

Video Article

Studying Pancreatic Cancer Stem Cell Characteristics for Developing New Treatment Strategies

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Keywords: Medicine, Issue 100, Pancreatic ductal adenocarcinoma, cancer stem cells, spheres, metformin (met), metabolism

Date Published: 6/20/2015

Citation: Lonardo, E., Cioffi, M., Sancho, P., Cruz, S., Heeschen, C. Studying Pancreatic Cancer Stem Cell Characteristics for Developing New Treatment Strategies. *J. Vis. Exp.* (100), e52801, doi:10.3791/52801 (2015).

Abstract

Pancreatic ductal adenocarcinoma (PDAC) contains a subset of exclusively tumorigenic cancer stem cells (CSCs) which have been shown to drive tumor initiation, metastasis and resistance to radio- and chemotherapy. Here we describe a specific methodology for culturing primary human pancreatic CSCs as tumor spheres in anchorage-independent conditions. Cells are grown in serum-free, non-adherent conditions in order to enrich for CSCs while their more differentiated progenies do not survive and proliferate during the initial phase following seeding of single cells. This assay can be used to estimate the percentage of CSCs present in a population of tumor cells. Both size (which can range from 35 to 250 micrometers) and number of tumor spheres formed represents CSC activity harbored in either bulk populations of cultured cancer cells or freshly harvested and digested tumors^{1,2}. Using this assay, we recently found that metformin selectively ablates pancreatic CSCs; a finding that was subsequently further corroborated by demonstrating diminished expression of pluripotency-associated genes/surface markers and reduced *in vivo* tumorigenicity of metformin-treated cells. As the final step for preclinical development we treated mice bearing established tumors with metformin and found significantly prolonged survival. Clinical studies testing the use of metformin in patients with PDAC are currently underway (e.g., NCT01210911, NCT01167738, and NCT01488552). Mechanistically, we found that metformin induces a fatal energy crisis in CSCs by enhancing reactive oxygen species (ROS) production and reducing mitochondrial transmembrane potential. In contrast, non-CSCs were not eliminated by metformin treatment, but rather underwent reversible cell cycle arrest. Therefore, our study serves as a successful example for the potential of *in vitro* sphere formation as a screening tool to identify compounds that potentially target CSCs, but this technique will require further *in vitro* and *in vivo* validation to eliminate false discoveries.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52801/>

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid tumours. It is currently the 4th most common cancer-related death in Western society and is predicted to rise to the 2nd most frequent cause within the next decade (~400,000 deaths per year worldwide)³. At time of diagnosis 90% of patients present with advanced disease, which has a 5-year survival of less than 5%. This survival rate has disappointingly remained unchanged in the past 50 years despite increasingly intensive research activities⁴. Of the remaining 10% of patients who have potentially 'curable' disease through surgical resection, 80% will die from recurrence within 5 years. For many years the standard of care for advanced disease has been gemcitabine monotherapy, but this only confers a marginal survival advantage⁵. Small improvements in short-term survival have been achieved by the addition of erlotinib⁶ or capecitabine⁷, however the survival benefit is in the order of weeks with the median overall survival still ~6 months. Recently, more encouraging results have emerged for gemcitabine/*nab*-paclitaxel⁸ and the *FOLFIRINOX* combination regime^{9,10}. These therapies improve median survival by a modest 2 and 4 months respectively, but are highly toxic and long-term survivors are still a rare exception. Although treatment offers the potential for improvement, these are toxic regimes to which many patients do not respond or only show incremental improvement in overall survival. As a consequence, there is an urgent need to supplement current therapies and to develop novel, most likely multimodal therapeutic approaches.

Tumor Heterogeneity

It is becoming increasingly evident that cancer heterogeneity is not only confined to distinct evolutionary subclones within each tumor¹¹, but also driven by phenotypic and functional heterogeneity and plasticity within each subclone¹². So-called cancer stem cells (CSCs) or tumor-promoting cells are responsible for intracлонаl heterogeneity¹³⁻¹⁶. Specifically, CSCs represent a subset of cancer cell, for which we and others have provided conclusive evidence, down to the single cell, that they represent the root of disease by giving rise to all differentiated progenies within each cancer subclone¹⁷. More importantly, these cells are essential for metastatic behavior and also represent an important source for disease relapse following treatment, even with relatively effective drugs capable of inducing initial tumor regression (e.g., *nab*-paclitaxel)^{15,18-20}. It is important to note that CSCs do not necessarily represent *bona fide* stem cells, nor do they arise from tissue stem cells in many instances, but

rather they have acquired certain features of stem cells. Most of these are functionally defined, for example CSCs are equipped with indefinite self-renewal capacity making them resistant to conventional chemotherapy, and show increased invasiveness which promotes metastatic activity.

Functional Cancer Stem Cell Phenotypes

The functional phenotype of CSCs is based on their ability to self-renew, which can be tested *in vitro* using serial sphere formation and colony formation assays respectively. Even more importantly, CSCs capable of self-renewal bear *in vivo* tumorigenicity which can be tested by limiting dilution *in vivo* assays as the ultimate functional readout, preferably during serial transplantation indicative of exclusive long-term tumorigenicity. Moreover, there is heterogeneity within the CSC compartment, with a distinct subpopulation of CSCs bearing the exclusive ability to give rise to metastases that is not just a direct consequence of their exclusive *in vivo* tumorigenicity. Indeed, metastatic CSCs acquire the ability to evade the primary tumor, survive anoikis and eventually translocate and seed secondary sites. These advanced functional abilities can be tested *in vitro* using modified invasion assays and *in vivo* using metastasis assays.

Targeting Cancer Stem Cells

We and others have provided convincing evidence that treatments focusing on the bulk tumor of differentiated PDAC cells, even in combination with stroma-targeting agents, do not have a major impact on tumor progression and subsequent outcome unless combined with a CSC-targeting strategy^{21,22}. Thus, based on the crucial functions of CSCs in disease progression and resistance to therapy, these cells should signify an essential component for any novel treatment approach^{18,20}, but will require a much more thorough understanding of the regulatory machinery of CSCs. Although CSCs and their more differentiated progenies bear identical genetic ground states with respect to genetic alterations, CSCs exhibit distinct and thus epigenetically determined gene expression profiles that share modules with pluripotent stem cells. Most of the genes involved in generating induced pluripotent stem cells (Nanog, Oct3/4, Klf4, Sox2) have not only been linked to cancer, but their expression is mostly restricted to the CSCs compartment. Moreover, the functional relevance of CSCs by loss-of-function experiments using genetic tools for targeting CSCs have now firmly established the CSC concept for several cancer types²³⁻²⁵. While most of these approaches are based on mouse models and thus are not easily transferable into the clinic, they do provide proof-of-concept for the potential clinical relevance of targeting CSCs in combination with bulk tumor cells.

Studying Cancer Stem Cells *In Vitro* to Identify Their Achilles' Heel

In order to identify new and clinically applicable ways for targeting CSCs, their features are regularly studied *in vitro* and sphere formation is widely used in this context. Originally developed for studying normal stem cell biology, including self-renewal and differentiation capacity, this assay was later adapted to study CSCs *in vitro* and has been used for investigating CSCs isolated from PDAC²⁰. We have found that tumor spheres formed from primary human PDAC cells bear all the distinct features of CSCs, therefore indicating they contain *bona fide* pancreatic CSCs²¹. Thus, the tumor sphere assay represents a powerful tool to screen for more effective therapies *in vitro*, but results need to be further evaluated in more stringent *in vivo* assays. Indeed, data generated with this *in vitro* assay should be treated with great caution as the assay can be subject to significant error. Highly standardized protocols, including automated counting of formed spheres, should be established to ensure reproducible and predictive data.

In this context, we recently used this assay to screen pancreatic CSCs derived from a diverse set of primary human PDACs and showed that these cells are highly vulnerable to metabolic reprogramming by anti-diabetic compound metformin. Previously, metformin had been demonstrated to inhibit cancer cell expansion by indirect activation of AMP-activated protein kinase (AMPK) signaling and subsequent inhibition of mTOR²⁶, resulting in reduced protein synthesis and cell proliferation²⁷. In addition to these effects on the bulk tumor population, we and others have found that metformin is able to target and actually eliminate the CSCs subpopulation in a number of solid tumors such as breast, esophageal cancer, glioblastoma and pancreatic cancer²⁸⁻³¹. Thus, metformin represents a promising and safe new therapeutic strategy for several cancers with currently unmet medical need. Moreover, using sphere formation as a way to enrich for CSCs, we showed that metformin's primary effects on pancreatic CSCs was independent of AMPK activation and mostly relied on its modest mitochondrial toxicity (via inhibition of complex I), which apparently was lethal for the subset of CSCs only. For the latter we were able to assess their cellular oxygen consumption and mitochondrial ROS production as indicators of the drug's toxicity at the cellular level. Subsequently, these *in vitro* data could be validated in preclinical mouse models and indeed resulted in significantly prolonged survival³¹. The methodology presented herein allows for the rapid generation of drug sensitivity profiles for CSCs, including studies on their effects on CSC metabolism. We now provide extended experimental details about the utilized complementary *in vitro* and *in vivo* procedures.

Protocol

Human PDAC tumors were obtained with written informed consent (Comunidad de Madrid, Spain (C.P. CNIO-CTC-11 - C.I. 11/103-E). Implantation of human pancreatic tumors into immunodeficient mice requires the approval of Institutional Review Board as well as Institutional Animal Care and Use Committee (IACUC). The procedures of xenograft pancreatic tumor mouse models must be conducted in accordance with the institutional and national regulations (here Ethics Committee of the Instituto de Salud Carlos III; Madrid, Spain; Protocol PA 34_2012).

1. Culture Media

1. Prepare Complete Medium.
 1. Supplement RPMI medium with 10% FBS, 100 units/ml penicillin/streptomycin. For 500 ml of RPMI add 55 ml of heat-inactivated FBS and 6 ml of penicillin/streptomycin (10,000 U/ml, 100X).
 2. Store the complete medium at 4 °C.
2. Prepare CSCs Medium.
 1. Supplement the serum-free DMEM/F12 medium with 100 units/ml penicillin/streptomycin and 2 mM glutamine.
 2. Store the CSC medium at 4 °C. B27 (1:50) and 20 ng/ml bFGF are freshly added to the medium before each experiment.

NOTE: The addition of B27 has been shown to increase tumor sphere formation and to sustain several passages of sphere cultures³². The medium composition is a crucial step and has to be tested for each cell type in culture. We recommend testing self-renewal and differentiation capacity of spheres and analyze expression of CSC markers by flow cytometry (*i.e.*, CD133+ and CD44+).

3. Prepare Extracellular Flux Assay Medium.
 1. This specific medium is basal DMEM 5030 containing vitamins, aminoacids and other supplements, but lacking glutamine, glucose, pyruvate and bicarbonate.
 2. For the current assay, supplement the medium with 2 mM glutamine, 8 mM glucose and 2 mM sodium pyruvate to a full mitochondrial metabolic activity.

NOTE: The absence of sodium bicarbonate is essential in order to accurately measure extracellular acidification of cells growing in culture, since regular medium containing bicarbonate will buffer pH variations during the assay. In addition, the use of a basal medium allows a tight control of the concentration of L-glutamine (as L-alanyl-glutamine) glucose, or sodium pyruvate necessary for different assay conditions and/or applications.

2. Sphere Forming Assay and Analysis

NOTE: All tissue culture protocols and manipulations must be performed using sterile techniques with great attention using clean, detergent-free, sterile glassware. Before use, pre-warm all medium and solution in a 37 °C water-bath (as an alternative, you can use medium and solutions pre-warmed at RT). Obtain Human PDAC tumors as previously detailed²¹.

1. Isolation of CSCs from Human PDAC (**Figure 1**)
 1. In a sterile biosafety cabinet transfer the human PDAC tissue to a 60 x 15 mm culture dish containing 1 ml of sterile 1X phosphate buffered saline (PBS). Mince the tumor into small pieces (1 - 5 mm) using a sterile scalpel and forceps.
 2. Add 1 ml of sterile 1X PBS to the culture dish and repeat the trituration step until the tissue is completely dissociated, regularly this requires 3-4 rounds. Transfer the tissue suspension (top up to a final volume of 5 ml with 1X PBS) into a sterile tube and mechanically homogenize it with a dissociator such as gentleMACS.
 3. Incubate the homogenized tissue with collagenase (use 2.5 mg/ml of collagenase in 1X PBS) for 60 min at 37 °C and then centrifuge for 5 min at 900 x g. Decant the supernatant and resuspend the cell pellets in 10 ml of complete medium. Filter the cell suspension through a 40 µm strainer and centrifuge for 5 min at 900 x g.
 4. Decant the supernatant and resuspend the pellet with 5 ml of red blood cell lysis buffer (ammonium-chloride-potassium, ACK), and incubate the
 5. Resuspend the pellet in CSCs medium, plate on a gelatin-coated dish, and incubate at 37 °C for 1 h. This step will remove most of the fibroblast cells that quickly attach to the plate.
 6. Remove the plate from the incubator and carefully recover cell suspension and quantify the number of viable (trypan blue-negative) cells using a hemocytometer. For this purpose, gently mix the suspension and pipette 20 µl of the suspension into an Eppendorf tube. Add 20 µl (1:1 ratio) of trypan blue to the cells in the microcentrifuge tube and mix well. Pipette approximately 10 µl of the mixture onto the hemocytometer.
 7. Count all clear cells within the four corner quadrants of the counting chamber for viable cell count. Note: The viability and yield of cells may vary considerably between tumor specimens. For PDAC samples viability regularly ranges between 45-70%, and tissue pieces from a macroscopic tumor sample with a diameter of 3 - 5 mm should yield to approximately 5x10⁶ viable cells.
2. Sphere Formation Assay and Metformin Treatment
 1. Take the required number of cells and add the appropriate volume of CSCs medium to prepare a cell concentration of 2,000 cells/ml. Do not keep the cell suspension on ice for no longer than 1h and mixed well prior to plating.
 2. Add 500 µl of 1X PBS to the first and last row of a 24-well plate for humidification in order to help minimize medium evaporation. Seed the cells into ultra-low attachment cells plates at a density of 2,000 cells per well in 1 ml of tumor sphere medium (2,000 cells/ml).

NOTE: The number of cells used for tumor sphere formation assays may vary between tumor types.
 3. Treat at least 4 wells with vehicle (negative control) and 4 wells with each allocated treatment, *e.g.*, 3 mM of metformin. Place the cells in an incubator set to 37 °C and supply the cells with 5% CO₂ for one week.

NOTE: The medium should not be changed in order to allow the undisturbed formation of tumor spheres, but can be topped up with growth factors on a daily basis as they are not stable in culture medium.
 4. Every other day add treatment, *e.g.*, 3 mM of metformin or vehicle to each of the wells. Assess the number of formed tumor spheres after 5 and/or 7 days by the use of an automated cell counter that allows for counting of larger structures (**Figure 2**).

NOTE: Only tumor spheres with at least 40 µm should be considered and can be categorized as small (40 - 80 µm) or large (80 - 120 µm), respectively. The results can be illustrated as the percentage of tumor spheres divided by the initial number of cells seeded (*e.g.*, 2,000 cells).
3. Serial Passaging of First Generation Tumor Spheres
 1. After 7 days of incubation harvest the tumor spheres using a 40 µm cell strainer and centrifuge them for 5 min at 900 x g at RT.
 2. Dissociate the pellet of tumor spheres to single cells using trypsin, and then expand the obtained single cell cells suspension again for another 7 days as described above (see 2.2).

3. Metabolism Analysis (ROS Production and Oxygen Consumption)

1. ROS Production
 1. In this protocol, we have used carboxy-DCFDA as an example to measure ROS production since it is an affordable, fast and widely used probe for ROS detection. However, there are several other probes and methodologies that can be used for this assay.

2. Treat tumor spheres with metformin as described in 2.2 for the allocated amount of time. At the end of treatment, harvest tumor spheres using a 40 μm cell strainer, centrifuge the cells at 900 x g for 5 min and resuspend the pellet in 1 ml of trypsin. Incubate for 20 min at 37 °C.
 3. Once cells are singularized, centrifuge at 900 x g and resuspend the cells in HBSS containing 2.5 μM carboxy-DCFDA. Incubate the cells for 20 min at 37 °C in the dark.
 4. Prior to flow cytometry analysis protect stained cells from light as carboxy-DCFDA can be photo-oxidized giving false positive results. Place tubes on ice until analysis.
NOTE: carboxy-DCFDA is fluorescent after reaction with a variety of reactive oxygen and nitrogen species, being detected in FL1 (ex. 488nm, em. 520nm).
2. Oxygen Consumption Measurement
1. For this protocol, we will use the Extracellular Flux Analyzer system from Seahorse Biosciences, as an example. Coat the desired cell culture plate with a solution of 22.4 $\mu\text{g}/\text{ml}$ of cell and tissue adhesive for 20 min at RT. Rinse the wells with water 2 - 3 times prior to seeding the cells.
 2. At the end of treatment, harvest tumor spheres using a 40 μm cell strainer, centrifuge the cells at 900 x g for 5 min and resuspend the pellet in 1 ml of trypsin. Incubate for 20 min at 37 °C.
 3. Plate singularized cells in the cell culture plate at a density of 30,000 cells/well for a 96 well plate (final volume: 150 μl). Resuspend the cells in Oxygen consumption assay medium containing 8 mM glucose and 2 mM sodium pyruvate.
 4. Centrifuge the cells to the bottom of the well by spin centrifugation of the wells until 100 x g has been reached and then let the centrifuge stop with brake off. Reverse the orientation of the plate and repeat the process. Transfer the plate to a 37 °C incubator without addition of CO₂ for 30 min.
 5. In the meantime, prepare the cartridge for the assay. Each well has 4 different injectors to load (25 $\mu\text{l}/\text{port}$):
 1. Port A: metformin. To obtain a final concentration of 3 mM in the well, prepare a 8x solution (24 mM) as the final volume will be 175 μl after injection of port A.
 2. Port B: metformin. To add 3 mM and obtain a final concentration of 6 mM in the well, prepare a 9x solution (27 mM) as the final volume will be 200 μl after injection of port B.
 3. Port C: metformin. To add 3 mM and obtain a final concentration of 9 mM in the well, prepare a 10x solution (30 mM) as the final volume will be 225 μl after injection of port C.
 4. Port D: rotenone 11 μM , to obtain 1 μM as final concentration in the well (11x). Note: rotenone completely inhibits any residual mitochondrial activity.
 6. Calibrate the cartridge and load the cell culture plate. Perform the assay with the standard protocol of mixing and measurement.
 7. Calculate the percentage of inhibition of the total mitochondrial respiration achieved by metformin as the percentage of inhibition obtained with rotenone (set as 100%).

4. Xenograft Model for Human Pancreatic Cancer in Immunocompromised Mice

NOTE: Order sufficient numbers of female athymic nude or other, more immunocompromised mouse models such as NOD-SCID, SCID-Beige, or NSG for the experiments³³. Autoclave all surgical instruments prior to the experiment and allow them to cool down to RT before use. For subcutaneous tumors we used a minimum of 4 mice per condition with one tumor per flank for a total of 8 tumors.

1. Xenograft Transplantation
 1. Prepare tumor pieces from human pancreatic cancer tissue specimens. Transfer the tumor into a Petri dish and dissect the tissue into small pieces (2 mm in diameter, $\sim 8 \text{ mm}^3$) using a scalpel and forceps to hold the tissue. Dip the obtained pieces into gelatinous protein mixture solution kept on ice.
 2. Anesthetize mice using 1 - 3% isoflurane or other inhalative or injectable anesthetics.
NOTE: Before starting any surgical procedure, ensure that the mice have completely lost consciousness by stimulating the abdominal skin or tows with a pair of splinter forceps. Apply eye ointment to prevent dryness.
 3. Once anesthesia has taken effect, wipe the back of the mice with ethanol-containing skin antiseptic. Administer buprenorphine (0.05 mg/kg BW) prior to surgery to ensure post-operative analgesia.
 4. Lift the back skin with forceps, make a 0.7 - 1.0 cm long incision with sterile micro scissors and then bluntly prepare a small subcutaneous pocket, in which a piece of patient-derived pancreatic cancer tissue is inserted ($\sim 8 \text{ mm}^3$).
 5. Close the wound using 1 - 2 skin staples. Remove them after 7 days. Return the mice to their cages and keep them on a heating pad or under a heating lamp until they are fully active again.
 6. Following tumor implantation, monitor the mice at least twice weekly for tumor growth and general signs of arising morbidity such as ruffled fur, hunched posture, and immobility.
 7. Once tumors have reached an average size of 200 mm^3 , mice are randomized to respective treatments. Administer vehicle or metformin daily via i.p. injections (150 mg/kg BW) or via the drinking water (150 mg/kg BW) with comparable treatment effects.
 8. Monitor tumor burden using a caliper and calculate tumor volume once a week (measurement of tumor volume = 1/2 length \times breath \times width).
 9. Sacrifice the mice once tumors have reached 1 cm in diameter or mice start showing any signs of severe pain or illness as specified above.

Representative Results

Metformin Selectively Targets Pancreatic CSCs

We first examined the functional effects of metformin treatment on the *in vitro* self-renewal capacity of CSCs. For this purpose, we conducted sphere formation assays using primary human pancreatic cancer cells isolated from PDAC tissues resected during surgery. We observed that metformin strongly decreased the size of formed spheres (**Figure 1A**). This was most likely by inhibiting the expansion of progenies of CSC, which still represent the bulk of cells found in spheres as they are only enriched for CSCs. Indeed, the longer spheres are cultured without passaging the more extensive the expansion of more differentiated progenies will be. Thus we found metformin to significantly decrease the number of spheres actually formed irrespective of their size, occurring in a dose-dependent manner suggesting strong inhibition of the self-renewal capacity of CSCs (data not shown). We found that metformin at 3 mM significantly decreased the number of spheres formed (**Figure 1B**). In order to more stringently study the long-term effects of metformin on the self-renewal capacity of CSCs, we subsequently passaged the formed primary spheres into secondary and tertiary spheres. Although only primary spheres were treated with metformin, the formation of spheres in the second and third passages was drastically and increasingly reduced, implicating metformin treatment had indeed irreversibly eliminated the majority of CSCs (**Figure 1C**). Thus, our data showed significant treatment effects for metformin on the self-renewal capacity of primary pancreatic CSCs and thus encouraged us to perform further mechanistic as well as *in vivo* studies.

Metformin Inhibits Mitochondrial Oxygen Consumption and Induces ROS Production

Since metformin was shown to act as a mitochondrial poison by partially inhibiting complex I activity, we next examined the acute effects of metformin on cellular oxygen consumption in sphere-derived cells. For this purpose, we selected cells derived from two representative primary PDAC tumors and measured the oxygen consumption over time following sequential injection of 3 mM metformin (three injections) and 1 μ M rotenone (one injection). As shown in **Figure 2A**, metformin injection induced a rapid and dose-dependent decrease in oxygen consumption, which varied considerable between the different tumors. We calculated the residual mitochondrial activity upon metformin treatment by injecting rotenone, a potent complex I inhibitor, which is capable of completely inhibiting mitochondrial oxygen consumption. Sensitivity to metformin in terms of mitochondrial oxygen consumption correlated with its capacity to induce ROS production defined as a displacement of the mean of the population after metformin treatment (**Figure 2B**, left panel), which can be easily quantified (**Figure 2B**, right panel). As expected, the primary cells that were most sensitive to inhibition of oxygen consumption also showed the strongest increase in ROS production upon metformin treatment. Thus, these metabolic *in vitro* experiments demonstrate that metformin targets CSCs by inhibiting their dependence on mitochondrial function, resulting in a subsequent increase in mitochondrial ROS production and eventually induction of apoptosis.

Metformin Stalls PDAC Progression *In Vivo*

We finally studied the effects of metformin *in vivo* using tissue xenografts derived from different patients with PDAC. We observed a significant reduction in tumor progression for metformin-treated mice as compared to the control group, but tumors never completely disappeared (**Figure 3**). Subsequently, during long-term follow-up all tumors eventually relapsed suggesting metformin resistance of cells surviving the early phase of treatment. Nevertheless, metformin treatment, even when applied as single agent, significantly extended survival in all mice.

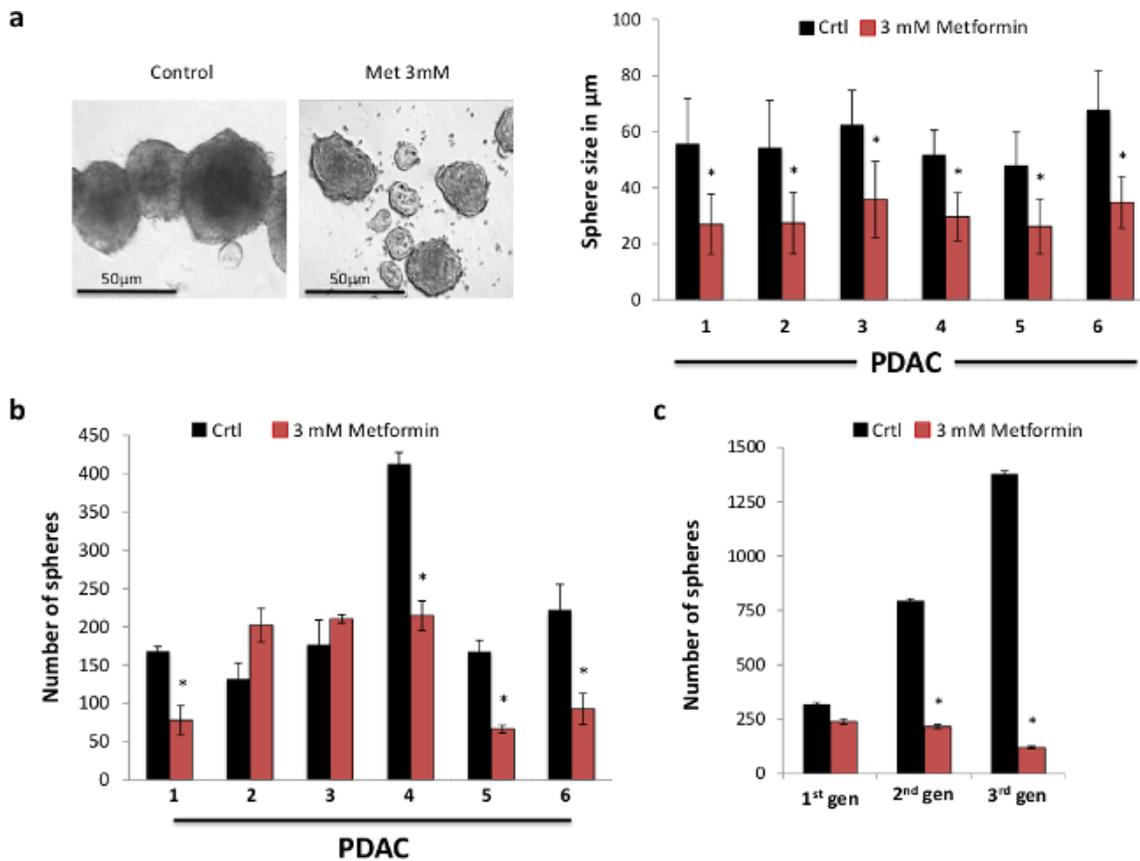


Figure 1. Metformin Selectively Targets the CSCs. (A) Metformin decreased the size of spheres. Representative images of spheres obtained after the treatment with the indicated doses of metformin for 7 days (right panel). Quantification of sphere size ($n \geq 6$) (left panel), the indicated numbers in abscissas axis represent individual tumor. (B) Sphere formation capacity in the presence or absence of metformin for 7 days ($n \geq 6$), the indicated numbers in abscissas axis represent individual tumor. (C) Representative graph of CSC self-renewal capacity in secondary and tertiary spheres of primary pancreatic cancer cells. The spheres were only treated during first generation sphere formation for a total of 7 days ($n = 6$). [Please click here to view a larger version of this figure.](#)

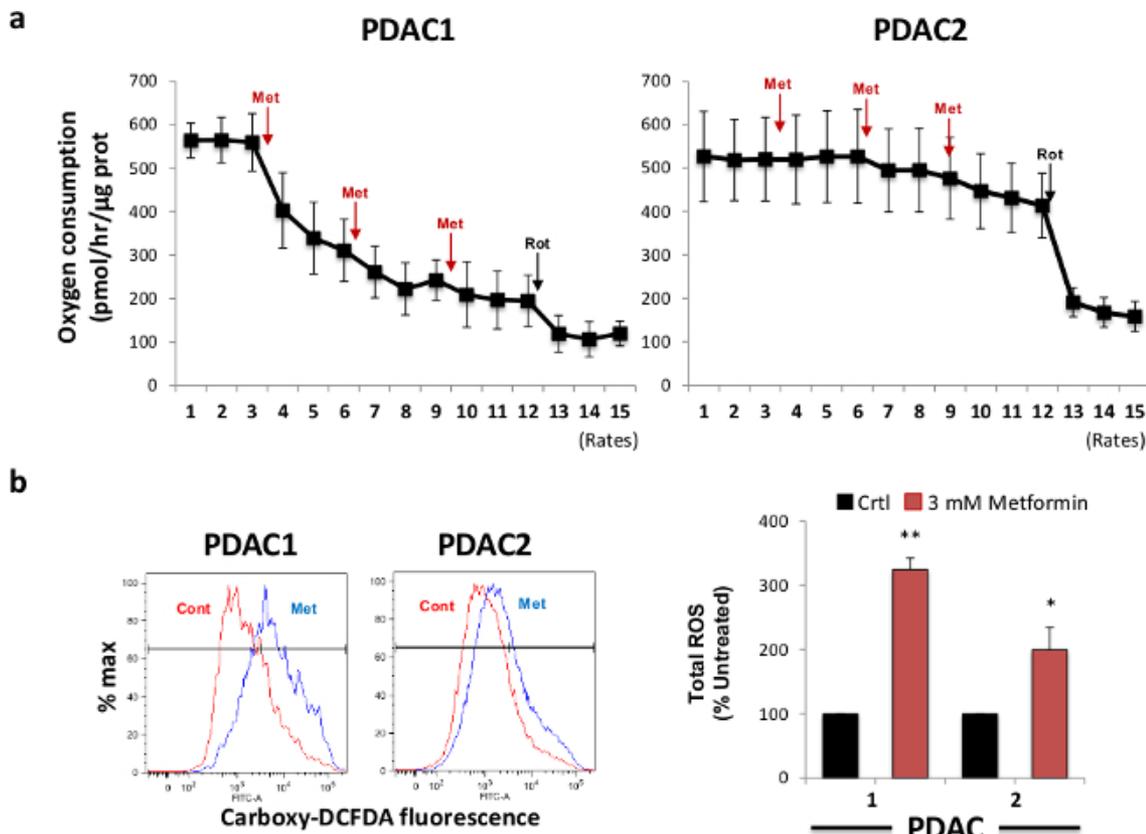


Figure 2. Metformin Treatment Inhibits Oxygen Consumption and Induces ROS Production. (A) Metformin addition inhibits oxygen consumption (OCR) of sphere-derived cells. Two different PDAC secondary spheres were treated with metformin and OCR changes were measured by extracellular flux analysis. Rotenone injection was used as a control for total mitochondrial OCR consumption. X-axis represents the oxygen consumption rate in pmol of oxygen/hr normalized by the protein content in each well. (B) PDAC spheres used in A were treated for 8 hr with metformin and ROS production was assessed by flow cytometry using carboxy-DCFDA. Left, representative flow cytometry plots. Right, summary of data from three independent experiments. [Please click here to view a larger version of this figure.](#)

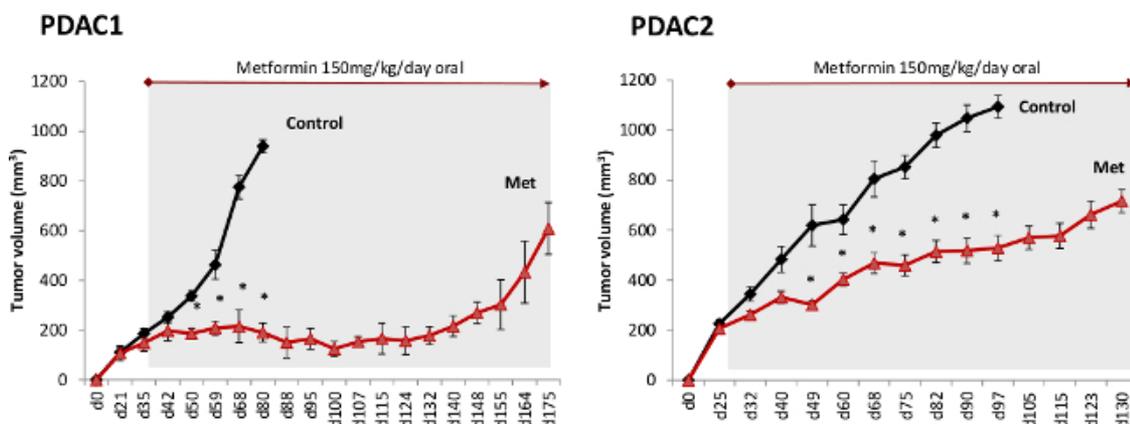


Figure 3. Metformin Stalls PDAC Progression *In Vivo*. Two different PDAC xenograft tissues were implanted into immunocompromised mice and treatment was allocated after initial tumor take was verified. Mice were treated with metformin (Met) added to the drinking water (150 mg/kg body weight; based on 5 ml of water consumption per day). [Please click here to view a larger version of this figure.](#)

Discussion

With the emergence and subsequent validation of the CSC concept for many tumors, the field of drug development has gained new momentum, with the potential for developing more efficient cancer treatments and subsequently reducing risk for disease relapse. However, the CSC field is still at an early state and more needs to be achieved in terms of understanding CSC origin and propagation, and their role in shaping the tumor architecture and promoting metastasis.

In this context, the use of reproducible and meaningful CSC assays as well as the informed interpretation of obtained data represents a crucial component for advancing our understanding of CSC biology. From many biological perspectives, sphere formation has emerged as an extremely

valuable assay; nonetheless users should be aware of its limitations in order to properly interpret their experimental results. An important aspect to consider even before the evaluation of sphere formation data is the origin of the investigated sample, since the results obtained from fresh patient-derived samples could be significantly different from those obtained from established cancer cell lines.

Classic sphere formation assays involve seeding cells at clonal density in ultralow-attachment plates and evaluating sphere formation in the presence of epidermal growth factor (EGF) and/or basic fibroblast growth factor (b-FGF) enriched, but serum-free medium^{21,34}. The culture medium composition is also an important issue to consider. In our experience we found that the DMEM/F12 supplemented with B27, bFGF and glutamine in the absence of serum was an optimal medium to grow, expand and maintain the CSC in undifferentiated conditions. We found that in this culture condition (medium and suspension) the cells express high amounts of cancer stem cell markers (including CD133+, CD44+ and CD24+) and do not express differentiation-associated proteins (CK-20 and CK-19).

One of the fundamental assumptions for these assays is that each sphere is clonal, *i.e.*, one sphere results from the expansion of a single stem cell. However, spheres are intrinsically dynamic structures that are prone to fuse even at low seeding density³⁵. Plating cells is a critical step in the protocol and the density of one cell/well can certainly circumvent the aggregation issue. But even for prospectively sorted progenitors, this regularly results in very low sphere formation due to low intrinsic sphere formation activity and can also be attributed to limited autocrine stimulation due to dilution effects. In our experience we observed that only 30% of cells plated are able to form spheres. We recommend using at least 100 cells/well to obtain consistent and reproducible results.

Other culture methods to growth CSC are based on solid or semi-solid 3D cultures (*e.g.*, based on matrigel, collagen or methylcellulose). These methods have been used to limit cell mobility and subsequent cell aggregation³⁶. However, each of these matrices bears their own limitations. For instance, matrigel is a soluble basement membrane isolated from the Engelbreth-Holm-Swan (EHS) tumor and although the extract resembles the complex extracellular tumor environment found in many tissues, it does contain multiple more or less defined growth factors which often renders mechanistic studies for specific regulatory elements very difficult. Another point of consideration is that sphere-forming capacity and stem cell functions are not interchangeable terms³⁴. While certain stem and progenitor cells have been shown to exhibit sphere-forming capability³⁷, failure to generate spheres *in vitro* does not exclude *in vivo* stem/progenitor function. For example, quiescent stem cells may simply not respond to the extracellular cues provided by the selected *in vitro* culture conditions. Thus, long-term *in vitro* and *in vivo* self-renewal capacity of purified cell populations, preferentially during serial passaging, should be assessed in order to prove or disprove stem cell function. In this context, the ability to prospectively and simultaneously propagate diverse populations of stem and progenitor cells is a crucial aspect of the sphere-forming assay and renders this assay highly valuable for the CSC field; as long as its limitations are kept in mind for the interpretation of the obtained data.

Sphere-forming assays have been widely used to retrospectively identify stem cells based on their capacity to self-renewal and differentiation at the single cell level *in vitro*. The discovery of markers that allow the prospective isolation of stem cells and their progeny from their *in vivo* niche allows the functional properties of purified populations to be defined. The combination of sphere formation assay and the FACS is a successful strategy to isolate cells from solid tissue or enrich specific subpopulations of PDAC in culture. For example, simultaneous expression of EpCAM, CD24 and CD44 defines a subpopulation of CSCs able to: self-renewal, differentiate and initiate a tumor, reflecting the heterogeneity of the original tumor. Hermann *et al.* showed that CD133+ cells possessed augmented proliferative capacity and CSC features¹⁵. Other markers have also been used for the characterization of CSCs: ALDH-1 (ALdehyde DeHydrogenase-1) expression is associated with highly tumorigenic cells in pancreatic cancer³⁸; cells able to exclude the DNA dye Hoechst 33342, named side population (SP) cells, have the proved capacity to initiate tumors³⁹. Recently we showed that a subcellular autofluorescence compartment characterizes a distinct population of human cells with exclusive CSC traits across different tumor types⁴⁰. Although none of these markers appears to selectively characterize a pure population of CSCs, more and more complex combinations of markers need to be used to FACS purify more refined populations of cells.

Many questions still remain about the precise role of CSCs in the origin, progression, and drug resistance of tumors. For example, one way to address the question of clonal evolution to define if and how a single CSC initiates a tumor, could be to analyze patient samples at different stages of the disease, and specifically follow the numbers of CSCs during and after treatment. By answering these questions, we can make meaningful progress of our knowledge of CSCs in solid tumors and develop drug therapies to ultimately prevent tumor progression and relapse.

Disclosures

Open access fees have been provided by Seahorse Bioscience

Acknowledgements

The present research was supported by an ERC Advanced Investigator Grant (Pa-CSC 233460), the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement No 256974 (EPC-TM-NET) and No 602783 (CAM-PaC), the Subdirección General de Evaluación y Fomento de la Investigación, Fondo de Investigación Sanitaria (PS09/02129 & PI12/02643), and the Programa Nacional de Internacionalización de la I+D, Subprograma: FCCI 2009 (PLE2009-0105; Ministerio de Economía y Competitividad, Spain). E. Lonardo was supported by Roche Fellowship. M. Cioffi was supported by the La Caixa Predoctoral Fellowship Programme.

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