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Irreversible electroporation shows efficacy against pancreatic carcinoma without systemic toxicity in mouse models.

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Running Title: IRE shows antitumor efficacy in pancreatic cancer

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Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) therapies show limited success. Irreversible electroporation (IRE) is an innovative loco-regional therapy in which high-voltage pulses are applied to induce plasma membrane defects leading to cellular death. In the present study we evaluated the feasibility of IRE against PDAC. IRE treatment exhibited significant antitumor effects and prolonged survival in mice with orthotopic xenografts. Extensive tumor necrosis, reduced tumor cell proliferation and disruption of microvessels were observed at different days post-IRE. Animals had transient increases in transaminases, amylase and lipase enzymes that normalized at 24h post-IRE. These results suggest that IRE could be an effective treatment for locally advanced pancreatic tumors.

Keywords: Pancreatic ductal adenocarcinoma, irreversible electroporation, orthotopic pancreatic cancer xenografts, bioluminescent imaging.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the predominant form of pancreatic cancer and is one of the most aggressive and devastating human malignancies in developed countries. It is the fourth leading cancer-related mortality with a median survival of 6 months and a 5-year survival of less than 5%. Pancreatic cancer is usually diagnosed at late stages and only 10-15% of patients present with operable disease. About 25% of unresectable PDAC are locally advanced and the rest are metastatic. The survival of non-resected patients is not fundamentally altered by any particular general therapy. Novel approaches for early detection and effective treatment will make the most significant impact on clinical management of this disease [1].

Radiofrequency (RFA) ablation of pancreatic neoplasms has been approached for the treatment of locally advanced, non-resectable and non-metastatic tumors and showed to be a feasible palliative treatment that led to tumor reduction and improved quality of life[2]. However the anatomical complexity in which the pancreatic ductal adenocarcinoma grows represents an increased difficulty to the procedure. Limitations of RFA, have already been reported for hepatocarcinoma treatment in terms of loco-regional recurrence and a higher rate of adverse events from procedure complications[3; 4]. All together highlights the need of more effective ablation techniques.

Electroporation is a non-thermal phenomenon in which cell membrane permeability to ions and macromolecules is increased by exposing the cell to high electric field pulses. If short pulses of low electric field magnitude are applied, such permeabilization will be reversible and the treated cells will be viable after the procedure. However, when such artificially induced permeabilization is too high cell homeostasis will be permanently disrupted, the process will be irreversible and cells will end up dying by necrotic or

apoptotic processes. The severity of the damage will be influenced by the pulse amplitude, pulse duration, number of pulses and frequency of pulses [5]. Reversible electroporation has been exploited to deliver chemotherapeutic drugs into the cells of solid tumors in a process termed electrochemotherapy [6] and to transfect tissues with DNA plasmids in a process termed electrogenetherapy [7]. In fact, our group has previously shown that the intratumoral electrogene delivery of the Herpes Simplex Thymidine Kinase gene followed by intraperitoneal ganciclovir administration reduces pancreatic tumor growth in mouse models [8]. Recently, irreversible electroporation (IRE) has been proposed as a method for solid tumor ablation and it is now being assayed clinically [9]. Animal models studies have shown that IRE can ablate substantial volume of tissues and the efficacy of IRE to target breast and hepatocarcinomas has already been evaluated and reported anti-tumor efficacy [10; 11]. Furthermore a Phase I clinical study to treat renal carcinomas with IRE has shown to be a safe technique that can offer some potential advantages over current ablative techniques [12].

In the current work we have investigated for the first time the feasibility of IRE to target pancreatic tumors in orthotopic pancreatic xenografts. We provide evidences of profound necrosis induced in the tumors and of increased median survival of IRE treated mice bearing tumors. Moreover we show a rapid recovery of pancreatic function from a transient serum increase in some physiological parameters.

2. Materials and Methods

2.1. Cell Lines

The human pancreatic adenocarcinoma cell line BxPC-3 was obtained and maintained as described previously [13]. Luciferase-expressing cells BxPC-3-Luc were established by transducing the parental cells (BxPC-3) with luciferase recombinant retrovirus. Briefly, pLHCLuc retroviral vector was transfected by the calcium/phosphate-DNA precipitation method into the amphotropic packaging cell line Phoenix Ampho (ATCC, Rockville, MD), and 48 hours after transfection, viral supernatant was collected, passed through 0.45- μ m filters, and used to transduce BxPC-3 cells. Twenty-four hours later, cells were selected in 0.2 mg/ml hygromycin, cloned and tested for luciferase expression.

2.2. Orthotopic Human Pancreatic Cancer Xenografts and IRE surgical procedure

Animal procedures met the guidelines of European Community Directive 86/609/EEC and were approved by the Local Ethical Committee. Orthotopic human pancreatic cancer xenografts were generated as previously described [14]. Briefly, 5×10^5 BxPC-3-Luc cells were injected into the pancreas of 8-week-old male athymic nude mice (Harlan Interfauna Iberica, Spain), in a final volume of 50 μ l. Animals were randomly divided in two groups: untreated control group (n=16) and IRE-treated group (n=24). Tumors were electroporated when reached 10^6 - 10^7 photons/s, measured by *in vivo* bioluminescent system (IVIS50, Xenogen), that correlates with 2-5 mm in diameter and 4-5 weeks after BxPC-3-Luc implantation. For IRE procedure, mice were anesthetized with a mixture of isoflurane and oxygen, and buprenorphine was used as analgesic. Then, a laparotomy incision in the left dorsal side of the mouse was performed to expose the BxPC-3-Luc tumor within the tail of the pancreas. Tumor was measured and positioned between the electrodes and IRE pulse train was applied (see next

section for details). Abdominal muscle layer was closed with interrupted suture and the overlying skin was closed using Autoclips® (Stoelting Europe, Ireland).

A subset of untreated mice were sham operated. To this end mice were submitted to surgery, exposed to the conductive gel, and positioned within the electrodes. No effects on tumor growth related to the procedure were observed.

To assess the effect of IRE treatment on tumors, animals were euthanized at different time points after IRE-treatment and pancreas/tumor was removed. For histological studies half part of pancreas/tumor was frozen in OCT (Akura Finetek, Zoeterwoude, the Netherlands), and the other half part was paraffin-embedded after fixation overnight in 4% phosphate-buffered formaldehyde. Histology sections were reviewed by a pathologist.

2.3. Irreversible electroporation treatment

After exposing the pancreas, tumor nodules were identified visually or by palpation. Then, a partially conductive gel (Aquasonic 100 Sterile, Parker Laboratories, Fairfield, NJ, USA) was applied and the nodule was gently squeezed between two parallel plate electrodes as schematically displayed in Fig. 1C. Two electrode setups were employed depending on the size of the tumor nodule. For nodules with a diameter below 5 mm a commercial stainless steel electrode pair shaped as tweezers was employed (code 45-0118, BTX Instrument Division, Harvard Apparatus, Holliston, MA, USA). For larger nodules it was employed a custom developed electrode setup consisting of two 21×12 mm² aluminum plates obtained from a commercial electroporation cuvette (code 165-2086, Bio-Rad Laboratories, Hercules, CA, USA) mounted on a plastic caliper (D00377 Duratool, Premier Farnell, London, UK). The distance between the plate electrodes was measured with caliper and was annotated for calculating the voltage to be programmed in a high voltage pulse generator connected to the electrode setup.

High voltage pulses required for irreversible electroporation were delivered by a commercial square wave electroporation generator (ECM830, BTX Instrument Division, Harvard Apparatus, Holliston, MA, USA). The generator was programmed to deliver sequences of ten pulses of 2500 V/cm (i.e. distance between plates in mm \times 250 V) with a duration of 100 μ s and repetition frequency of 1 Hz. The whole electroporation treatment consisted of ten of those ten pulses sequences (i.e. 100 pulses in total). Between those sequences, a manual pause of 10 seconds was introduced for allowing Joule heat to dissipate. Total treatment time was 200 seconds or slightly larger as some of the pauses between the sequences were made larger for repositioning the electrode setup.

It is worth noting that the partially conductive gel was not only applied for improving the electrical contact between the electrodes and the tissues but also for improving the electric field homogeneity as previously described [15].

2.4. Immunostaining

Hematoxylin–eosin staining was performed on paraffin-embedded samples to evaluate tumor and pancreas morphology. For immunohistochemical analyses five-micrometer paraffin-embedded sections were deparaffinized, rehydrate and treated with 10 mM citrate buffer (pH 6.0). Tissue sections were incubated with anti-human Ki-67 antibody clone MIB-1 (Dako, Denmark) or anti-active caspase-3 antibody clone C92-605 (BD Pharmingen, USA) for 16h at 4°C. Bound antibodies were detected with LSAB+ system-HRP (Dako, Denmark). Tissue sections were counterstained with Harris's hematoxylin and mounted with EUKITT® (Sigma-Aldrich, USA). Images were captured with a microscope (Leica DM6000 B) and digital camera (Leica DFC300 FX; Leica Microsystems) and processed with Leica Application Suite software.

Frozen tissue sections (ten-micrometer thick) were air-dried during 30 min and fixed in cold acetone for 5 min, acetone/chloroform 1:1 treatment was applied for 5 min and then acetone during 5 min. Sections were incubated with anti-mouse CD31 antibody

clone MEC 13.3 (BD Pharmingen, USA) for 16h at 4°C. Alexa Fluor 488 goat anti-rat antibody (Molecular Probes, USA) was used as a secondary antibody. Nucleus were counterstained with 5 ug/ml bis-benzimide (Hoechst 33342; Sigma) and visualized under a fluorescent microscope (Observer/Z1; Zeiss). The fluorescent images were captured using a digital camera (AxioCamMRm; Zeiss).

2.5. Bioluminescence Assay and Quantification

Animals were anesthetized with a mixture of isoflurane and oxygen preparation and the substrate D-Firefly-Luciferin (Xenogen, Alameda, CA) was administered intraperitoneally (32 mg/kg). Luciferase activity was visualized and quantified using an *in vivo* bioluminescent system (IVIS50; Xenogen) and Living Image 2.20.1 Software overlay on Igor Pro4.06A software (Wavemetrics, Seattle, WA) was used, as previously described [16]. Luciferase activity was quantified from non-saturated images, measuring the total amount of emitted light recorded by the CCD camera.

2.6. Biochemical parameters analyzed

Liver and pancreas IRE-associated damage was evaluated measuring the serum levels of AST, ALT, amylase, lipase and glucose. Briefly, blood samples were collected in untreated and IRE-treated animals at different time points: before (t=0) or after 1 h, 6 h, 24 h, 7 days and 14 days of electroporation. Serum parameters were determined in an Olympus AU400 Analyzer, as previously described [17].

2.7. Survival studies

Survival studies were performed to analyze time-to-event probability using the SPSS software. The survival curves (Kaplan-Meier curves) obtained were compared for the different treatments. Animals that were alive at the end of the experiment or used for histological analysis, were included as right censored information. A log-rank test was

used to determine the statistical significance of the differences in time-to-event. A p value of less than 0.05 was considered statistically significant.

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3. Results

3.1. Irreversible electroporation of pancreatic tumors increases survival of mice with orthotopic tumors

We developed an orthotopic pancreatic cancer model by implantation of BxPC-3-Luc cells in the body of the mouse pancreas. Bioluminescence analysis was used to monitor tumor engraftment and tumor growth (Fig. 1A). Histological studies showed areas of normal pancreas in vicinity to the tumor and regions in which tumor cells were infiltrating the pancreatic parenchyma (Fig. 1B). Eighteen mice bearing pancreatic tumors received tumor IRE following the procedure shown in Fig. 1C. IRE was applied when luciferase counts from tumor cells were within the range of 10^6 - 10^7 photons/s. Such luciferase counts corresponded to tumor nodules of $95,79 \pm 29,59$ mm³ and a tumor weight of 0.27 ± 0.03 g. Luciferase expression was monitored once a week in treated and untreated mice. Four days post-IRE, treated animals presented significantly lower luciferase activity than control mice. For most of the animals luciferase values remain low (50%) or undetectable (44%), suggestive of reduced tumor progression or tumor eradication; however in a small subset of mice (19%) 30 days post-IRE treatment luciferase activity increased, indicative of tumor regrowth (Fig. 2A and 2B). IRE treatment prolonged mouse survival and increased the median survival time from 42 days in untreated mice, up to 88 days in the IRE-treated group (Fig. 2C). At the end of the experiment 25% of mice presented complete tumor eradication.

3.2. IRE treatment of pancreatic tumors produces major pathological alterations

To assess the pathological effects of IRE in the tumors and to evaluate potential pancreatic tissue damage, animals were treated with IRE and at days 1, 7 and 14 post-IRE animals were euthanized and pancreas and tumor tissues were processed for gross morphology, histological analysis and immunohistochemical studies. Gross

findings of the isolated tissues visualized tumors in untreated animals as a mass of white bright color. Post-IRE treated tumors at days 1 and 7 revealed an intense brown area covering the tumor suggestive of blood accumulation. At day 14 a yellow mass of strong intensity in the periphery was observed defining a well-demarcated tumor (Fig. 3A).

Tissue histological examination post-IRE treatment revealed an extensive necrotic area, already evident at day 1. In some of the tumors there were no signs of remnant tumor whereas in some cases residual viable tumoral cells were still observed (Fig. 3B). Regions of non-viable epithelium of the pancreatic parenchyma were also observed in some animals, probably due to the inclusion of a portion of normal pancreas between the plate electrodes during the IRE procedure. At day 1 post-IRE a large number of red blood cells were visualized suggesting vascular disruption as a consequence of the procedure. At day 7 post-IRE treatment extensive areas of necrotized tissue with the presence of lymphocytic infiltrates and histiocytes were observed that was remarkable by day 14 (Fig. 3B).

We next analyzed the effect of IRE treatment on tumoral cell proliferation using Ki67 staining. Strong immunoreactivity was observed in untreated tumors while most of the treated tumors analyzed were Ki67 negative at all the time-points. In particular tumors remnant proliferating cells, positive for Ki67 were identified, at day 1 post-IRE probably indicating incomplete IRE effect (Fig. 4A).

To study a possible effect of IRE-inducing apoptosis we analyzed by immunodetection activated caspase-3, an early and specific apoptotic marker. No caspase-3 positive cells in any of the analyzed tumors were detected in the necrotic area or the vicinity of the tumor indicating that IRE in this pancreatic tumor model was not activating apoptotic cell death. Isolated caspase-3 positive cells could be identified in untreated tumors, corresponding to normal tumor development (Fig. 4A) and at day 14 some lymphocytic cells in the tumor burden also stained positive (data not shown).

We next analyzed the effect of IRE on tumor vascular endothelium by CD31 (an endothelial marker) immunodetection. Anti-CD31 staining nicely showed microvessel-density in the BxPC-3Luc xenograft model. Disruption of the vascular architecture was clearly observed since day 1 post-IRE and was present at all time-points post-IRE treatment (Fig. 4B).

3.3. Rapid recovery from low toxic effects associated to IRE pancreatic tumor treatment

To ascertain the safety of the tumor IRE procedure we evaluated serum biochemical parameters of liver and pancreatic function. Liver damage was assessed by measuring serum AST and ALT levels at different time-points after IRE application. As shown in fig. 5A and 5B an increase in the serum levels of liver transaminases was detected at 1h and peaked at 6h. At one day post-treatment ALT values were already within the normal range and AST levels almost completely recovered. At days 7 and 14 post-IRE treatment ALT and AST values were both within the reference range. Pancreatic function was followed at the indicated periods by analyzing serum amylase and lipase and measuring serum glucose levels. A transient increase in both amylase and lipase enzymes was detected at 6h post-IRE that completely normalized at 24h. Normal values were also detected at days 7 and 14 post-IRE treatment (Fig. 5C and 5D).

A 2-fold increase in the glucose levels was detected in the first hour post-IRE, followed by a slight hypoglycemia at 6h that resolved at 24h. The transient increase in the glucose levels in the first hour could be an indication of the glucose release from dying tumor cells. Normal glucose values were maintained at 7 and 14 days post-treatment (Fig. 5E).

These data indicate that IRE pancreatic tumor treatment generates minimal damage in the first hours post-treatment that resolves at 24h.

4. Discussion

In the present study we investigated the efficacy of irreversible electroporation to achieve antitumoral effects in a pancreatic cancer mouse model. We demonstrate efficacy of IRE for the treatment of pancreatic cancer by showing potent reduction in tumor growth from day 4 post-IRE with increased mice survival and complete tumor regression in the 25% of animals at 90 days post-treatment.

These data have been achieved in a preclinical orthotopic model based on the pancreas inoculation of a BxPC-3 pancreatic cancer cell line modified to express the luciferase gene. Several works have already demonstrated the successful use of bioluminescence to measure tumor biometrics after antitumor therapy in orthotopic pancreatic xenografts and highlight the utility of the technology to monitor tumor response in longitudinal studies [18; 19; 20]. In the present work we show for the first time the potential of the method to evaluate the antitumor effects of IRE.

We observed the efficacy of IRE in BxPC-3 xenografts, an aggressive tumor model when implanted orthotopically [19]. The response to IRE was detected very early, already at one day post-treatment major necrosis and limited viable cells with proliferation capacity trapped within the ablated area were observed. Microvascular architecture of the tumor was also disrupted with a rapid release of red blood cells and the appearance of a bloody tumor. At days 7 and 14 post-IRE necrosis was much more evident with the presence of an inflammatory cell reaction.

Efficacy of IRE has also been recently reported in a rat model of hepatocellular carcinoma with a necrotic response similar to the one we observed [10]. On the contrary they reported extensive caspase-3 activation suggesting an apoptotic cell death at one day post-IRE that was no longer visible later on. In our model we did not detect caspase-3 activation in the tumor cells at any of the periods analyzed. This is not surprising because they are two very different tumor models grown in different tissues with substantial particular characteristics of the tumor cells that could result with

different susceptibility to apoptotic cell death upon IRE. Nevertheless we can not discard that the IRE protocol that we applied presents with different kinetics on the response to cell death and that caspase-3 activation could be an earlier event in our model and escaped from our analysis. In fact both IRE procedures show marked differences in terms of the type of electrodes used and the number and frequency of applied pulses.

The IRE procedure that we used in addition to ablate the tumor area produced some damage to the adjacent parenchyma, also showing small necrotic areas. Nevertheless the effects on pancreas and liver functionality were only transiently affected. Most damage occurred in the first to 6h and at one day post-IRE, despite necrosis in small pancreatic areas, enzyme serum levels were normalized. Thus no major adverse effects were observed in treated mice proving for the low toxicity of the procedure. Our data is in agreement with a recent study in which IRE was applied to the pancreas of swine pigs and only transient increases in pancreatic enzymes were observed [21]. In that study, as in ours, all animals tolerated the procedure without immediate complications and no associated cardiac dysrhythmias.

The preclinical data generated in this study indicates that IRE leads to an increase in mice survival (from 42 days in untreated mice, up to 88-days in the IRE treated group) and a 25% of complete tumor regression. These remarkable antitumoral effects highlight the potential, of IRE as a future treatment modality in a subset of patients with pancreatic neoplasms. IRE procedure would be of interest in cases of locally advanced non-resectable solid masses, with an eligibility criteria similar to that employed for other ablative techniques such as radio frequency (RFA). RFA is currently performed for locally advanced pancreatic neoplasms in a combined therapeutic plan. Improvement in the quality of life due to the achievement of pain relief has been reported [22; 23], nevertheless significant results on increased survival are still missing [2]. The anatomical complexity of the pancreatic and peripancreatic regions in which the

pancreatic ductal adenocarcinoma grows makes the procedure of RFA more challenging than in other tumors. IRE is counted as a potential approach without the known complications of RFA or other conventional thermal ablative methods, being vascular damage leading to hemorrhages among the most frequent complications. Interestingly the application of IRE to a major blood vessel, the carotid artery has been reported to structurally preserve the vessel with no evidence of aneurysm, thrombus formation or necrosis. Obviously, IRE provoked an ablation of cells but left intact the cellular matrix that provides a good scaffold for the formation of new tissue [24].

Another major complication of ablation techniques is that nerves in thermal ablative zones can not regenerate. Noticeable, recent data shows that after IRE the nerve can achieve complete recovery [25].

Although additional studies are required, the work we present in the current study shows for the first time the potential of IRE in the treatment of pancreatic tumors and it can be visualized as an alternative approach to RFA. Some of the relevant advantages that IRE present are that it is a very rapid procedure and could potentially be applied to treat different nodules located in separated areas by a unique surgical procedure. Moreover the low toxic effects of the therapy could encourage the application of more than one round of IRE treatment in the subset of tumors that may present with partial responses.

In conclusion, our preclinical study shows the feasibility of IRE as a therapeutic modality to treat non-metastatic pancreatic tumors.

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Figure Legends

Fig. 1 Orthotopic human tumor model and schematic representation of IRE

technique. A. Representative images of tumor aspect (left panel) and luciferase signal (right panel) of orthotopic pancreatic BxPC-3-luc tumors. Black arrow indicates tumoral masses within the pancreas and white arrow indicates luciferase positive area, corresponding to the tumoral masses. B. H&E staining of representative orthotopic BxPC-3-Luc tumors. Left and right scale bar: 100 μm and 25 μm , respectively. White arrows indicate tumoral cells invading healthy acinar cells. C. Scheme of the IRE application procedure (left panel). Photograph showing the IRE tweezerrodes placement around the pancreatic tumor (right panel).

Fig. 2 Survival analysis of IRE-treated tumor-bearing mice.

BxPC-3-Luc tumor-bearing mice were irreversible electroporated when reached 10^6 - 10^7 photons/s. A. Representative images of bioluminescent emission from untreated or IRE-treated BxPC-3-Luc tumor-bearing mice, at different time points. B. Luciferase quantification of bioluminescent emission images from untreated (n=7) and IRE-treated (n=12) BxPC-3-Luc tumor-bearing mice, before (pre-IRE) and after 4, 14 and 20 days of IRE treatment. Results are expressed as photons per second. Values are represented as mean \pm SEM. *P < 0.01. C. Kaplan-Meier analyses survival curve (log-rank test, p < 0.01).

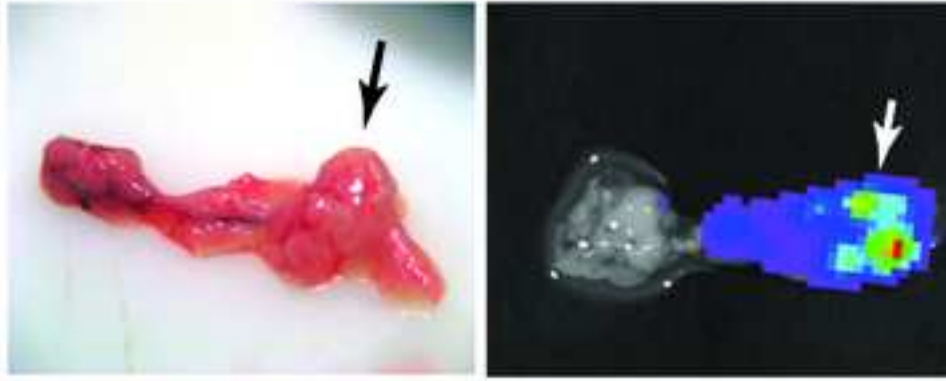
Fig. 3 Analysis of IRE treatment on BxPC-3-Luc orthotopic tumors.

A. Representative tumors from untreated (n=2) and IRE-treated mice, showing the macroscopic effect of electroporation at day 1 (n=2), 7 (n=4) and 14 (n=4). B. H&E staining of BxPC-3-Luc tumors untreated and IRE-treated at 1, 7 and 14 days. Black arrows indicate hemorrhagic reaction, green arrow indicates islands of tumoral cells immersed into necrotic tissue and yellow arrows point at lymphocytic infiltrates. Scale bar 400 μm (top panel) and 50 μm (bottom panel).

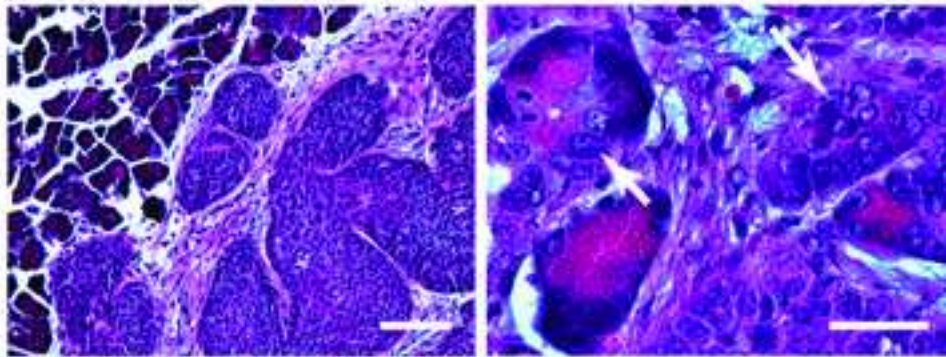
Fig. 4 IRE treatment effects on proliferation, apoptosis and vascular architecture in BxPC-3-Luc orthotopic tumors. Immunohistochemical analysis in untreated and IRE-treated mice at 1 (n=2), 7 (n=4) and 14 (n=4) days after treatment. A) Representative images of anti-Ki67 and anti-active caspase-3 immunostaining. Scale bar; 100 μ m. B) Representative images of anti-CD31 immunoreactivity (top panel scale bar: 400 μ m; bottom panel scale bar: 100 μ m).

Fig. 5 Liver and pancreatic function studies in IRE treated mice. Analysis of ALT (A), AST (B), amylase (C), lipase (D) and glucose (E) serum levels in IRE-treated mice before (n=18) and after 1h (n=6), 6h (n=6), 24h (n=7), 7 d (n=12) and 14 d (n=9) IRE treatment. Graphic insets correspond to the first 24h after IRE-treatment. Dash lines indicate reference values from untreated BxPC-3-Luc tumor-bearing mice.

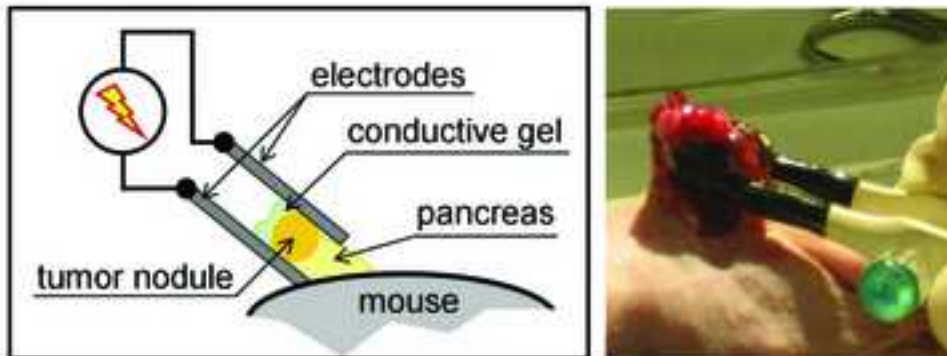
A

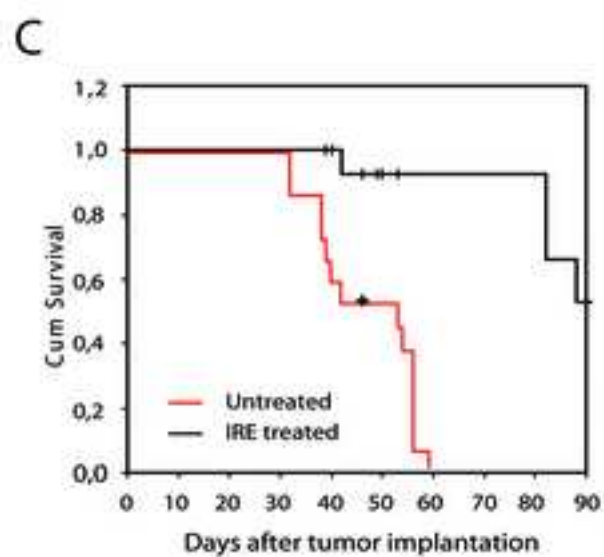
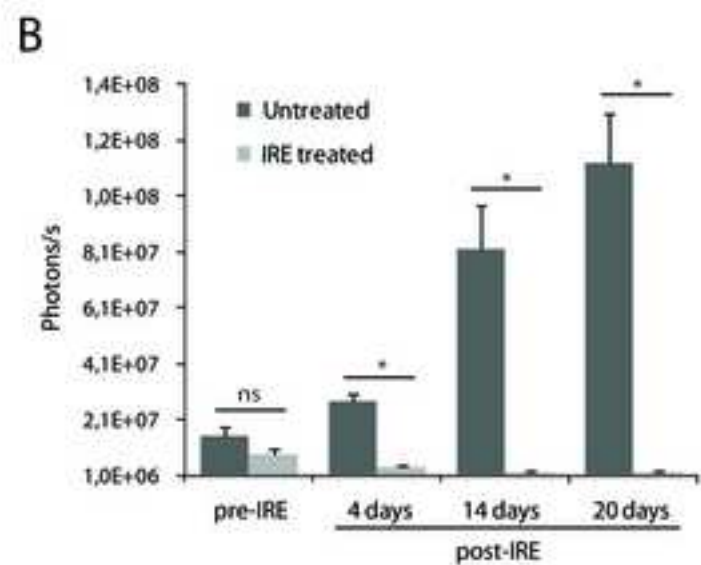
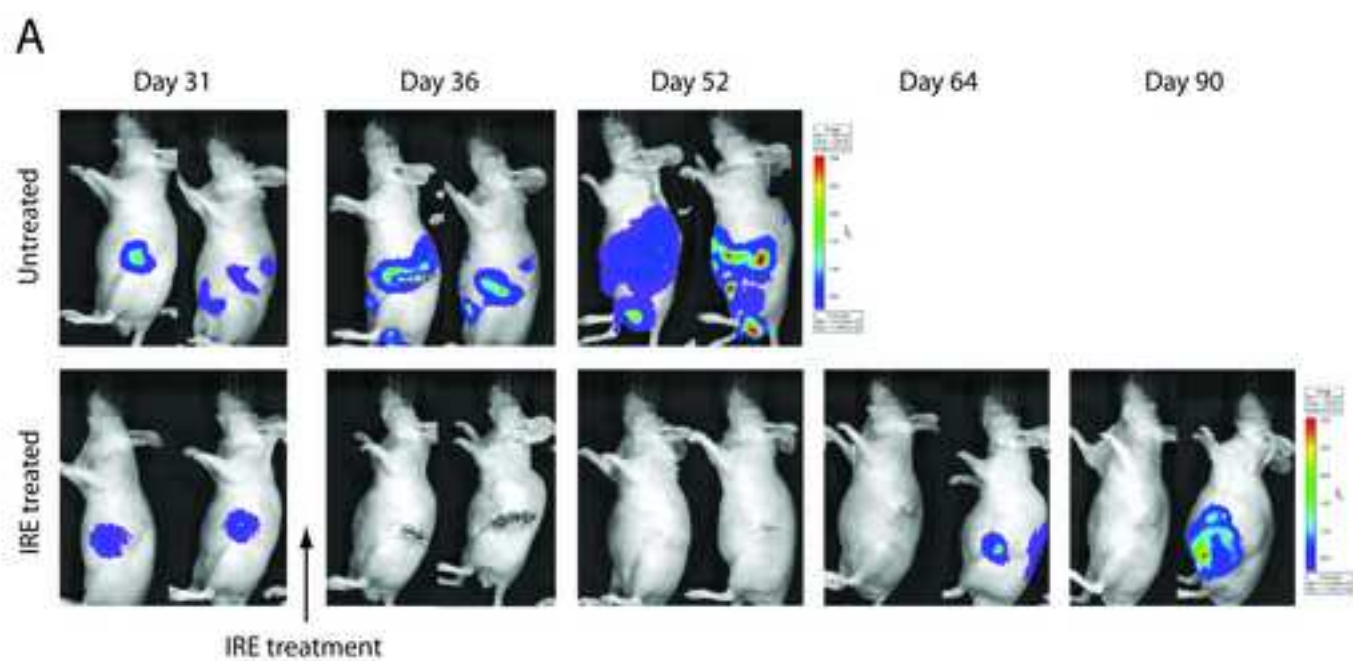


B

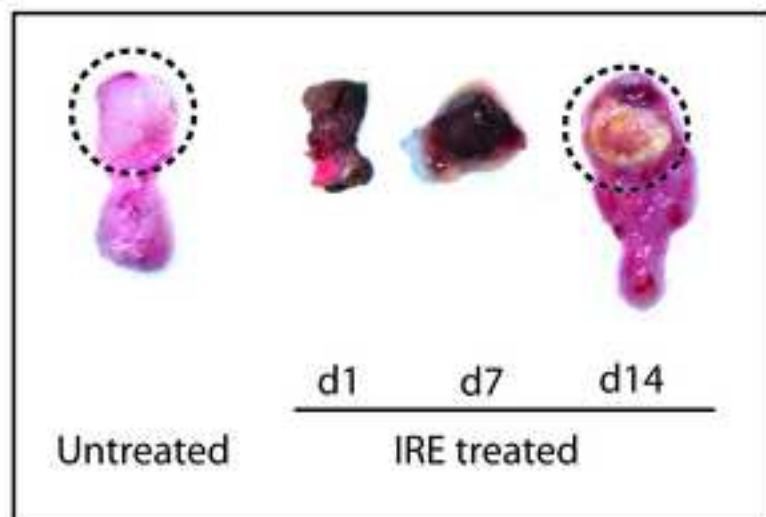


C





A



B

