Mouse Modeling for Human Pancreatic Cancer

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Modeling Human Pancreatic Cancer

- History of cancer
- Pancreatic cancer etiology
- Mouse models for human PC-xenograft, carcinogen-induced, genetically engineered mouse models (GEMMs), 3-D organoids
- Lessons from GEMMs

Cancer History

- DNA structure was proposed by Drs. Francis Crick and James Watson in 1953.
- "Two-hit hypothesis": multi-mutation theory on cancer-first published by Carl Nordling in 1953, popularized by Alfred Knudson in 1971 and also known as "Knudson’s Hypothesis".
- 1971, President Richard Nixon declared war on cancer.
- 1976, Drs. J. Michael Bishop and Harold Varmus discovered the first oncogene (Nobel Prize winners, 1989).
- 1986, Dr. Robert Weinberg discovered the first human tumor-suppressor gene (TSG).
- 1994, Dr. Mark Skolnick linked TSG to familial breast and ovarian cancer, confirmed by Dr. Mary-Claire King the same year.
- 1984, oncogenic KRAS was discovered as an oncogene in lung cancer, and subsequently in PC in 1988.
- 1996, DPC4/HMAD4/SMAD4 was discovered by Dr. Scott Kern.

Epidemiology of Pancreatic Cancer

- Pancreatic cancer is relatively rare (the eleventh most common cancer in respect to incidence). >53,070 Americans will be diagnosed with pancreatic cancer in 2016.
- The 3rd leading cause of cancer death due to poor prognosis (~41,780 deaths estimated for 2016)- a five-year survival rate is ~8% (2005-2011).
- The highest rates of pancreatic cancer tend to occur in the developed countries.
- The risk factors includes aging, current smoking (OR: 2.20), heavy drinking (>3 drinks/day), obesity (body mass index >30kg/m²), diabetes (>3 years), family history of PC (OR:1.6), non-O ABO genotype, certain SNP alleles, chronic inflammation.
- Pancreatic cancer is a genetic disease.

Cancer Statistics 2016

(Siegel et al, CA Cancer J CLIN 2016)

- A total of 1,685,210 new cancer cases and 595,690 deaths from cancer are projected to occur in the United States in 2016.
- Death rates peaked in 1991 (215.1 per 100,000 population) and up to 2012 have declined 23% (166.4 per 100,000).
- The reduction in the overall cancer death rates translates to the avoidance of approximately 1.7 million deaths from cancer between 1991-2012.
- Over the 10 years of available data (2006-2009), death rates continue to decline for all 4 major cancer sites (lung, colorectum, breast, and prostate).
- In contrast to declining trends for the major cancers, joinpoint analysis indicates that from 2003 to 2012, death rates rose in both sexes for cancers of the anus, liver, and prostate.
- Overall, cancer is the second leading cause of death following heart disease, which accounted for 24% of total deaths. However, cancer is the leading cause of death among adults aged 40 to 79 years.
The pancreas is made up of three cell lineages-islet, acinar, and ductal epithelial cells.

Pancreatic Cancer

- >95% are derived from exocrine cells, including PDAC, medullary carcinoma, acinar cell carcinoma, pancreatoblastoma, etc.
- The majority of human pancreatic cancer are presented as pancreatic ductal adenocarcinoma (PDAC) that progress from PanIN (pancreatic intraepithelial neoplasm) lesions.
- <5% are pancreatic neuroendocrine tumors (PNET).
PanIN vs. IPMN

PanIN (pancreatic intraepithelial neoplasia)
- Progresses to pancreatic ductal adenocarcinoma (PDA).
- Microscopic papillary or flat noninvasive.
- Arise from intralobular ducts.
- Columnar-to-cuboidal cells with varying amounts of mucin and degrees of cytologic and architectural atypia.
- Ducts less than 5 mm in diameter.
- Five-year survival following resection is less than 20%.

IPMN (intraductal papillary mucinous neoplasm)
- Progresses to non-invasive or invasive carcinoma.
- Grossly visible, noninvasive, mucin-producing predominantly papillary or rarely flat, epithelial neoplasm.
- Arising from the main pancreatic duct or branch ducts.
- Lesions greater than 1 cm in diameter.
- Five-year survival following resection of IPMN with invasive cancer (43%) or without invasive cancer (77%).
- GNAS, RNF43, STK11, PIK3CA

Invasive Cancer

- If a tumor is found to be malignant, its extent or spread is measured by a process called staging. The stages of pancreatic cancer are:
  - Stage I: Very small tumors limited to the pancreas (12-14%).
  - Stage II: Larger tumors localized to the pancreas (5-7%).
  - Stage III: The cancer has spread to the lymph nodes, although not necessarily to distant organs (7%).
  - Stage IV: The cancer has metastasized to the liver, lung, stomach, or more distant organs such as the spleen or bone (5%).

The Importance of Cancer Genetics Profiling

- Early Detection: High risk patients (familial and sporadic) and follow-up/recurrence.
- Biomarkers: Prognosis, diagnosis, staging.
- Therapeutic targets.
- Chemoprevention.
- Animal modeling.

Genetics of Cancer

- Tumor suppressor genes normally function to restrain cell proliferation, and loss of their activity may lead to unrestrained cell growth (broken brake pedal).
- Oncogene: Encodes for proteins which, when overexpressed or activated by mutation, possess transforming properties (gas pedal stuck in the "on" position).
- Mismatch Repair Gene: Check the fidelity of DNA replication. When inactivated, errors which normally occur during DNA replication are not corrected (Drunk mechanic).

Mutational Profile of Pancreatic ductal adenocarcinoma (PDA)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Location</th>
<th>Frequency</th>
<th>Mutation Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNAS</td>
<td>12q13.14</td>
<td>&gt; 90%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>RNF43</td>
<td>1p36.3</td>
<td>1-4%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>STK11</td>
<td>13q12.3</td>
<td>7%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>3q25.3</td>
<td>19-31%</td>
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<td>3q25.3</td>
<td>19-31%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>E2F1</td>
<td>9.137</td>
<td>3%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>TERT</td>
<td>5q35-q33.3</td>
<td>4%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>KIF12A</td>
<td>3q27.3</td>
<td>3%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>PDLIM4</td>
<td>12q13.14</td>
<td>2%</td>
<td>som. &gt; germ.</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>9p21.31</td>
<td>1%</td>
<td>som. &gt; germ.</td>
</tr>
</tbody>
</table>

Global genomic analysis of pancreatic cancer

- 24 pancreatic cancer, 23,219 transcripts, 20,661 genes, ~10^6 SNP-pancreatic cancer harbors 63 alterations on average, majority are point mutations.
Global genomic analyses of pancreatic cancer
- 63 alterations can be defined by 12 core signaling pathways.

- KRAS, TP53, SMAD4, CDKN2A, ARID1A, ROBO2 were confirmed and candidate drivers
  KDM6A and PREX2 were identified—adding chromatin modification, nucleosome remodeling,
  and axon guidance signaling.

Genomic analyses and subtypes of pancreatic cancer
(Bailey et al, Nature 2016)
- 456 pancreatic ductal adenocarcinomas identified 32 recurrently mutated genes that aggregate into 10 pathways:
  KRAS, TGF-β, WNT, NOTCH1, ROBO/SLIT signaling, G1/S transition, SWI-SNF, chromatin modification,
  DNA repair and RNA processing.

- Expression analysis defined 4 subtypes: (1) squamous; (2) aberrantly differentiated endocrine exocrine (ADEX);
  (3) pancreatic progenitor; and (4) immunogenic that correlate with histopathological characteristics.

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  (3) pancreatic progenitor; and (4) immunogenic that correlate with histopathological characteristics.

Majority of mutations occur before metastasis
- Sequencing the genomes of 7 pancreatic metastases.
- A total of 426 somatic mutations in 388 different genes were identified among ~221 millions base pairs sequenced, corresponding to an average of 61 mutations per index metastatic lesion (range 41–77).
- Two categories of mutations were: ‘founder’ mutations (mutations present in all samples from a given patient; mean of 64%, range 48–83% of all mutations per patient). All other mutations were characterized as ‘progressor’ mutations.
- Parental, non-metastatic clones from primary tumors give rise to distal metastatic clonal populations.

Late diagnosis, not its intrinsic aggressiveness, causes high mortality in PC patients
(Yachida et al, Nature 2010; Yachida et al, Oncogene 2013)
- Using Ki-67 as a marker to calculate cell doubling time, as well as the accumulation of passenger mutations to estimate number of passages, it was deduced that it takes at least a decade from the initiating mutation to the birth of a parental non-metastatic clone.
- At least five more years are required for the acquisition of metastatic ability and patients die an average of two years thereafter.
**PanINgram**

- KrAs
- Tagged pancreatic cells
- Inflammation
- Invaded and entered bloodstream early, before frank malignancy could be detected (Rhim et al, Cancer Cell 2012).
- Circulating tumor cells (CTC) were captured in 7/21 (33%) patients with cystic lesions and no clinical signs of cancer, 8/11 (73%) with PDA, and 0/19 (0%) of control (Rhim et al, Gastroenterol 2014).

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**Desmoplasia-Friend or foe?**

- Current model: The stroma is protective and is exerted at the PanIN stage (Ozdemir et al, Cancer Cell 2014, Rhim et al, Cancer Cell 2014)

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**Hurdles in PC Patient Care**

- Low incidence.
- Most of PC patients die within 6 months of diagnosis.
- Only 15-20% of PC are surgically resectable.
- Resected tumors have lots of normal stromal tissue contamination.
- Early PanIN samples are rare.
- Complexity of mutations
- Desmoplasia complication
- Metastasis in most patients

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**The Needs in PC Patient Care**

- Early Detection-High-risk patients (familial and sporadic) and follow-up/recurrence.
- Biomarkers-Prognosis, diagnosis, staging.
- Therapeutic targets
- Chemoprevention
- Animal modeling

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**Mouse Modeling for Human Pancreatic Cancer**

- A model that recapitulates its human counterpart in tumorigenesis (in both histological progression and genetic mutations).
- A model that allows spontaneous tumor development and yet with predictable time line.
- A model that has an intact microenvironment and yet allows metastasis.
Generating Models for PC

- Xenografts (human PC cells into mice)
  - Subcutaneous
  - Orthotopic
  - Patient-derived xenografts
- Carcinogen administration
  - BOP into hamsters
  - DMBA into mice & rats
  - Azaserine into rats
- Genetic Engineering
  - Oncogenes: mutant Kras, TGFalpha, SHH
  - TSG: p16, Smad4, p53, TGFbRII, Stk11, etc.
- 3-D organoids

Orthotopic: Implantation into the pancreas

Subcutaneous Xenograft

Carcinogen administration

- BOP into hamsters
- DMBA into mice & rats
- Azaserine into rats

Genetic Engineering

- Oncogenes: mutant Kras, TGFalpha, SHH
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3-D organoids

Orthotopic: Implantation into the pancreas

Patient-derived xenografts
Carcinogen induced

Mouse Models for PC

- Xenografts (human PC cells into mice)
  - Pros: Easy & cheap, short-term, high penetrance, easy to quantify tumor burden.
  - Cons: No metastasis, no PanIN, lacks intact TME.
- Orthotopic
  - Pros: Metastasis & cheap, short-term, PanIN in some, high penetrance, better mimicking human PDAC histologically.
  - Cons: Labor-intensive, PanIN in some, more mouse-to-mouse variability, more difficult to quantify tumor burden, lacks intact TME.
- Patient-derived xenografts
  - Pros: Same as other xenografts plus the potential for personalized medicine.
  - Cons: Same as other xenografts

Carcinogen administration

- BOP into hamsters, DMBA into mice & rats, Azaserine into rats
  - Pros: Easy & cheap, PanIN in some models, simulate environmental assaults, intact TME.
  - Cons: Unknown genetic profile, difficult to monitor progression, few carcinogens have been studied in mice.

Genetically-Engineered Mouse Models

- Strategies
  - Transgenics
  - Knock-in
  - Knock-out
- Targeted genes
  - Oncogenic: mutant Kras, TGFalpha, SHH; GNAS
  - TSG: p16, SMAD4, p53, TGFbRII, STK11, etc.
- Targeted cell types
  - Pancreatic progenitor cells
  - Acinar cells
  - Ductal epithelial cells
  - Centroacinar cells
- Pro & Con
  - Pros: Best mimicking human PC at genetic & histologic levels, intact TME, PanIN development, allows pathway analyses, progression to PDA and metastasis.
  - Cons: Expensive, time-consuming, labor-intensive, requires extensive knowledge on gene targeting, may have limited tumor complexity, may harbor secondary (not engineered) mutations.
How to target pancreatic ductal epithelium?

Existing Mouse Models for Pancreatic Cancer

Hruban et al, Can Res 2006; 66:95-106

**LSL-Kras^{G12D}; Pdx1-Cre and LSL-Kras^{G12D}; p48-Cre oncomice**

(Hingorani et al, Cancer Cell 2003)

- The first mouse model that develops PanINs that simulate human precursor lesions.
- It utilizes both knock-in and conditional activation technologies.
- Conditional activation of Kras^{G12D} at physiological level.
- The phenotypes of LSL-Kras^{G12D}; Pdx1-Cre and LSL-Kras^{G12D}; p48-Cre are very similar.

**Mutational Profile of Pancreatic ductal adenocarcinoma (PDA)**

Pdx1-Cre;LSL-Kras^{G12D}; p53^{R172H/+} mice have accelerated tumor progression, but the same histology-PanIN to PDA

(Hingorani et al, Cancer Cell 2005)

Pdx1-Cre; LSL-Kras^{G12D}; Smad4^{−/−} mice preferentially develop mucinous cystic lesions in the pancreases (MCN vs IPMN)

Bardessy et. al., Can Dev 2006; Izeradjene et al, Cancer Cell 2007
**P16/INK4a Conditional Knock-out Mice**

- **Why p16?**
  - Inactivated in virtually 100% of pancreatic cancer.
  - Germline mutations lead to increased risk for pancreatic cancer, melanoma, and head and neck cancer.
- **What is p16?**
  - An inhibitor of the cyclinD-Cdk4 and cyclinD-Cdk5 kinase complexes that down-regulate Rb.
- **Why conditional knock-out?**
  - P16 conventional knock-out mice die from lymphoma, sarcoma, melanoma etc. at early ages (Sharpless et al, Nature 2001).

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**p16 inactivation works synergistically with oncogenic Kras in promoting pancreatic tumorigenesis**

Medium survival of p16−/−; LSL-KrasG12D; Pdx1-Cre mice and p16loxp/loxp; LSL-KrasG12D; Pdx1-Cre (PKP) mice are 4 and 6 months respectively.


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**PKP mice developed PDAC and frequent metastasis**

- PDAC is the major histologic presentation.
- Frequency of metastasis increased with age: 44% in mice younger than 4 months and 49% in those older than 4 months.
- Metastasis is observed in all non-thriving mice (n=14) and involves liver, LN, stomach, lung, testis, spleen, etc.
Mouse Modeling for Human Pancreatic Cancer

- A model that recapitulates its human counterpart in tumorigenesis (in both histological progression and genetic mutations).
- A model that allows spontaneous tumor development and yet with predictable time line.
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Mouse Model #2-IPMN/Pancreatic Ductal Adenocarcinoma

AKP GEMM-
Acvr1bflox/flox;LSL-KrasG12D;Pdx1-Cre mice

The histologic presentations of the LSL-KrasG12D oncomice differed for each tumor-suppressor gene


Mutational Profile of Pancreatic ductal adenocarcinoma (PDA)

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<th>Genomic Location</th>
<th>Frequency (%)</th>
<th>Mutation Status</th>
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<tbody>
<tr>
<td>Acvr1B</td>
<td>2p12</td>
<td>95%</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Apaf1</td>
<td>19p13.3</td>
<td>95%</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Jkb</td>
<td>6p21.33</td>
<td>4</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Jkb</td>
<td>1p36.33</td>
<td>4</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Jkb</td>
<td>10p15.11</td>
<td>2</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Jkb</td>
<td>1q41-q42</td>
<td>7</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Jkb</td>
<td>11q13.1-q13.2</td>
<td>6</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Jkb</td>
<td>18q21-q21.1</td>
<td>5</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

Mutated in 2% of sporadic pancreatic ductal adenocarcinomas.

Su et al., PNAS: 98(6):3254-7, 2001

Inflammation and ADM were observed in the pancreata of Acvr1bf/f;Pdx1-Cre mice

ALK4/ACVR1B, a bona-fide tumor-suppressor gene

- Activin type 1 receptor B- part of the TGFbeta receptor family.
- Sequenced the genomic DNA of 29 (34% LOH) pancreatic cancer xenografts and 5 (45% LOH) pancreatic cancer cell lines genomic DNA in the ALK4 locus.
- Mutated in 2% of sporadic pancreatic ductal adenocarcinomas.
AKP mice exhibited shortened survival and IPMN histologic phenotype

Qiu et al., Gastroenterology 2015

PanIN vs. IPMN

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<td>lesions greater than 1 cm in diameter</td>
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The inactivation of Acvr1b favors the expansion and progression of IPMNs in AKP mice

Notch4 may be involved in the early development of IPMN

RNA-Seq analysis confirmed the involvement of Notch4 in the early development of IPMN

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Increase</th>
<th>Adj. p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch4</td>
<td>5.80</td>
<td>0.005</td>
</tr>
<tr>
<td>Wdfy1</td>
<td>4.73</td>
<td>0.004</td>
</tr>
<tr>
<td>Zdhhc14</td>
<td>10.27</td>
<td>0.005</td>
</tr>
<tr>
<td>Gbp1</td>
<td>35.37</td>
<td>0.048</td>
</tr>
</tbody>
</table>

AKP vs. KP cells - 1130 genes were differentially expressed between the two genotypes (adjusted p-value <0.05).
NOTCH4 expression was significantly upregulated in AKP than KP tumor cells, but there was no statistical significant differences in the expressions of Notch1, Notch2, or Notch3 genes.
A list of NOTCH4-specific target genes were extracted from the original data from Verhein et al. to identify differentially expressed genes between the Notch4 knockout and wild-type samples. Three of the Notch4 target genes (Zdhhc14, Wdfy1, and Gbp1) were also upregulated along with Notch4 as expected, while none of the canonical NOTCH target genes such as Hes and Hey family were differentially expression between AKP and KP tumor cells.
p16 play pivotal roles in the IPMN to invasive cancer progression


Alk4-/ IPMN/MCN or TGFbR2-/

Qiu et al, Gastroenterology, 2015

Mouse Model #3-Chronic Pancreatitis

STP GEMM-Smad4floxflox;MT-TGFalpha;p48-Cre mice

TGFalpha
- Ligand of EGFR
- MT-TGFα (Sangren et al, Cell 1990)
  - Rat TGFα driven by metallothionein promoter, inducible by ZnSO4.
  - Hyperplasia of adult liver, pancreas, stomach, small intestine, cecum, colon, coagulation glands.
  - Fibrosis and metaplasia of adult pancreas.
- EL-TGFα (Sangren et al, Cell 1990; Wagner et al, Gastroenterology 1998)
  - Rat TGFα driven by elastase promoter.
  - Hyperplasia, fibrosis, and metaplasia of adult pancreas (29%, tumor-free 410d).
  - Increased nuclear staining of p53.
- EL-TGFα/p53-null (Wagner et al, Gene&Dev 2001)
  - CK19+ pancreatic cancer, metastasis to liver and lung (77.3%, tumor-free 45-120d).
  - Biallelic inactivation of p16 (33%), LOH of SMAD4.
**Smad4 deficiency cooperates with MT-TGFα to promote pancreatic fibrosis and PanIN development**

Garcia-Carracedo et al, PLOS One, 2015

**STP mice develop features similar to human chronic pancreatitis transitioning to preneoplasia**

**PanIN, IPMN, & CP mouse models**

- KP GEMM-LSL-KrasG12D;Pdx1-Cre mice
  - Develop mPanIN and PDA (Hingorani et al, Cancer Cell 2003)
- PKP GEMM-p16<sup>flox/flox</sup>;LSL-KrasG12D;Pdx1-Cre mice
  - Accelerated development of mPanIN, PDA, and 100% metastasis (Qiu et al, Oncotarget, 2011)
- AKP GEMM-Acvr1<sup>bflox/blox</sup>;LSL-KrasG12D;Pdx1-Cre mice
  - Expansion of IPMN, progression to PDA, and some are associated with metastasis
- MT-TGFalpha
  - Develop metaplasia and chronic pancreatitis in females (Sandgren et al, Cell 1990; Liao et al, J of Carcinogenesis 2006)

**The Utilities of GEMMs**

- Tumorigenesis
- Cancer stem cells/tumor initiating niche.
- Early Detection/Biomarkers-Prognosis, diagnosis, staging.
- Metastasis
- Therapeutic targets/Chemoprevention

**The Needs in PC Patient Care**

- Early Detection-High-risk patients (familial and sporadic) and follow-up/recurrence.
- Biomarkers-Prognosis, diagnosis, staging.
- Therapeutic targets
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- Animal modeling

**3-D Organoids**

Achin to ductal metaplasia culture-Maor et al, Development 2006; Qiu et al, Gastroenterology 2015

Primary pancreatic duct epithelial cell cultures-Aghnunag et al, Cancer Res 2006
3-D Organoids

- **Pro & Cons**
  - **Pro**: Allows personalized medicine, duplicates PanIN and PDA histology and genetics, bypassing the question of cell origin.
  - **Con**: Lacks TME (if xenografted), require transforming growth factor β (TGF-β) pathway inhibitors (A83-01 and Noggin), R-Spondin1 and Wnt/β-catenin-conditioned media, EGF, and PGE2 for propagation; potential issue of cell origin.

**Boj et al., Cell 2015**

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**Future Directions**

- **Early Detection**
  - Exosomes
  - Circulating tumor DNA (ctDNA)
  - Circulating tumor cells (CTCs)

- **Combination therapies**
  - FOLFIRINOX
  - Nab-paclitaxel (Abraxane) plus Gemzar
  - Other target therapies (angiogenesis, metabolism, KRAS & other oncogenes, immunotherapies, autophagy, etc)
  - Immunotherapy/vaccine

- **Metastasis**
  - Tumorigenesis
  - Clonal expansion/tumor heterogeneity
  - Treatment options

- **Cell Origin**
  - PanIN vs. IPMN
  - Ductal vs. Acinar

**Boj et al., Cell 2015**