for malignant BD-IPMN than clinical parameters such as nodule size or dilatation of a branch duct. Based on the above findings, MSX2 expression was found to be a better predictive factor for carcinoma of IPMN compared to the clinical parameters that were previously reported to be relevant to malignant IPMN.

Table 4 | Predictive factors for malignant IPMN by multivariate analysis (Satoh et al., 2010).

Parameter	Adjusted odds ratio (confidence interval)
Age (≥70)	0.38 (0.06 – 2.35)
Sex	0.51 (0.08 – 3.28)
Branch (≥30 mm)	2.90 (0.46 – 18.26)
Main pancreatic duct (≥6 mm)	2.01 (0.24 – 16.65)
Nodule (≥6 mm)	2.99 (0.39 – 22.65)
CEA (>5)	0.65 (0.08 – 5.22)
CA19-9 (>37)	6.91 (0.12 – 394.4)
MSX2 expression	8.19 (1.4 – 47.9)*

*$P < 0.02$ *(Log rank regression analysis).*

MSX2 AND CHEMORESISTANCE OF PDAC

Carcinoma tissues are known to consist of a heterogeneous cellular population containing a minor population of permanent proliferating cells and a major population of differentiated cells with limited proliferation potential. Among the permanent proliferating cells, so-called cancer stem cells (CSCs) are considered to be responsible for the initiation, metastasis, chemoresistance and recurrence of tumor (Reya et al., 2001). Recently, the induction of a breast CSC phenotype by the forced expression of Snail and Twist, which leads to EMT, has been demonstrated (Mani et al., 2008). Since increased expression of MSX2 induced EMT and enhanced the metastasis of PDAC cells, MSX2 is likely to have pivotal role in maintaining the characteristics of CSCs. Thus, the involvement of MSX2 in chemoresistance, which is one of the characteristics of CSCs in PDAC, was investigated (Hamada et al., 2012). To assess the association between MSX2 and chemoresistance, MSX2-expressing and down-regulated PDAC cells were treated with gemcitabine or 5-FU. The survival of MSX2 expressing PDAC cells was approximately twofold greater than that of control cells, while MSX2 down-regulated cells showed 30–50% decreases in cell viabilities after gemcitabine or 5-FU treatment. Furthermore, forced or reduced expression of MSX2 in PDAC gave rise to increased or decreased numbers of side population (SP) cells, which have been shown to be associated with CSCs (Dean et al., 2005; Golebiewska et al., 2011), respectively. Interestingly, the chemoresistance of PDAC cells by MSX2 expression was abolished when ATP-binding cassette transporter ABCG2, identified as one of the MSX2 target genes by cDNA microarray as described above, was down-regulated by siRNA transfection. Based on these findings, it is suggested that MSX2 enhances the chemoresistance through ABCG2 induction and the increase in the CSC phenotype.

CLINICAL APPLICATION FOR DIAGNOSIS OF PDAC BY MEASURING MSX2 EXPRESSION LEVEL

Endoscopic pancreatic duct brushing is a convenient diagnostic method for strictures in the main pancreatic duct or in the second branch. However, the diagnostic sensitivity of this method for PDAC is shown to be low (40–70%) (McGuire et al., 1996; Vandervoort et al., 1999; Pugliese et al., 2001; Uchida et al., 2007). Since MSX2 expression is limited to neoplastic duct cells in the adult pancreas, the detection of MSX2 could be a useful marker for the diagnosis of PDAC. Therefore, the

expression level of MSX2 mRNA was investigated in 95 endoscopic brushing samples from stricture of the pancreatic duct to determine whether MSX2 expression could distinguish malignant from benign pancreatic diseases and improve the diagnostic yield of brush cytology (Satoh et al., 2011). The samples were collected when ductal strictures were found during ERCP using cytology brushes with 0.025–0.035 inch guide wire. QRT-PCR was carried out on each sample by adding the same amount of total RNA.

In 13 of 95 patients (13.7%), cytological brushing could not be done because the guide wire could not be passed through the ductal stricture. In the remaining 82 patients, endoscopic brushing was successfully carried out and satisfactory specimens were obtained from all cases. Final diagnoses were PDAC ($n = 57$) and chronic pancreatitis (CP, $n = 25$). The sensitivity of routine brush cytology for PDAC was 47.4% (27/57) with 100% specificity and 63.4% diagnostic accuracy (**Table 5**). MSX2 mRNAs in brushing samples were successfully detected and quantified by normalization to the respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) copy number. The mean expression level of MSX2 mRNA was significant higher in PDAC samples than benign strictures (**Table 6**). The MSX2 expression level was judged positive when it was equal to or higher than the cut-off value which was defined by the receiver operating characteristic curve. Using this cut-off value, the sensitivity, specificity for malignancy, and accuracy for diagnosis were 73.7, 84.0, and 79.3%, respectively (**Table 5**). The diagnostic sensitivity for PDAC and the accuracy by analyzing the MSX2 expression levels were much higher than those by cytological examination. The diagnostic sensitivity or accuracy by the evaluation of MSX2 expression compared favorably to other markers such as telomerase (Ohuchida et al., 2005) or MUC1 (Wang et al., 2007) and is similar to the combination analysis of the DNA concentration of methylated cyclin D2, NPTX2, and TFPI2 promoter in brush cytological samples (Parsi et al., 2008). Although the K-*ras* mutation was reported to be more frequently found in brush samples (Van Laethem et al., 1995; Pugliese et al., 2001), it is difficult to use the the K-*ras* mutation as a tool to differentiate PDAC from CP since this mutation is also frequently detected in CP (Yanagisawa et al., 1993). For example, the K-*ras* mutation was frequently detected in both cancer (87%) and pancreatitis (40%) when brush samples from 34 cases of PDAC and 11 of CP were analyzed (Pugliese et al., 2001). In this context, the evaluation of the MSX2 expression level could be a useful tool for differentiating PDAC from CP when a stricture is found in the pancreatic duct.

Table 5 | Comparison between cytology and MSX2 measurement in brush samples (Satoh et al., 2011).

	Sensitivity (%)	Specificity (%)	Accuracy (%)
Cytology	47.4	100	63.4
MSX2 evaluation	73.7	84.0	79.3

Table 6 | MSX2 expression levels in ERCP brush samples (Satoh et al., 2011).

	Number of samples	Mean MSX2 expression level*	*P*-value#
PDAC	57	0.012 ± 0.0024	<0.0001
CP	25	0.0026 ± 0.0004	–

**MSX2/GAPDH (copy number/μl, mean ± standard error).*

#Mann–Whitney U-test.

CONCLUSION

Recent studies have clarified the functions of MSX2 in pancreatic tumor development. MSX2 appears to enhance the malignant phenotype of PDAC by stimulating cell proliferation, the induction of EMT and an increase in the characteristics of CSCs (**Figure 1**). MSX2 also plays an important role in enhancing the aggressiveness of BD-IPMN, indicating that this gene may be a good therapeutic target in pancreatic tumors. Moreover, measurement of the MSX2 expression level in endoscopic brush

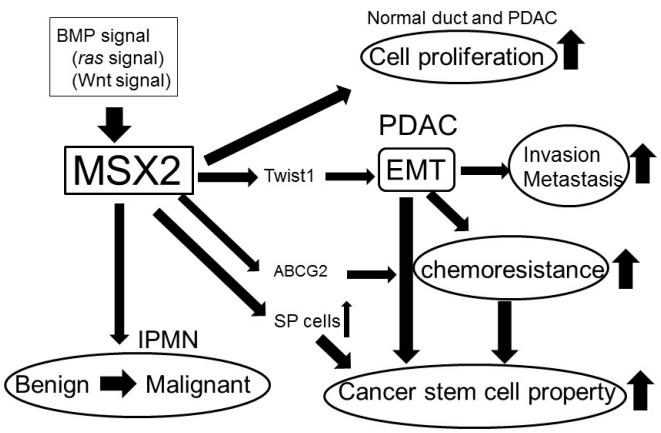

FIGURE 1 | Summary of the roles of MSX2 in pancreatic tumor development.

samples enabled the differentiation of malignant strictures in the pancreatic duct from benign ones, suggesting that the evaluation of MSX2 could be applied clinically for the diagnosis of malignant pancreatic tumor.

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Cytoplasmic expression of LGR5 in pancreatic adenocarcinoma

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Background: CD133 has been identified as a cancer stem cell marker for pancreatic ductal adenocarcinoma. Although leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5), a marker of intestinal stem cells, has been shown to be on a higher level of the stem cell hierarchy than CD133, the expression and function of LGR5 in pancreatic cancer tissue remains unclear. This study investigated tissue expression of LGR5 and CD133 in resected pancreatic cancer tissue.

Methods: LGR5 and CD133 expression was immunohistochemically examined in 9 patients with pancreatic ductal adenocarcinoma who underwent resection.

Results: LGR5 was expressed in the cytoplasm of pancreatic cancer cells in 4 of 9 cases. CD133 was not detected in cancerous tissue. In non-neoplastic tissue, LGR5 was expressed in the basolateral membrane of a subset of endocrine cells. Conversely, CD133 was expressed in the apical membrane of small duct cells. Co-localization of LGR5 and CD133 was not found in either neoplastic or non-neoplastic tissue. LGR5 expression in pancreatic cancer cells showed no statistically significant correlation with survival after surgery.

Conclusion: We have demonstrated that LGR5 is expressed in the cytoplasm of pancreatic adenocarcinoma cells, and the basolateral membrane of a subset of endocrine cells of the human pancreas. Further investigation is required to clarify any prognostic significance of LGR5 expression.

Keywords: LGR5, CD133, cancer stem cell, endocrine cell

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is a highly aggressive disease usually diagnosed in an advanced stage and for which effective therapies remain lacking. Increasing evidence suggests that stem cells play a decisive role not only in the generation of complex multicellular organisms, but also in the development and progression of tumors (Clevers, 2011). Many tumors have been shown to harbor a subset of distinct cancer cells that bear stem cell characteristics, termed cancer stem cells (CSCs). CSCs are hypothesized to be exclusively responsible for tumor initiation, propagation, and metastasis. In addition, CSCs are thought to be highly resistant to chemo- and radiotherapy.

To date, several CSC markers of pancreatic cancer cells have been identified. A highly tumorigenic CD44+CD24+EpCAM+ cell subpopulation, displaying typical stem cell features, could initiate tumors at low cell numbers, using a xenograft model of immunocompromised mice for human pancreatic cancer cells (Li et al., 2007). CD133 (Hermann et al., 2007), aldehyde dehydrogenase 1a1 (Jimeno et al., 2009), and c-Met (Li et al., 2011) have since been identified as other CSC markers of pancreatic cancer cells.

We have previously demonstrated that corticosteroids induce regeneration of acinar cells in patients with autoimmune pancreatitis (Ko et al., 2010). In that study, we showed that the presence of CD133-positive ductal cells correlate with the regeneration of acinar cells, and thus, play an important role in organ regeneration. These data indicated that CD133 is a good marker for pancreatic stem/progenitor cells.

Clevers and colleagues identified Wnt target gene, leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) as a marker for the intestinal stem cells from which all cellular linages of gastrointestinal epithelium are derived (Barker et al., 2007; Barker and Clevers, 2010). A recent study suggests that LGR5 is on a higher level of the stem cell hierarchy than CD133 (Snippert et al., 2009).

We hypothesized that LGR5 is a stem cell marker of pancreatic cancer cells on a higher level of the stem cell hierarchy than CD133. However, LGR5 expression and its function in pancreatic cancer cells remain unclear. The present study therefore, investigated the tissue expression of LGR5 and CD133 in resected pancreatic cancer tissue.

PATIENTS AND METHODS

PATIENTS

Nine of 109 patients with PDA who had undergone pancreatic resection at Aichi Cancer Center Hospital between 2005 and 2010 were included in this pilot study to explore a possible implication of LGR5 for survival. Five patients had a short survival of 1-year or less, and four survived longer than 3 years. The study protocol was approved by our institutional review board. The study was conducted in accordance with the Declaration of Helsinki.

IMMUNOHISTOCHEMISTRY

Surgically resected tissues were fixed in 10% formalin and embedded in paraffin. Sections were deparaffinized, permeabilized, and used for immunohistochemistry. Antigen retrieval was performed by heating in 0.01 M citrate buffer (pH 6.0) in a microwave. The primary antibodies used for immunohistochemistry included rabbit polyclonal antibody to LGR5 (ab75732; Abcam, Cambridge Science Park, UK) and mouse monoclonal antibody to CD133 (MB9-3G8; Miltenyi Biotec, Germany). Dilutions for all antibodies followed the manufacturer's recommendations. Immunoreactions were intensified using Envision plus reagent (DAKO, Carpinteria, CA). Immunolabeling was visualized using 3,30-diaminobenzidine (DAB) as substrate for horseradish peroxidase. Sections were counterstained with Mayer's hematoxylin. LGR5 and CD133 immunostaining in the neoplastic tissue was compared to immunoreactivity in the non-neoplastic tissue as an internal positive control. When the non-neoplastic tissue was overstained or understained, immunohistochemical staining was repeated to achieve appropriate status.

STATISTICAL ANALYSIS

Disease-free and overall survival was analyzed using the Kaplan-Meier method and log-rank analysis. Hazard ratios were estimated using of a Cox proportional- hazards model. All statistical tests were two-sided, and statistical significance was defined for values of $P < 0.05$. JMP version 9.0.3 software (SAS Institute, Cary, NC) was used for all statistical analyses.

RESULTS

BASELINE CHARACTERISTICS OF RESECTED PATIENTS WITH PDA

Nine patients (4 men, 5 women) were enrolled in this study (**Table 1**). Median age was 64 years (range, 44–72 years). Seven patients underwent pancreaticoduodenectomy and 2 underwent distal pancreatectomy. Tumor grade according to the World Health Organization (WHO) classification was Grade 1 in two patients and Grade 2 in seven patients. The final stage according to 7th edition of Union for International Cancer Control (UICC) classification was stage IIA in one, IIB in six, and IV in two patients.

EXPRESSION OF CD133 IN PANCREATIC CANCER TISSUE

All pancreatic cancer cells were negative for CD133 (**Table 2**, **Figure 1A**). Conversely, CD133 was expressed at the apical membrane of small pancreatic duct cells in the non-neoplastic tissue around the cancer tissue (**Table 2**, **Figure 1B**).

Table 1 | Baseline characteristics of patients with resected pancreatic adenocarcinoma.

Case	Sex	Age (years)	Tumor location	Surgery	TNM categories			Stage	Tumor grade
					T	*N*	*M*		
1	M	64	Tail	DP	3	1	1	IV	1
2	F	54	Head	PD	3	1	0	IIB	1
3	M	72	Tail	DP	3	1	0	IIB	2
4	F	56	Head	PD	3	1	0	IIB	2
5	M	44	Head	PD	3	1	0	IIB	2
6	M	71	Head	PD	3	1	0	IIB	2
7	F	56	Head	PD	3	0	0	IIA	2
8	F	67	Head	PD	3	1	1	IV	2
9	F	65	Head	PD	3	1	0	IIB	2

PD, pancreaticoduodenectomy; DP, distal pancreatectomy.

EXPRESSION OF LGR5 IN PANCREATIC CANCER TISSUE

Next, we investigated expression of LGR5 in resected pancreatic cancer tissues. In 4 of the 9 cases, LGR5 was weakly positive in the cytoplasm of pancreatic cancer cells (**Table 2**, **Figure 2A**). LGR5 was strongly positive in the basolateral membrane of a subset of remaining endocrine cells in non-neoplastic tissue surrounding the pancreatic cancer tissue (**Table 2**, **Figure 2B**). On the other hand, LGR5 was negative in the apical membrane of the remaining small pancreatic duct cells that were positive for CD133 (**Figure 2B**).

CORRELATION OF LGR5 AND PROGNOSIS OF PANCREATIC CANCER

We investigated correlations between LGR5 expression in pancreatic cancer cells and survival after surgery. Median overall survival has not been reached in the LGR5+ group and 10.4 months in LGR5− group [hazard ratio (HR), 0.52; 95% confidence interval (CI), 0.08–2.99; $P = 0.52$]. Median disease-free survival has not been reached in the LGR5+ group and 5.4 months in LGR5− group (HR, 0.13; 95% CI, 0.04–1.42; $P = 0.13$) (**Table 3**).

DISCUSSION

Central to the CSC concept is the observation that not all cells in tumors are equal. The CSC concept postulates that, similar to the growth of normal proliferative tissue such as bone marrow, skin or intestinal epithelium, the growth of tumors is fueled by a limited number of dedicated stem cells that are capable of self-renewal (Clevers, 2011). This stem cell hypothesis has recently been explored in PDA (Hermann et al., 2007; Li et al., 2007, 2011; Jimeno et al., 2009).

In the present study, CD133 was expressed at the apical membrane of small pancreatic duct cells in the non-neoplastic tissue around the cancer tissue. These results are consistent with our previous data. Hermann et al. reported that human pancreatic cancer tissue contains CSCs defined by CD133 expression that are exclusively tumorigenic and highly resistant to standard chemotherapy (Hermann et al., 2007). They showed that in the invasive front of pancreatic tumors, a distinct subpopulation of CD133+CXCR4+ CSCs determined the metastatic phenotype

Table 2 | Expression of LGR5 and CD133 in pancreatic tissue and survival time.

Case	LGR5		CD133		Survival time			
	Cancer cells	Non-cancer cells	Cancer cells	Non-cancer cells	OS (days)	Censored	DFS (days)	Censored
1	+	−	−	−	306	no	221	no
2	−	−	−	−	111	no	36	no
3	+	+	−	−	2081	yes	2081	yes
4	+	+	−	+	247	no	201	no
5	−	+	−	+	1283	no	552	no
6	+	−	−	−	2061	yes	2061	yes
7	−	+	−	+	2081	yes	1522	no
8	−	+	−	−	292	no	163	no
9	−	+	−	+	315	no	102	no

OS, overall survival; DFS, disease free survival.

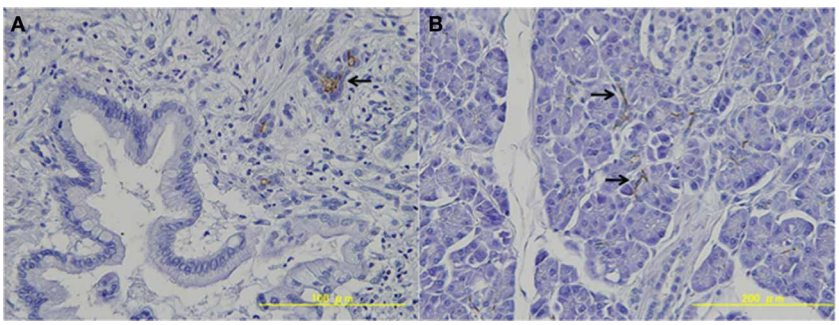

FIGURE 1 | Expression of CD133 in pancreatic tissue. (A) Pancreatic cancer cells were negative for CD133. **(B)** CD133 was expressed at the apical membrane of the small pancreatic duct cells in the non-neoplastic tissue (arrows).

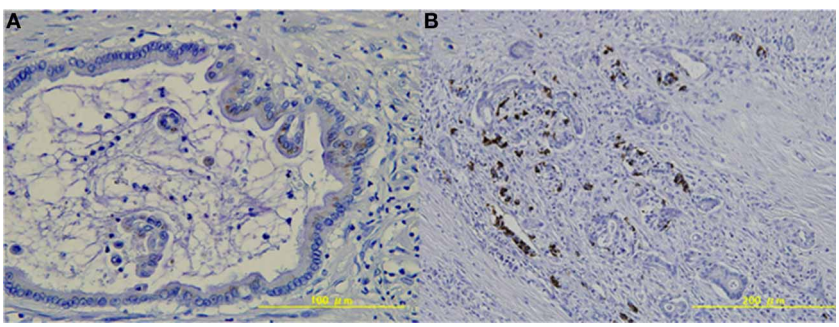

FIGURE 2 | Expression of LGR5 in pancreatic tissue. (A) LGR5 was weakly positive in the cytoplasm of pancreatic cancer cells. **(B)** LGR5 was strongly positive in the basolateral membrane of the remaining endocrine cells in non-neoplastic tissue.

of the individual tumor. However, pancreatic cancer cells were negative for CD133 in our study. One possible explanation for this discrepancy is the sensitivity of an anti-CD133 antibody. The results of immunohistochemical analysis can vary in frequency from antibody to antibody (Sauter et al., 2009). We used clone MB9-3G8 (Miltenyi Biotec, Germany), which is widely used and recognized as reliable antibody against CD133 (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). In our previous study, we used the same antibody and showed clear immunostaining in the pancreas tissue (Ko et al., 2010). The present results are consistent with our previous study. Another explanation could be the condition of formalin-fixed tissues, because immunohistochemical positivity depends on the methods and times of tissue fixation (Sauter et al., 2009).

Table 3 | Correlation between LGR5 expression and survival.

	LGR5 expression	
	(−) $n = 5$	(+) $n = 4$
Median OS	10.4	not reached
HR (95% CI)	0.52 (0.08 − 2.99, $P = 0.52$)	
Median DFS	5.4	not reached
HR (95% CI)	0.13 (0.04 − 1.42, $P = 0.13$)	

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval.

Stem cells of the mouse small intestine, colon, and stomach can be identified by the specific expression of LGR5, a G protein-coupled receptor of unknown function. LGR5+ intestinal stem cells are long-lived, proliferating continuously and generating all the cell types present in the gut. The onset of intestinal tumorigenesis is driven in most cases by activation of Wnt signal pathways. Mouse LGR5- positive cells give rise to intestinal tumors with higher efficiency than other intestinal cell populations upon mutational activation of the Wnt pathway (Barker et al., 2009). Therefore, LGR5- positive cells are thought to represent candidates for CSCs of colorectal cancer. Snippert et al. reported that CD133 marks intestinal stem cells, as well as transit-amplifying progenitors (Snippert et al., 2009). The expression of LGR5 in pancreatic tissue has not been investigated. The present study showed that LGR5was expressed in the cytoplasm of some pancreatic cancer cells. LGR5 was also positive in the basolateral membrane of the remaining endocrine cells surrounding the pancreatic cancer tissue. Immunoreactivity of LGR5 in endocrine cells was stronger than that in pancreatic cancer cells. On the other hand, LGR5 did not co-localize with CD133 in pancreatic cancer tissue.

Simon et al. recently studied the prevalence, histoanatomical distribution and tumor biological significance of LGR5 in tumors of the human gastrointestinal tract (Simon et al., 2012). That study found that LGR5 expression was positive in all 17 cases (100%) with PDA tissue and in 12 of 17 cases (71%) in non-neoplastic tissue. Localization of LGR5 expression was observed as mainly cytoplasmic, but a sporadic membrane or core membrane accentuated expression occurred. Localization of LGR5 in pancreatic cancer cells is consistent with our study. However, the positive rate for LGR5 was higher than in our result (4 of 9, 44%). One possible explanation for this discrepancy is the use of different antibodies against anti-LGR5. They used anti-LGR5—antibody generated by themselves, while we used a commercial polyclonal antibody against LGR5. We have tested reliability of several antibodies against LGR5 prior to this study. Therefore, we used ab75732 (Abcam, Cambridge Science Park, UK) as an antibody against LGR5 in the present study. Immunoreactivity of LGR5 for endocrine cells was consistent with our previous study (Ko et al., 2013). Another possible explanation is the differences in ethnic background. Although LGR5expression has not been studied across ethnic groups, proportions of other colorectal CSC markers vary according to ethnic background (Leavell et al., 2012).

The CSC hypothesis predicts that stem cells are responsible for tumor initiation and preferentially drive tumor growth. Patients with LGR5+ colorectal cancer and gastric cancer reportedly show shorter survival than patients with LGR5− (Merlos-Suarez et al., 2011; Simon et al., 2012). On the contrary, using mouse models of glioma cells, Barrett et al. described that high expression of ld1 identifies tumor cells with high self-renewal capacity, while low ld1 expression identifies tumor cells with proliferative potential but low self-renewal capacity (Barrett et al., 2012). Their results argue against stringent interpretation of the CSC hypothesis. In our study, hazard ratios for OS and DFS with LGR5- positive were 0.52 and 0.13, respectively, however, there were no statistically significant differences. Moreover, stage distribution varied among LGR5- positive (stage IIB 3, IV 1) and negative (stage IIA 1, IIB 3, IV 1) groups, because cases were not matched for stage. Inadequate statistical power with small sample size ($n = 9$) and an absence of case-control study design are limitations of this study. Further studies with adequate statistical power and stage-matched cases are needed to verify the prognostic implications of LGR5 in pancreatic cancer, if any.

Wnt signaling plays an important role in the activation of the mammalian target of rapamycin (mTOR) pathway to stimulate intestinal polyp formation (Fujishita et al., 2008). Activation of the mTOR pathway has also been implicated in the proliferation of pancreatic neuroendocrine tumors. A recent study suggests that Wnt/β-catenin signaling contributes to the pathogenesis and growth of neuroendocrine tumors (Kim et al., 2013). LGR5 was expressed in the basolateral membrane of a subset of endocrine cells of the pancreas in our study. Although LGR5 function in endocrine cells and neuroendocrine tumor cells remains unclear, LGR5 might represent a putative stem cell marker of matured neuroendocrine cells and neuroendocrine tumor of the pancreas (Ko et al., 2013). Recent study suggests that LGR5 is expressed in the remaining islets and in ductal cancer cells in cancerous pancreas, therefore, pancreatic islets beta cells contain cells-of-origin of PDA that express their unique markers in the PDA tumor cells (Amsterdam et al., 2013).

In conclusion, we have demonstrated that LGR5 is expressed in the cytoplasm of pancreatic cancer cells and the basolateral membrane of endocrine cells of the pancreas in patients with PDA. Further investigations are required to clarify the biological functions of LGR5 and its possible application as a stem cell marker for pancreatic exocrine and endocrine tumors.

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