Chapter-3

Characterization of a hamster model of pancreatic cancer desmoplasia
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6.1 Characterization of a hamster model of pancreatic cancer desmoplasia

6.1.1 Background and rationale

Previous studies have employed in vivo models of genetically modified mice or xenograft models of pancreatic cancer, these models have several limitations that make them unsuitable to study novel therapeutics against PC. For example, xenograft models lack all the cellular components of a fibrotic tumor because of the absence of an effective immune system. Genetically modified mouse models have long periods of tumor latency that limits their usefulness in testing new therapeutics. Therefore, there is a need for new animal models that overcome the shortcomings of existing models, particularly to test new treatment strategies.

The chemically induced model for PDAC in Syrian golden hamsters (Mesocricetus auratus) was shown to be similar in morphologic appearance and biological behaviour to that seen in humans [147]. Moreover, the chemically induced hamster models using N-nitroso-bis(2-oxopropyl)amine (BOP) have also shown to harbour point mutations in the K-ras gene [148]. Different labs have established and characterized multiple PC cell lines from carcinogen induced pancreatic tumor in hamsters [149, 150]. Some of these cells lines have been frequently used to generate allograft tumors in Syrian hamsters. Particularly, the commercially available HapT1 hamster PC cell line derived from DIPN-induced pancreatic tumor has been widely used as an in vitro model in PC related studies [150]. Carcinogen induced hamster PC shows desmoplastic reactions; however, till date no studies have reported the characterization and/or use of hamster PC model as a pre-clinical model for cancer-associated desmoplasia. Therefore here we aim to characterize HapT1 derived homologous tumors as a model of pancreatic cancer desmoplasia.
6.1.2 Methodology

6.1.2.1 Orthotopic injection of HapT1 cancer cells in to hamster pancreas

Prior to conducting any animal studies, all protocols were approved by the Institutional Animal Ethical Committee (Institute of Life Sciences, Bhubaneswar, India). To develop the desmoplastic pancreatic tumor, HapT1 PCCs \((8 \times 10^6)\) were orthotopically injected into the pancreases of Syrian golden hamsters. Animals were anesthetized with intraperitoneal (i.p.) administration of a cocktail mixture of ketamine \((150 \text{ mg/kg})\) and xylazine \((10 \text{ mg/kg})\). After sterilizing the incision site, an approximately 1cm long incision was made near the position of spleen. Gently, the splenic lobe of the pancreas (Figure 1A) along with the spleen was pulled up to the incision site, and around \(8 \times 10^4\) HapT1 cells in 50 µl volume were injected into the splenic lobe of the pancreas using a 30-guage needle. After resetting all the pre-moved organs, the abdomen was closed with absorbable catgut sutures. The skin incision was closed with interrupted sutures of non-absorbable material. The skin suture material was removed at approximately five days after the surgical procedure. The animals were monitored daily.

6.1.2.2 Tissue processing and haematoxylin and eosin staining

Tissue samples were collected and preserved in 10% formalin buffer solution at room temperature. For histopathological analysis, tissues were processed for paraffin embedding, and multiple 5-micron sections were prepared. For haematoxylin and eosin staining, slides were deparaffinized and hydrated with deionized water followed by haematoxylin (Sigma) staining for three minutes and eosin for two minutes. Next, slides were thoroughly washed in H₂O and dehydrated through sequential alcohol grading then cleared in xylene and mounted with permanent mounting media (Vector Lab). Stained slides were observed under a Leica DM500 light microscope, and representative images were taken at 10x and 40x magnifications.
6.1.2.3 Aniline blue staining
For aniline blue staining, we modified the conventional trichrome staining protocol. Briefly, slides were deparaffinized and hydrated with deionized H$_2$O followed by incubation in pre-heated Bouin’s solution (Sigma) for 15 minutes. Next, slides were cooled at room temperature and washed under running tap water. Tissue sections were then incubated in working phosphotungstic-phosphomolybdic acid solution (Sigma) for five minutes followed by aniline blue (Sigma) for 10 minutes and rinsed in 1% acetic acid (SRL) for 2 minutes. Finally, slides were thoroughly washed with deionized H$_2$O and dehydrated through sequential alcohol grading. They were then cleared in xylene and mounted with permanent mounting media (Vector Lab). Stained slides were observed under a Leica DM500 light microscope, and images were taken at 10x and 40x magnifications. The percentage of collagen stained area (aniline blue) was quantified using the automated ImageJ program in conjunction with the threshold plugin [151].

6.1.2.4 Toluidine blue staining
For toluidine blue staining, briefly, the slides were deparaffinized and hydrated with deionized H$_2$O, followed by staining with toluidine blue solution pH 2.3 (Merck) for three minutes. They were subsequently washed with deionized H$_2$O three times. Slides were dehydrated quickly using 95% and 100% ethanol and then cleared in xylene and mounted with permanent mounting media (Vector Lab). Stained slides were observed under a Leica DM500 light microscope, and images were taken at 10x and 40x magnifications.

6.1.2.5 Immunohistochemistry
For immunohistochemistry (IHC), mouse monoclonal anti-αSMA, rabbit monoclonal anti-vimentin, and rabbit polyclonal anti-fibronectin primary antibodies were used to detect the cellular and extracellular proteins (Table5). Briefly, slides were
deparaffinized and hydrated with deionized water. Antigen retrieval was performed in acidic pH citrate buffer (Vector Lab) by incubating slides in a steam cooker for 20 minutes. Slides were then washed twice with PBS for five minutes, followed by endogenous peroxidase quenching in 3% H$_2$O$_2$ (SRL) for 15 minutes. Nonspecific binding was blocked by incubating the slides with horse serum for 30 minutes, followed by overnight incubation with primary antibody α-SMA, vimentin, and fibronectin at 4°C in a humidified chamber. Details of antibody dilutions are provided in Supplementary Table 1. Slides were then washed twice with PBS for five minutes and incubated with biotinylated anti-rabbit/mouse IgG secondary antibody for 45 minutes. Diaminobenzidine (Vector Lab) was used to detect the immunoreactivity. Slides were subsequently counterstained with haematoxylin (Sigma), dehydrated through sequential alcohol grading, cleared in xylene, and mounted with permanent mounting media (Vector Lab). Stained slides were observed under a Leica DM500 light microscope, and images were taken at 10x and 40x magnifications. For all type of histopathological evaluations, the stained slides were assessed by a qualified veterinary pathologist (SKP).

Table 5: List of primary antibodies used for Immunohistochemical analysis:

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Antibody</th>
<th>Company</th>
<th>Catalog No.</th>
<th>Dilution for IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Anti-α-SMA (Mouse Monoclonal)</td>
<td>Sigma</td>
<td>A2547</td>
<td>1 : 2000</td>
</tr>
<tr>
<td>02</td>
<td>Anti-Ki67 (Rabbit Monoclonal)</td>
<td>Vector Laboratories</td>
<td>VP-RM04</td>
<td>1 : 50</td>
</tr>
<tr>
<td>03</td>
<td>Vimentin (Rabbit Monoclonal)</td>
<td>Cell Signalling Technology</td>
<td>D21H3</td>
<td>1:100</td>
</tr>
<tr>
<td>04</td>
<td>Fibronectin (Rabbit Polyclonal)</td>
<td>abcam</td>
<td>ab2413</td>
<td>1 : 50</td>
</tr>
</tbody>
</table>
6.1.3 Results

6.1.3.1 Morphology and composition of HapT1 primary and metastatic tumors

Necropsy of all the animals in this study showed 100% tumor uptake. All the tumors were firm, round, and slightly reddish in colour (Figure 12A). Further, all animals had incurred metastasis to one or multiple sites/organisms (Figure 12B). Microscopically, the primary tumors were found to consist of multiple duct-like structures (pseudo ducts; Figure 12C), and the proportion of duct-like structures was greater in 24-day-old tumors compared with the 14-day-old tumors (data not shown). The tumors had centrally located necrotic zones and peripheral proliferative zones. Necrotic regions were laden with infiltrated immune cells (data not shown). Some immune cells were also visible in the non-necrotic regions of all the tumors. Spindle-shaped myofibroblast-like cells and the deposited extracellular matrix were clearly visible around all the individual duct-like structures (Figure 12C). At multiple places, the stromal structures surrounding the cancer cells acted as a barrier between infiltrated immune cells and cancer cells (Figure 12C). As reported for human PC, the secondary or metastatic tumors harvested from multiple hamsters clearly showed the presence of fibrotic stroma (Figure 12D); irrespective of the site of metastasis, all the metastatic tumors were desmoplastic; activated myofibroblast-like cells and immune cells were also present. [152]
Figure 12: Gross morphology and histopathology of HapT1 orthotopic tumor: A) Diagram of the orthotopic implantation of HapT1 cancer cells in hamster pancreases. Representative macroscopic view of a HapT1 orthotopic tumor present inside the splenic lobe of a hamster pancreas (dotted circle). The splenic lobe of the pancreas (pa) and spleen (sp) are also shown in the image. B) Representative gross images of various organs that showed metastatic tumor growth are shown (arrowhead). C) Representative images of H&E stained sections of HapT1 orthotopic primary tumors from three different tumor sections (a, b, and c). An image from primary pancreatic tumor sections shows the presence of pseudo-duct-like structures (arrow) and deposition of dense extracellular matrix proteins/desmoplastic reaction around them (star). At some places, extracellular matrix proteins act as a barrier between tumor-infiltrated immune cells and cancer cells (c). D) H&E stained sections of HapT1 metastatic tumors clearly show the presence of a desmoplastic reaction within the tumor stroma of metastatic tumors (arrowhead). (N) normal tissue area; (Ca) cancerous regions; (I) immune cells (scale bar= 50µm).
6.1.3.2 Molecular and cellular characterization of HapT1 primary and metastatic tumors

For further molecular and cellular characterization of the desmoplastic reactions present in HapT1 tumors, this study examined the expression of two major extracellular matrix proteins (i.e., collagen and fibronectin) and two important cell types known to promote PC-associated fibrosis (i.e., myofibroblast-like and mast cells). Staining with aniline blue (one component of MS trichome stain) showed dense collagen deposition in all the primary and metastatic tumors (Figure 13A; data not shown). However, the normal pancreatic tissue present in closer proximity to the primary tumors had markedly low levels of collagen (Figure 14). Figure 13A shows immunohistochemical (IHC) staining of tumors derived from HapT1 cell lines and confirms the area that stained positive for collagen also stained positive for fibronectin. Moreover, fibronectin was also present in the metastatic tumors (Figure 13A; data not shown). IHC analysis of tumor sections also revealed that HapT1 tumors contained myofibroblast-like cells, which is based on the fact that they stained positive for α-SMA and vimentin. The myofibroblast-like cells were identified in the areas with desmoplastic reactions (Figure 13B). A similar observation was made in all the secondary/metastatic tumor tissues (Figure 13B; data not shown). Additionally, the toluidine blue staining revealed the presence of mast cells (MCs) in different zones of the primary tumors (Figure 15), as reported in human PC tissues.[153] Multiple MCs were present close to the blood vessels; their number was increased in areas with a greater number of blood vessels. These observations also corroborate a previous report.[153]
Figure 13: Non-cellular and cellular components of HapT1 orthotopic tumor: A) Representative images of primary and metastatic tumor tissue sections (liver) showing the presence of collagen and fibronectin, as detected by aniline blue staining and IHC analysis, respectively (scale bar = 50µm). Morphometric analysis of images obtained from aniline blue stained sections shows the extent of collagen deposition in primary (pancreas) and corresponding metastatic tumors (liver). The percentage of aniline blue stained area estimation was done in tissues obtained from two different animals (with liver metastasis). Presented data obtained from one animal (n = 5; five different fields/tissue) shows the representative pattern of collagen deposition in corresponding primary and metastatic tumors. Data presented as mean + SE. B) IHC analysis for α-SMA and vimentin as markers of activated myofibroblast-like cells followed by quantification of α-SMA positive cells shows the presence of a similar number of activated myofibroblast-like cells in both primary and corresponding metastatic tumors. Presented data obtained from one animal (n = 5; five different fields/tissue) show the representative pattern of the abundance of α-SMA positive cells in corresponding primary and metastatic tumors (scale bar = 50µm). Data presented as mean + SE.
Normal pancreatic tissue
HapT1 tumor tissue

Figure 14: Masson’s Trichrome staining of HapT1 tumor and normal pancreatic tissue:
Masson’s Trichrome staining shows the extent of collagen deposition (blue) in HapT1 tumor and normal pancreatic tissue of the same animal. In normal pancreas, blood vessels and ducts have clear collagen staining around them; however, in other parts of the tissue the collagen level is very less or undetectable. On the other hand, HapT1 tumor tissue has an extensive deposition of collagen throughout the tumor parenchyma.

Figure 15: Presence of mast cells in HapT1 orthotopic tumor: Representative images of toluidine blue-stained primary tumor sections showing the presence of mast cells (violet color; arrows) in peri-tumor, intra-tumor border and intra-tumor center regions of HapT1 orthotopic tumors (scale bar= 50µm). Most of the mast cells were present closer to the blood vessels (bv).
6.1.4 Discussion

Currently, a significant proportion of studies in human PC employ the orthotopic implantation of PCCs in athymic mice. However, there is experimental evidence that syngeneic tumors are clearly more reliable compared with those developed in athymic mice.[154] In our current study, the presence of extracellular matrix proteins (e.g., collagen and fibronectin) and stromal cells (e.g., myofibroblast-like and mast cells) in HapT1 tumors mimics the desmoplastic reactions seen in human PDAC (Figure 13, 14 and 15). Moreover, the presence of similar types of extracellular matrix proteins and their extent of deposition in metastatic tumors corroborates findings in human PDAC patients.[152] In syngeneic experimental tumors like the HapT1 orthotopic tumor, due to the functional immune system of the host, the inflammatory milieu in tumor microenvironment is more robust compared with tumors grown in immune-compromised hosts. Moreover, this could be the major cause behind enrichment of a large number of myofibroblast-like cells and immune cells in HapT1 tumors. In HapT1 tumor tissues, the presence of Ki67-positive stromal or myofibroblast-like cells indicates a certain level of proliferative activity of these cells (Figure 16). Hence, recruitment of myofibroblast-like cells and/or their further proliferation in tumor microenvironment might have contributed to the overall population of these cells.

Accumulating evidence suggests that mast cells promote the progression of PC.[153, 155, 156] In a 2015 study, Masso-Valles D et al. showed that Ibrutinib, a mast cell inhibitor, restricted fibrosis associated with PDAC and extended the survival time of animals bearing these tumors.[157] Tumor tissue derived from the HapT1 cells contained numerous mast cells (Figure 15). Whether this could be employed in the future to investigate the effect of mast cell inhibitors on PC fibrosis and growth remains an unanswered question.
Figure 16: Proliferative nature of HapT1 tumor stromal cells in vivo. Primary HapT1 tumor tissue sections were stained with a human Ki67 specific antibody. Analysis of stained slides showed cross reactivity of this antibody with hamster protein. Most of the cancer cells (with round and bigger nucleus) are positive for Ki67 (typical nuclear staining); and some stromal cells (spindle shaped cells; arrow head) are positive for Ki67.