Development of new therapeutic strategies targeting cancer associated fibroblasts (CAFs) in pancreatic ductal adenocarcinoma

PhD thesis

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Budapest

2018

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most frequent type of pancreatic tumors. It is an extremely aggressive disease with diagnosis at advanced stages and highly refractory to most treatments. By 2030 it will become the second leading cause of cancer death in developed countries.

PDAC is associated with a very poor prognosis, with a 5-year survival rate of only 6% and a median survival of less than 6 months. This low survival rate is the result of its aggressive features and its late diagnosis due to the lack of early symptoms and early biomarkers. Therefore, at the time of detection 80% of patients have locally advanced or metastatic PDAC and less than 20% of the patients are eligible for resection. Moreover, PDAC biology contributes to early recurrence, distant metastasis and resistance to chemotherapy and radiotherapy. A very important player in this poor prognosis is the extensive stromal reaction (desmoplasia) resulting in a hypovascular and hypoxic microenvironment and evasion of tumor immunity.

This stroma is mainly composed (around 90%) of cancer-associated fibroblasts (CAFs). CAFs secrete extracellular matrix proteins (ECM) as well as soluble factors (chemokines, cytokines) that stimulate cancer progression. Furthermore, it has also been reported that CAFs mediate drug resistance and immunosuppression. Hence, CAFs may represent an important target for anti-cancer therapy.

This concept has been recently challenged by two independent studies in which elimination of the stroma in genetically engineered mouse (GEM) PDAC models resulted in more aggressive tumors and reduced survival. However, CAFs are a heterogeneous population, thus, it is conceivable that different CAFs may have differential pro- and anti-tumorigenic roles. Therefore, a better understanding of CAF biology and the distinct roles of each subpopulation of CAFs in PDAC progression may allow designing novel therapeutic strategies based on their selective reprogramming to thwart pro-tumorigenic effects without having to resort to their physical elimination.

In this study, we have characterized a CAF subpopulation with protumorigenic properties in PDAC mouse model, defined by the expression of the platelet-derived growth factor receptor alpha (PDGFRα), while we also observed that PDGFRα+ normal pancreatic

fibroblast (NPFs) are anti-tumorigenic. The comparative transcriptome analysis of fibroblasts present in normal pancreata showed that the most differentially overexpressed gene in CAFs was *Saa3*, a member of the acute-phase Serum Amyloid A (SAA) apolipoprotein family found associated with high density lipoproteins in plasma. Expression of SAA members is induced in injured tissues and cells including atherosclerotic plaques, rheumatoid synovitis and in certain tumor cells.

We describe that Saa3 plays a key role in inducing the pro-tumorigenic properties of PDGFRα+ CAFs in pancreatic tumor bearing mice. In addition, we also specify that the pro-tumorigenic activity of Saa3 is regulated by the Membrane Palmitoylated Protein 6 (Mpp6), a member of the peripheral membrane-associated guanylate kinases (MAGUK). Moreover, we identified and functionally validated further targets differentially overexpressed in CAFs including: *Lumican* (*Lum*), *Haptoglobin* (*Hp*) and *Hyaluronic acid synthase 1* (*Has1*), as well as *Mesothelin* (*Msln*). We generated genetically engineered mouse models (GEMMs) by CRISPR/Cas9 gene editing technology in order to study their role and their potential therapeutic value in PDAC development.

Methods

PDAC mouse model to study CAFs

The K-*Ras*$^{+/LSLG12Vgeo}$;*Trp53*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre (KPeCY) PDAC mouse model was used to study the tumor stroma and to isolate CAFs. This strain was generated by crossing K-*Ras*$^{+/LSLG12Vgeo}$, the *Trp53*$^{lox/lox}$ alleles with the bitransgenic *Elas*-tTA/*tetO*-Cre strain. The Cre recombinase expression is driven by the acinar cell specific *Elastase* promoter and is under the negative control of the doxycycline inducible Tet operon (Tet-off system).

Cell isolation

Primary fibroblasts were first isolated either by tissue outgrowth from fresh tissue samples collected from tumor-bearing KPeCY or WT mice. To utilize a more physiological method we selected a subset of fibroblasts by the cell surface marker PDGFRα+. We FACS sorted PDGFRα+/EYFP-/CD45-/CD31- stromal cells from PDAC tumors of KPeCY mice as well as from normal pancreata of control *Elas*-tTA/*tetO*-Cre;*Rosa26*$^{+/LSLEYFP}$ animals.

Gene expression profiling

We analyzed transcriptome profiles of distinct CAF populations isolated by two different methods. To characterize better the cells isolated by outgrowth from normal (WT) pancreas and PDACs we performed RNA sequencing (RNAseq) of NPFs (n = 3) and CAFs (n = 3). To understand the genetic bases responsible for the tumor-promoting activity of PDGFRα+ CAF subpopulation compared to the PDGFRα+ NPFs, we performed a comparative study of the transcriptome profiles of freshly isolated CAFs (n = 5) with those of NPFs (n = 3) by RNA sequencing analysis. We performed functional analysis by gene ontology and gene set enrichment pathway analysis (GSEA).

***In vitro* validation**

We selected potential targets for validation at RNA level by quantitative RT-PCR. Gene expression was validated in PDGFRα+ NPFs, PDGFRα+ CAFs and tumor cell samples isolated by cell sorting. Cell lines were generated to functionally study these cells.

Subcutaneous and orthotopic allografts

Immunodeficient NU-*Foxn1*^{nu} mice (females, 5-weeks-old) were purchased from Harlan Laboratories. Tumor (0.5x10^{6}) cells only or in combination with (0.5x10^{6}) CAFs or NPFs

were injected in PBS:Matrigel (1:1) into dorsal flanks of the mice. Growth was measured every 3 days until humane end point. Orthotopic injection was performed by surgery under anesthesia (4% Isofluorane) utilizing the same number of cells for injection as in the subcutaneous model. Tumor growth was monitored by micro-ultrasound. Mice were sacrificed 3-weeks-post-injection.

GEMMs generated

Therapeutic strain: K-*Ras*$^{+/FSFG12V}$;*Trp53*$^{frt/frt}$;Elas-tTA/tetO-FLp(o);*Egfr*$^{lox/lox}$;*c-Raf*$^{lox/lox}$;Ub-CreERT2 PDAC mouse strain was generated by crossing these alleles in our laboratory. This model takes advantage of a dual recombinase system (Cre/LoxP and Flp/Frt) which allows temporal and spatial separation of tumor development and target elimination.

Saa3 germline knockout mice: the *Saa3 null* allele was generated by homologous recombination using a BAC vector, where a LacZ gene cassette and a Neomycin resistance cassette at the ATG transcription start site replaces the entire protein coding sequence. *Saa3 null* mouse sperm was used for in vitro fertilization of KPeCY females.

CRISPR germline knockout mice: the CRISPR/Cas9 system is an efficient gene-editing technology. This method allows rapid generation of multiple knockout models by injection of mRNA of the Cas9 protein and the guide RNA of the target genes. sgRNA sequences were validated in vitro for the selected targets. sgRNA and Cas9 mRNA were microinjected into the cytoplasm of the one-cell state embryos derived from females of the therapeutic strain. Next, the mouse embryos were transferred into foster mothers and chimeras were born. Genotyping strategy was set to select and validate knockout mice.

Human samples

Primary tumors were obtained from the Tumor Bank of the Hospital ‘Virgen de la Arrixaca’ Murcia, Spain. Specific informed consent for tumor implantation in mice was obtained from all patients, and the study received the approval of the CNIO Ethics Committee.

DKFZ human PDAC dataset: a different set of primary tumors were used to isolate cell populations by cell sorting were obtained from PDAC patients at the University Hospital Heidelberg, Germany in collaboration with the German Cancer Research Center (DKFZ).

Objectives

Therefore, the following objectives were set for this thesis work:

1. Comparison of the gene expression profiles of cancer associated fibroblasts (CAFs) in PDAC to normal pancreatic fibroblasts (NPFs) to find specific targets that can help to reprogram the CAFs to counteract their pro-tumorigenic properties.
2. Functional validation of the pro-tumorigenic properties of the selected targets.
3. Study the role of the selected targets in vivo in PDAC development through the incorporation of germline knock-out alleles to the PDAC mouse model.

Hypothesis

We hypothesized that reprogramming of CAFs to become phenotypically closer to normal pancreatic fibroblast (NPF) characteristics, would restrain cancer progression and would not have the negative effects of stroma elimination.

Results

Gene expression profiling of CAFs

To investigate our hypothesis, we isolated CAFs and NPFs by two different methods from KPeCY PDAC mouse model, by outgrowth and by cell sorting, and compared their expression profile in order the better understand their role in tumor development. Differential expression study identified 117 commonly upregulated genes in the two CAF populations. Nevertheless, among the most upregulated genes we found several of them belonging to the acute-phase response proteins, where *Serum amyloid A3* presented the highest expression levels. Moreover, GSEA analysis displayed numerous pathways shared by these two CAF populations including Complement Cascade as the most significantly upregulated one, as well as Cytokine-Receptor Interaction and Innate Immune Response related pathways, such as NFκB. However, it has to be taken into consideration that CAFs isolated by outgrowth method do not represent a pure fibroblast population and may undergo gene expression alterations affected by culture conditions. This can explain the differences between the differential expression analysis of CAFs.

Hence, we decided to further characterize PDGFRα+ subpopulation of CAFs to avoid contamination by other cell types and to study a more physiological scenario of freshly isolated CAFs.

Pro-tumorigenic CAFs and anti-tumorigenic NPFs

To determine whether PDGFRα+ CAFs can promote tumorigenesis, we compared their tumor supporting capabilities with those of NPFs using *in vivo* assays. EYFP+ sorted PDAC tumor cells ($0.5x10^6$) isolated from tumor-bearing KPeCY mice were subcutaneously inoculated alone or in combination with CAFs ($0.5x10^6$) or NPFs ($0.5x10^6$) into the flanks of immunocompromised mice. Whereas CAFs stimulated tumor growth by as much as 75%, NPFs inhibited the proliferation of the pancreatic tumor cells. These results illustrate the pro-tumorigenic activity of the PDGFRα+ subpopulation of CAFs used in this study.

Target selection and *in vitro* validation

We selected and validated candidate genes in PDGFRα+ CAFs and NPFs obtained from our gene expression profiling studies, that fulfilled our criteria. Among those, druggability, significant overexpression in CAFs but no or low expression in NPFs; functional relevance in PDAC development, as well as in human disease were the more

important for selection. Validation of overexpression of candidate genes in CAFs by qPCR narrowed down our list, which finally resulted in the selection of several genes: *Serum Amyloid A 3* (*Saa3), Hyaluronan Synthase 1 (Has1), Lumican (Lum), Haptoglobin (Hp)* and *Mesothelin (Msln)*. Saa3 and Hp are acute-phase response inflammatory proteins associated to chronic inflammation. Lum and Has1 have important role in fibrosis and ECM remodeling, the latter is responsible for producing hyaluronic acid, the matrix component that defines structure and physical properties of the stroma (25). Whereas, Mesothelin is a tumor antigen and is proposed as a reliable marker of pancreatic cancer (26), however its function has not been addressed properly. We hypothesized, that functional studies of these genes would help to better understand to role of CAFs in PDAC development either in immunosuppression or in physically induced therapy resistance.

***In vivo* target validation and generation of knockout mouse models**

shRNA mediated knock down of these targets revealed functional role of the selected genes in tumor stroma crosstalk. We co-injected tumor cells with CAFs, in which expression of either *Saa3, Has1* or *Lum* was downregulated, as well as CAFs infected with control shRNA, subcutaneously in the flanks of immunodeficient mice. Tumor growth monitoring exhibited reduction in tumor size for all the three genes silenced in CAFs compared to CAFs treated with control shRNA, where the highest difference was obtained with CAFs with reduced Saa3 activity.

Therefore, to characterize the role of our top candidate, *Saa3* in PDAC development we generated knockout mice by conventional gene targeting. To investigate to role of other selected target genes we took advantage of the novel and fast gene editing technology, CRISPR/Cas9, and generated knockout mouse models. By this method, it is also possible to induce mutations simultaneously and to efficiently ablate genes at the same time.

We injected guide RNA of *Has1* into one cell state embryos derived from our *"therapeutic strain"*, which resulted in 4 knockout chimeras with the ability to transmit the modification germ line. However, several crosses were utilized in order to finalize a mouse strain for characterization. In addition, multiple mutational events and their identification has to be taken into consideration when using CRISPR/Cas9 system. Development of single mutated *Has1* KO mouse model resulted in 44% efficiency.

Moreover, we challenged the capacity of the system, to generate triple mutant mice to eliminate *Lumican, Haptoglobin* and *Mesothelin* at the same time. Indeed, the three sessions of microinjection resulted in 25 chimeras, from which 3 were single-, 5 double-

and 2 triple-mutant mice that were able to transmit the modification to the next generation. Future studies will address the functional role of these genes in PDAC development.

PDAC development in Saa3 germline knockout mice

Germline elimination of *Saa3* had no significant effect on overall PDAC development as reflected by the lack of survival benefit. However, *Saa3 null* tumors exhibit stroma remodeling including reduced fibrosis and ECM, infiltrating macrophages and increased vessel density. Collective reduction of the physical barrier and improved vascularization of the tumors suggested that drugs could reach easier and could act more efficiently against tumor cells. Even though we observed marginal improvements in therapeutic efficacy with combination treatments of standard of care Gemcitabine and stroma targeting agents, tumors remained less responsive presenting additional evidence that vascularization and tumor stiffness are not the only players in therapy resistance of PDAC.

Complete ablation of Saa3 protein reduces liver metastasis incidence

On the other hand, these structural changes in the tumor composition probably also provided a suitable environment for tumor cells to escape from primary tumor sites. In addition, *Saa3 null* tumors were less differentiated and more invasive, as suggested by a higher proliferation index and increased numbers of pancreatic cancer stem cells Moreover, *Saa3 null* tumor cells displayed enhanced migratory phenotype. We observed an unexpected abundance of *Saa3 null* tumor cells in the liver during the early stages of pancreatic tumor development, constituting as much as 15% of all liver cells. Yet, these cells were not able to form metastasis showing the important role of Saa family in the process of metastatic spreading.

CAFs support tumor growth via Saa3/Mpp6 axis in pancreatic cancer mouse model

Orthotopic co-injection of *Saa3 null* CAFs with *Saa3* competent tumor cells in the pancreas of *nude* mice significantly reduced tumor size. The inhibitory effect of *Saa3 null* CAFs was even more pronounced than that induced by NPFs. These results were also observed *in vitro* using tumor organoids, ruling out a putative role of a third cellular partner in this cross-talk. These reconstruction experiments recapitulate the results obtained with *Saa3 null* mice in which both CAFs and tumor cells are devoid of *Saa3*. Thus, explaining why PDAC development is unaffected in *Saa3 null* mice.

Comparative analysis of *Saa3* competent and *Saa3 null* CAFs revealed minor changes in their transcriptome. Yet, we identified three overexpressed genes in *Saa3* deficient CAFs.

Of particular interest was *Mpp6*, a member of the peripheral MAGUK family of proteins primarily involved in controlling epithelial cell polarity. Mpps also function in tumor suppression and receptor clustering by forming multiprotein complexes containing distinct sets of transmembrane, cytoskeletal, and cytoplasmic signaling proteins.

Interestingly, *Mpp6* overexpression appears to be responsible for the loss of pro-tumorigenic effect of *Saa3 null* CAFs. Indeed, knock down of *Mpp6* expression in these mutant CAFs restored their pro-tumorigenic properties as determined in co-injection studies with *Saa3* proficient pancreatic tumor cells in *nude* mice. Importantly, the expression levels of *Mpp6* were unaffected by the presence or absence of Saa3 in tumor cells, suggesting that the functional relationship between Saa3 and Mpp6 might be limited to CAFs. Understanding the molecular pathways implicated in the inhibitory role of Mpp6 on the pro-tumorigenic effect of CAFs should unveil novel therapeutic opportunities.

Most relevant features of *Saa3* competent and *Saa3 null* KPeCY mice

Features of KPCY mice	***Saa3 WT***	***Saa3 KO***
Stroma reorganization		
• ECM	+++	+
• Vascularization	+	++++
• Macrophage number and infiltration	+	++++
• Other immune cell infiltration	+	+
CAFs		
• Tumor growth support (nude mice assays)	+++	–
• Wound healing property (*in vitro*)	++	+++
Tumor cells		
• Differentiation	++	+
• Cancer stem cells (CSCs)	+	+++
• Migratory properties (*in vitro*)	+	++++
Liver metastasis		
• Macro-metastasis	++	–
• Micro-metastasis	++	+
• Migratory tumor cells	+	++++

SAA1 in human PDAC

To validate our findings in human disease, we isolated hCAFs and hNPFs from PDAC patient samples and adjacent normal tissues by outgrowth method. We compared their expression profile by RNAseq and verified significant enrichment between human and mouse CAF expression profiles. Indeed, the most upregulated gene sets were Cytokine-

Receptor Interaction and Complement Cascade pathways in hCAFs compared to hNPFs, similar to our observation in mouse CAFs.

We have interrogated whether our observations of *Saa3* in GEM PDAC tumor models could be translated to the human scenario. *SAA3* locus is a non-expressed pseudogene. On the other hand, the acute-phase SAA1 protein has structural and functional characteristics that closely resemble those of murine Saa3, suggesting that SAA1 and Saa3 could be orthologue proteins.

Indeed, *SAA1* is overexpressed in human CAFs compared to NPFs. Moreover, high levels of *SAA1* expression in the stromal component of human PDAC tumors correlate with significantly worse survival regardless of whether the tumor samples contain "normal" or "activated" stroma. However, high *SAA1* expression in tumor samples with low stroma content also correlate with slightly increased survival, suggesting that SAA1 may have a pro-tumorigenic effect when is highly expressed in stroma, but a possible anti-tumorigenic effect when overexpressed in tumor cells. Such dual role for SAA proteins depending on cellular context has already been reported by other investigators.

Finally, *MPP6* levels inversely correlated with *SAA1* expression in human PDAC samples. Therefore, our results support the concept that murine Saa3 may serve as a model to study the role of the human acute-phase SAA1 protein in pancreatic cancer and to develop novel therapeutic strategies against this potential target.

Conclusions

1. Cancer Associated Fibroblasts (CAFs) represent a heterogeneous population of fibroblasts in PDAC. PDGFRα+ subpopulation of CAFs display strong inflammatory signature with particular emphasis on innate immune response, cytokine and competent cascade signaling compared to PDGFRα+ normal pancreatic fibroblasts (NPFs).
2. PDGFRα+ CAFs are pro-tumorigenic, while PDGFRα+ NPFs are tumor inhibitory when co-injected subcutaneously with mouse pancreatic tumor cells into nude mice and in co-culture *in vitro* studies with organoids.
3. Comparative transcriptional profiling of PDGFRα+ CAFs identified *Saa3* as the top upregulated gene and shRNA mediated functional validation qualified it as a potential target in CAFs.
4. Other candidate genes, like *Haptoglobin, Lumican, Has1* and *Mesothelin* were chosen for *in vivo* studies since they fulfill our criteria of target selection: overexpression in CAFs, low expression in NPFs, functional and human relevance in PDAC. Knock-out alleles of these gene have been incorporated in the therapeutic PDAC strain by CRISPR/Cas9 gene editing strategy: single *Has1* knockout and the triple combination of *Haptoglobin, Lumican* and *Mesothelin*.
5. Germline elimination of *Saa3* in a PDAC mouse strain does not seem to affect tumor development, as reflected by the same number and type of PanIN lesions present induced by the *K-Ras* oncogene in the context of *p53* activity and by the lack of significant survival difference in mice that develop PDAC in a *p53* deficient background. However, tumors lacking *Saa3* exhibit an undifferentiated phenotype.
6. *Saa3* elimination induces stroma remodeling, namely: extracellular matrix reorganization, vessel density increase and macrophage infiltration. These stromal changes do not result in a significant therapeutic benefit when tumor-bearing mice are treated with the standard of care Gemcitabine alone or in combination with the macrophage depleting agent Clodronate, or VEGF blocking antibody.
7. Saa3 is a mediator of the pro-tumorigenic properties of PDGFRα+ CAFs as shown by significant decrease in tumor growth when *Saa3 null* CAFs and *Saa3*

expressing tumor cells are co-injected orthotopically in the pancreas of nude mice. Same results were observed in co-culture with organoids *in vitro*. However, anti-tumorigenic effect of *Saa3 null* CAFs is lost when tumor cells also lack *Saa3* expression.

8. Anti-tumorigenic properties of *Saa3 null* CAFs are mediated by the upregulation of the tight junction protein membrane-associated guanylate kinases family member 6 (Mpp6) as revealed by the transcriptional profiling of *Saa3 null* and competent CAFs and validated by orthotopic tumor reconstruction experiments. Mpp6 silencing in *Saa3 null* CAFs restored their tumor growth supporting properties.
9. *Saa3 null* tumor cells displayed migratory phenotype *in vitro* and *in vivo*, as illustrated by the high number of disseminated tumor cells in the liver of the PDAC mouse strain. Nonetheless, these tumor cells were unable to proliferate and form metastatic outgrowth.
10. SAA1, the human orthologue of mouse Saa3, is overexpressed in PDAC samples and in human CAFs. Moreover, this overexpression correlates with significantly worse survival when pancreatic stroma signatures are transcriptionally separated. SAA1-MPP6 expression negatively correlates in human CAFs. Therefore, our PDAC mouse model can help to study therapeutic opportunities that could be translated to patients.

Despite all the efforts invested in preclinical and clinical studies to find efficient treatment PDAC remains an incurable disease with slowly progressing therapeutic advances. Therefore, it is very important to search for novel strategies based on studies focusing on PDAC pathobiology that could help to design therapeutic approaches.

Altogether, this study gives new insights into CAF biology and could provide important therapeutic implications in the treatment of pancreatic cancer.

Publications

Publications related to this work:

Djurec M, Graña O, Lee A, Troulé K, Espinet E, Cabras L, Navas C, Blasco MT, Martín-Díaz L, Burdiel M, Li J, Liu Z, Vallespinós M, Sanchez-Bueno F, Sprick MR, Trumpp A, Sainz Jr B, Al-Shahrour F, Rabadan R, Guerra C, Barbacid M. (2018) Saa3 is a key mediator of the protumorigenic properties of cancer-associated fibroblasts in pancreatic tumors. *Proc Natl Acad Sci* 115(6):E1147–E1156.

Publications not related to this work:

Sanclemente M, Francoz S, Esteban-Burgos L, Bousquet-Mur E, **Djurec M**, Lopez-Casa P, Hidalgo M, Guerra C, Drosten M, Musteanu M, Barbacid M. (2018) c-RAF Ablation Induces Regression of Advanced Kras/Trp53 Mutant Lung Adenocarcinomas by a Mechanism Independent of MAPK Signaling. *Cancer Cell* 33(2):217–228.e4.