Targeting quiescent tumor cells via oxygen and IGF-1 supplementation

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Abstract

Conventional chemotherapy targets proliferating cancer cells, but most cells in solid tumors are not in a proliferative state. Thus, strategies to enable conventional chemotherapy to target non-cycling cells may greatly increase tumor responsiveness. In this study, we employed a 3D tissue culture system to assay diffusible factors that can limit proliferation in the context of the tumor microenvironment, with the goal of identifying targets to heighten proliferative capacity in this setting. We found that supra-physiological levels of insulin or IGF-1 in combination with oxygen supplementation was sufficient to initiate proliferation of quiescence cells in this system. At maximal induction with IGF-1, net tissue proliferation increased 3- to 4-fold in the system such that chemotherapy treatment could trigger a 3- to 6-fold increase in cytotoxicity, compared to control conditions. These effects were confirmed in vivo in colon cancer xenograft models with demonstrations that IGF-1 receptor stimulation was sufficient to generate a 45% increase in tumor cell proliferation, along with a 25-50% increase in chemotherapy-induced tumor growth delay. While oxygen was a dominant factor limiting in vitro tumor cell proliferation, we found that oxygen supplementation via pure oxygen breathing at 1 or 2 atmospheres pressure (mimicking hyperbaric therapy) did not decrease hypoxia in the tumor xenograft mouse model and was insufficient to increase tumor proliferation. Thus, our findings pointed to IGF-1 receptor stimulation as a rational strategy to successfully increase tumor responsiveness to cytotoxic chemotherapy.

Introduction

The microenvironment of solid tumors has long been identified as a source of resistance to chemotherapy and radiotherapy. Sustained expansion of the tumor cell population and aberrant
neovascularization leads to the creation of an environment that differs from normal tissues. Deregulated proliferation and survival of tumor cells result in increased separation of blood vessels (1, 2) and unstable perfusion (3-6), in turn leading to poor efficiency in the distribution and removal of molecules supplied by the blood (7-11). For example, tumor cells can be located up to 15-20 cells away from the nearest blood vessel (more in some cases) while in most normal tissues cells are within just a few cell layers of a vessel (12). The diffusion gradient of molecules supplied by the blood creates subpopulations of cells within the tumor that will differ in proliferation status and response to chemotherapy. This phenomenon is well illustrated in corded HCT116 xenografts, when gradients in oxygenation and proliferation are mapped in relation to tumor vasculature (13). As illustrated in FIG 1A, S-phase cells in HCT116 xenografts can be observed nearer blood vessels while quiescent cells predominate in areas further from vessels. Despite uncontrolled proliferation being a hallmark of cancer, human tumors often exhibit proliferative indices as low as 5-10% (14). Even micro-metastases a quarter of a millimeter in size will possess regions of hypoxia and nutrient deprivation leading to tumor cell quiescence (10, 15). Previous studies examining the microregional activity of paclitaxel and gemcitabine tissue mapping found that quiescent cells located far from blood vessels recovered more readily following treatment (13, 16).

While numerous factors have been identified from in vitro studies that modify tumor cell proliferation, their relative importance in the context of the tumor microenvironment is still poorly understood. Limitations in the supply of oxygen, signaling molecules and key nutrients such as glutamine as well as tumor acidification have all been implicated as causes of tumor cell quiescence (17-19). Despite oxygen being a critical factor in the regulation of proliferation and survival it is unclear if other factors may initiate quiescence in solid tumors prior to oxygen
becoming limiting (15, 20, 21). To date stimulating tumor cell proliferation for therapeutic gain using strategies that focus on single factors have largely been unsuccessful and it might be that identifying more than one factor will be required (22-24). Tumor-derived quiescence is likely due to the interrelation of many diffusible factors, an issue that is compounded by the difficulty of independently studying diffusion limitations of molecules supplied by the blood and those released from cells (25).

In this study we have evaluated the relative importance of a panel of diffusible molecules as potential regulators of proliferation and quiescence in the context of solid tumors. Initial screening work was carried out using an *in vitro* 3D tissue model and followed up in studies in tumor xenografts. Combinations of candidate factors identified from the screen were evaluated both in terms of their ability to stimulate proliferation and to re-sensitize quiescent cells to chemotherapy. Since any strategy that increased tumor cell proliferation between treatments would be more likely to fail, tumors were transiently stimulated prior to chemotherapy. By taking this approach it was hoped to favorably alter the balance between tumor debulking by chemotherapy and re-growth between treatments. In theory such a strategy would also maximize an anti-proliferative drug’s therapeutic ratio as normal tissues are likely less responsive to stimulation because they possess intact regulatory pathways controlling cell growth and, due to their efficient vasculature, they will already have access to the diffusible molecules that tumor cells have become deprived.

**Materials & Methods**

*Monolayer & 3D tissue-disc culture* - HCT116 & HT29 cells were obtained from the ATCC in 2001 and cell type was confirmed for each experiment via 3D tissue morphology and architecture as seen in histological sections. Mycoplasma status was tested on a monthly basis
using Hoechst 33342 labeling of DNA. Cells were cultured in MEM (HyClone, Logan, Utah) with 10% FBS (Gibco/BRL, Burlington, ON) under 5% O2 & 5% CO2. 3-D tissue discs were grown by seeding 1 x 10^6 cells into collagen-coated tissue culture inserts (CM 12 mm, pore size 0.4μm, Millipore, Nepean ON) as previously described (26). Inserts were then incubated for 16 hours to allow cells to attach to the porous membrane prior to being submerged in media and transferred to stirred growth vessels (FIG 1D).

**Closed-off 3D tissue-disc assay** – Upon reaching approximately 125 μm in thickness HCT116 3D tissue-discs were transferred from growth vessels (FIG 1D) to penetration vessels (FIG 1E) and immersed in stirred MEM with 10% FBS. The growth media was then supplemented with additional factors and the discs incubated for 24 hours. Supplemented factors included 22 mM D-glucose (Sigma-Aldrich), 2 or 18 mM L-glutamine (Sigma-Aldrich), 0.6 mM L-arginine, 0.1 mM uridine & inoline (Sigma-Aldrich), 1% ITS cocktail (10 μg/ml insulin, 5.5 μg/ml transferrin & 0.0067 μg/ml selenium, Gibco #41400) or 10 μg/ml insulin alone (insulin from bovine pancreas, Sigma-Aldrich #I0516). 2 hours prior to completion 100 μM BrdUrd (Sigma-Aldrich) was added to the reservoir to label S-phase. Tissue-discs were frozen and embedded in O.C.T. compound (Tissue Tek) in preparation for cryosectioning.

**Open 3D tissue-disc assay** - HCT116 or HT29 tissue-discs 200-300 μm in thickness were maintained in growth vessels (FIG 1D) immersed in stirred MEM with 10% FBS. Media was then supplemented with additional oxygen, glucose and insulin or in later experiments LR3-IGF1 (Long R3IGF-1, #855803C, SAFC Global). 2 hours prior to completion of each supplementation period 100 μM BrdUrd and 50 μM pimonidazole (Hypoxyprobe-1 Kit, Chemicon International Inc., Temecula, CA, USA) were added to the reservoir to label S-phase and hypoxic cells. Tissue-discs were frozen and embedded in O.C.T. compound in preparation for cryosectioning.
For cell survival experiments the 3D tissue discs were supplemented with 100 ng/ml LR3-IGF1 under normal or high oxygen conditions (5 or 95% O₂) for 12 hours prior to a 6 hours cytotoxic chemotherapy exposure. Following treatment, tissue discs were rinsed and returned to fresh media and incubated for an additional 24 hours prior to dissociation using 0.25% trypsin EDTA (Sigma-Aldrich) for 15 min followed by 5 min DNAase treatment (1.5 mg/ml Deoxyribonuclease I from bovine pancreas, Sigma-Aldrich). Cells were then counted and plated for colony forming assay.

**Mice and Tumors:** Female NOD.CB17-Prkdcscid/J mice were bred and maintained in our institutional animal facility in accordance with the Canadian Council on Animal Care guidelines. Experiments are approved by the Animal Care Committee of the University of British Columbia. Mice were allowed free access to standard laboratory rodent food and water and were used between 8 & 14 weeks of age, typically weighing between 20 & 30 g. HCT116 cells (8 x 10⁶ cells in 50 μl) or HT29 cells (5 x 10⁶ cells in 50 μl) were implanted sub-cutaneously (s.c.) into the sacral region and tumors grown to 150 mm³ as calculated from caliper measurement of three orthogonal diameters (a,b,c) using the formula volume = π/6(abc).

**Tumor mapping experiments** – Mice were administered 1 mg/kg LR3-IGF1 by s.c. injection at times 0, 4, 8 & 12 hours alone or in combination with pure oxygen breathing under 1 or 2 atmospheres pressure. Hyperbaric work was carried out using a pressurized acrylic chamber (27). At the 12-hour time point mice were administered 1000 mg/kg BrdUrd (Sigma-Aldrich) and 60 mg/kg pimonidazole by intra-peritoneal (i.p.) injection and euthanized 2 hours later. Five minutes prior to carbon dioxide euthanasia and tumor harvest mice were administered 35 μl of 0.6 mg/ml DiOC₇(3) (Sigma-Aldrich) in 25% DMSO to demarcate perfused vessels. Tumors were then excised, weighed and immediately frozen.
Tumor growth delay experiments – Mice were administered 1 mg/kg LR3-IGF1 by s.c. injection at times 0, 4, 8 & 12 hours prior to cytotoxic chemotherapy. Paclitaxel at 30 mg/kg or 120 mg/kg gemcitabine was then administered i.p.. Both the LR3-IGF1 and chemotherapy regimens were repeated once weekly for three weeks, except paclitaxel in HCT116 which was administered for two weeks only. Tumor volumes were measured 3 times per week.

Immunohistochemistry: DiOC7(3), CD31, pimonidazole & BrdUrd: Prior to immunostaining slides were imaged for DiOC7(3) tissue fluorescence to visualize blood flow. Cryosections were then fixed in a 1:1 mixture of acetone-methanol for 10 minutes at room temperature. Vasculature was stained using a 1:400 dilution of anti-PECAM/CD31 (BD Pharmingen clone MEC13.3) and 1:200 fluorescent Alexa 546 anti-rat secondary (Invitrogen, Burlington, ON, CA). Hypoxia was detected via bound pimonidazole adducts using a 1:400 polyclonal rabbit-anti-pimonidazole (Hypoxyprobe-1 Kit, Chemicon International Inc., Temecula, CA, USA) and a 1:200 Alexa 488 anti-rabbit secondary. Slides were imaged for fluorescence and then transferred to distilled water for 10 min and then treated with 2 M HCl at room temperature for 1 hour followed by neutralization for 5 min in 0.1 M sodium borate. Slides were then washed in distilled water and transferred to a PBS (phosphate buffered saline) bath and BrdUrd incorporated into DNA was detected using a 1:500 dilution of monoclonal mouse anti-BrdUrd (clone BU33) followed by 1:200 dilution of anti-mouse peroxidase conjugate antibody (Sigma-Aldrich) and 1:10 dilution of metal enhanced DAB substrate (Pierce, Rockford, IL). Slides were counterstained with hematoxylin, dehydrated and mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA). For 3D tissue discs, the CD31 staining was skipped and a fluorescent secondary, anti-mouse Alexa-546, was used to detect the BrdUrd primary. Tissue discs were stained with Hoechst 33342 to label cell nuclei.
Image acquisition: The imaging system consisted of a robotic fluorescence microscope (Zeiss Imager Z1, Oberkochen, Germany), a cooled, monochrome CCD camera (Retiga 4000R, Q Imaging, Vancouver, BC, Canada), a motorized slide loader and x-y stage (Ludl Electronic Products, Hawthorne, NY, USA) and customized ImageJ software (public domain program developed at the U.S. National Institutes of Health) running on a Macintosh computer (Apple, Cupertino, CA, USA). The robotic system allowed for tiling of adjacent microscope fields of view of entire tumor cryosections up to 1-2 cm² were captured at a resolution of 0.75 μm/pixel.

Image analysis of tumor xenografts: Using ImageJ and user supplied algorithms, images of DiOC$_7$(3), CD31, BrdUrd & pimonidazole staining from each tumor section were overlaid and areas of necrosis and staining artifacts manually removed. CD31 positive regions were identified by selecting all pixels 10 standard deviations above the tissue background levels. CD31 positive regions that were less than 10 μm² in size were considered artifacts and automatically removed from the analysis. BrdUrd positive staining was identified by selecting pixels that were 5 standard deviations above tissue background levels. Measuring the distance from each point in the tissue to the nearest CD31 positive object and noting if it was BrdUrd positive or negative was used to determine the relation between proliferation and distance to the nearest detected blood vessel. The data was tabulated so as to determine the fraction of BrdUrd positive pixels of the total number pixels found at each distance to a blood vessel. Pimonidazole staining was assessed via similar methods, using average signal intensity rather than the fraction of pixels above threshold.

Image analysis of 3D tissue discs: Using ImageJ and user supplied algorithms, images of BrdUrd, pimonidazole and Hoechst staining from each tissue section were overlaid and staining artifacts manually removed. Tissue edges were traced manually and then BrdUrd and
pimonidazole were mapped in relation to distance from tissue edges. Three tissue sections 5-7 mm in length were taken from each disc.

Statistical Analysis: One-Way ANOVA tests were performed using Prism software (GraphPad, La Jolla CA). Significance of differences between multiple groups was compared using a Bonferroni post test analysis.

Results

Activity-based screen using HCT116 3D tissue-discs identifies that supra-physiological insulin supplementation can reverse diffusion limited proliferation. The ability of a selection of media supplements to sustain proliferation on the temporarily closed-off side of 3D tissue-discs was assayed over a 24-hour period. FIG 1F shows analysis of BrdUrd labeling in tissue located against the clamped-side of the disc, 75-150 μm in from the media-side. Prior to being closed-off, the clamped-side had access to media and exhibited equal proliferation to the open-side. Of the panel of supplements tested only the 1% ITS cocktail or 10 μg/ml insulin alone was found to maintain active S-phase status in the tissue on the closed-side. 10 μg/ml insulin is ~100 times greater than physiological blood concentrations and at this level it also acts to efficiently stimulate the IGF-1 receptor and downstream AKT/mTOR pathway leading to proliferation (28-31). Follow-up studies found similar activity following 100 ng/ml LR3-IGF1 stimulation. FBS, glucose, glutamine and DNA precursor supplements uridine and inoline were not able to sustain proliferation following the 24-hour period of closure.

Supra-physiological insulin in combination with oxygen supplementation yields greatest increase in proliferation in HCT116 3D tissue-discs. A second screen was carried out to determine the effect of supplements on cells in the normally quiescent zone of thicker 3D tissue-discs. HCT116 discs were grown to 200-300 μm and then, without closing-off the discs, normal growth
conditions were supplemented with insulin, glucose, amino acids and oxygen (5, 20 & 95% O2) for a 24-hour period. Immunostaining results for S-phase and hypoxic profiles within tissue cryosections tissue are shown in FIG 2 (also see supplemental data 1 for a comparison with IGF-1 stimulation). FIG 3 summarizes analysis of the interrelation of insulin, oxygen, glucose and proliferation within the discs. The maximum depth of proliferation within the discs was seen to increase with increasing oxygen, panels A-C, and stimulation following insulin was more effective at higher oxygen levels, panels D-F. Results indicate that maximal proliferation occurred when both oxygen and insulin were supplemented. Under combined insulin and oxygen supplementation the high level of proliferation 100-150 μm into the tissue ruled-out the supply of other diffusible molecules as being dominant factors limiting proliferation within the tissue. Oxygen and insulin supplementation alone were enough to sustain proliferation within the tissue near levels seen on the tissue edge. Panels G & H compare average proliferation and disc thickness following the 24-hour supplementation period. In the discs under the approximately physiological level of 5% oxygen, glucose supplementation did not increase proliferation (panel G) but did increase the maximum depth that cells were able to survive within the tissue prior to becoming necrotic (panel H).

Time-course of induction of proliferation following LR3-IGF1 and oxygen supplementation. In preparation for translation of findings to tumor xenograft-based studies, work was carried out to look at the time-course of induction of proliferation in quiescent tissue using LR3-IGF1, a synthetic analogue of IGF-1 which is not bound by IGF binding proteins in the blood and remains in an active form (32, 33). FIG 4 shows the time course of stimulation in oxygenated regions near the edge of tissue, 0-50 μm, versus 50-100 μm intermediate zones and more hypoxic areas 100-150 μm into the tissue. As with previous work using insulin, direct
stimulation of the IGF-1 receptor using LR3-IGF1 in combination with oxygen supplementation was able to initiate proliferation in normally quiescent areas. Maximal induction occurred 16-24 hours following initiation at which point overall tissue proliferation increased 3±0.4-fold (HCT116) and 4±1-fold (HT29) as measured using an S-phase endpoint (BrdUrd incorporation). Under 5% oxygen a transient increase in proliferation occurred predominantly in regions 50-100 μm from the edge of the tissue but not in more hypoxic areas further inwards. This transient stimulation 8-16 hours following media supplementation explains the increase in disc thickness seen in FIG 3H despite that proliferation levels appeared normal at 24 hours as was seen in FIG 3G.

**LR3-IGF1 & oxygen supplementation in 3D tissue-discs sensitizes tissue to chemotherapy.** 300 μm thick HCT116 & HT29 3D tissue-discs were stimulated with LR3-IGF1 for a 12-hour period under 5 or 95% O₂ followed by a 6-hour chemotherapy treatment period. FIG 5A shows the result for overall surviving fraction of cells from HT29 discs treated under normal versus oxygen and LR3-IGF1 supplemented conditions. Surviving fraction in chemotherapy-treated discs was seen to decrease ~6-fold in under combined stimulation compared to discs under normal conditions (SF 0.71 ± 0.08 reduced to 0.10 ± 0.03). Cell kill was greater when chemotherapy was given 12 hours following initiation of stimulation compared to when given simultaneously. Similar results were seen for treated HCT116 discs (SF 0.28 ± 0.1 reduced to 0.046 ± 0.007). The effect of LR3-IGF1 pre-stimulation on cell kill for a panel of chemotherapy agents in discs under 5% O₂ is shown in FIG 5B where surviving fraction is seen to be decreased by 3-6 fold.

**Effect of LR3-IGF1 & oxygen supplementation on proliferation status in HCT116 tumor xenografts.** LR3-IGF1 stimulation resulted in significant induction of proliferation in HCT116 xenografts while attempts to supplement oxygen via pure O₂ breathing under normobaric or...
hyperbaric conditions were not able to modify tumor oxygenation status or proliferation levels. FIG 6A shows proliferation and tissue oxygenation status in relation to tumor vasculature in HCT116 cryosections. BrdUrd staining, indicating S-phase cells, showed a significant increase following 12 hours of LR3-IGF1 supplementation. In control tumors proliferation was seen to cluster around certain vessels while other vessels exhibited low proliferation, quiescence rates gradually increased with distance from vessels. In contrast LR3-IGF1 treated tumors exhibited more consistently high proliferation rates near all vessels and proliferation tapered-off more abruptly on the border of hypoxic areas. Analysis of average BrdUrd and pimonidazole staining of viable tissue regions from whole cryosections, FIG 6B, indicated that S-phase cells increased by 45% (from 19% ± 2% to 27% ± 5%; mean ± s.d. n=6) in the LR3-IGF1 treated group compared to controls. Proliferation and hypoxia profiles in relation to blood vessels following the various strategies to increase proliferation are shown FIG 6C. Attempts to increase tissue oxygenation via pure oxygen breathing under 1 or 2 ATM for the duration of the 12 hour stimulation period, either alone or in combination with LR3-IGF1, were not effective at altering either final tissue hypoxia or proliferation status.

LR3-IGF1 supplementation increases response to chemotherapy in tumor xenografts. The combination of IGF-1 receptor stimulation with paclitaxel or gemcitabine treatment was assayed in HCT116 and HT29 tumor xenografts. Tumor growth delay following 30 mg/kg paclitaxel (q7x2) in HCT116 xenografts with and without a 12-hour LR3-IGF1 stimulation is shown in FIG 7A. Significant increases in the delays in time to reach 3-fold increase in volume were seen following LR3-IGF1 pre-stimulation for paclitaxel (48% increase in HCT116), and for gemcitabine (25% increase in HCT116 and 40% in HT29) as compared to un-stimulated mice, FIG 7B. No significant change in mouse weight was seen between normal mice and stimulated
mice. On its own LR3-IGF1 stimulation was not seen to alter tumor growth rates, consistent with the transient nature of the stimulation period of once per week for a 12-hour period (data not shown).

**Discussion**

This study shows that IGF-1 receptor stimulation could be successfully employed to increase response to chemotherapy, though the presence of hypoxic cells within the tumors likely remains a significant barrier to full re-sensitization of the tissue. A key to the success shown here appeared to be the determination of the appropriate conditions from studies using the engineered 3D tissue model which replicated the gradients in diffusible factors leading to tumor-derived quiescence (13, 16, 26, 34, 35). HCT116 and HT29 colorectal cell lines were chosen for this study as examples of related tumor types with differing levels of contact inhibition. HT29 cells express a 3-fold higher level of e-cadherin, a driver of contact inhibition of proliferation (36). HT29 cells have also been shown to be more resistant than HCT116 cells to strategies employing IGF-1 receptor inhibition to control tumor cell growth (37). When grown as solid tumor both cell types exhibited regions of chronic hypoxia in areas 100-150 μm from vessels and both displayed a reduction in proliferation with distance from blood vessels. A comparison of these findings in other cancer types displaying both differing microenvironmental profiles and growth factor dependencies could help to determine the degree to which these IGF-1 findings are widely applicable.

The 3D tissue model used here consisted of cancer cells grown into discs of tissue 30-40 cell layers thick to match typical diffusion distances seen in solid tumors (35, 38). Discs were grown in stirred media such that physiological conditions were maintained on the surfaces of each disc while gradients in oxygen and nutrients formed with depth into the tissue from either surface.
Under these conditions comparable gradients in proliferation were seen between the 3D tissue system and tumor xenografts. The initial screen using 3D-tissue discs found that proliferation rates within tissue could be maintained by a combination of oxygen and supra-physiological insulin or LR3-IGF1 supplementation. Their combined supplementation was found to reverse tumor-derived quiescence and re-sensitize the tissues to a panel of anti-proliferative chemotherapies. Other diffusible molecules such as amino-acids and glucose did not appear to limit proliferation when physiological levels were maintained at the surface of the discs. However, supplementing glucose did increase the maximum depth that cells could survive to prior to becoming necrotic.

Translation of in vitro findings from 3D tissue-discs to the xenograft systems indicated a good correlation between the two models. In both cases moderate increases in proliferation status could be achieved via IGF-1 receptor stimulation. Consistent with published data (39), the 5% oxygen exposure used for the 3D tissue-discs appeared closest to actual tumor oxygen levels as in both cases pimonidazole staining increased dramatically 50-100 μm into the tissue. The presence of unmapped blood vessels lying outside of the tissue section may explain why this increase did not appear as sharp in the tumors compared to the 3D tissue discs. These unmapped vessels would act to skew the analysis of the tissue oxygenation in regions that appeared to be distant from mapped vessels but are actually close to unmapped ones. In mice, pure oxygen breathing at 1 & 2 atmospheres pressure did not alter hypoxia profiles or proliferation at the end of the 12-hour stimulation period. Hence the larger effects predicted from oxygen supplementation in discs could not be achieved in the xenografts. Working above 2 atmospheres oxygen was not feasible in mice due to their low tolerance compared to larger animals for
prolonged exposures to hyperbaric oxygen and induction of pulmonary toxicity and bradycardia (40, 41).

Our results demonstrate that a significant re-sensitization of quiescent tumor cells to chemotherapy is achievable. A key step towards the successful translation of these findings may lie in follow-up studies to test hyperbaric oxygen strategies in larger animal models. Additionally, the ability to stimulate proliferation on a repeating schedule may be highly dependant on the duration of activity of the anti-proliferative drug combination that is being used. Hence further work looking at a larger panel of drugs and over longer treatment schedules is necessary. While a quiescent cell induction strategy has the potential to impact the response of any cancer, micro-metastases might be a good target for future development as the inherent difficulty of their detection makes them less amenable to curative radiotherapy or surgery.
References


Figure Legends

Figure 1. Screen for diffusible supplements that maintain proliferation within 3D tissue-discs. A| HCT116 xenograft cryosection showing gradients in S-phase cells (BrdUrd) in relation to tumor vasculature (CD31) & blood flow (DiOC7(3)). B&C| HCT116 3D tissue-disc showing hematoxylin and BrdUrd staining in relation to tissue edges. D| Growth vessel in which discs are open on both sides. E| Penetration vessel in which one side of each disc is closed-off. F| Effect of a panel of media supplements on proliferation on the far side of closed-off discs: (i) 2 X FBS, (ii) 2 X glutamine & arginine, (iii) 10 X glutamine, (iv) 5 X glucose, (v) 0.1 mM uridine & 0.1 mM inoline, (vi) 1 % ITS cocktail, (vii) 10 μg/ml insulin. Data show BrdUrd incorporation in cells on the closed-off side located 75 - 150 μm into the tissue (averages ± s.d., n=6). Scale bars show 150 μm. *, P<0.01.

Figure 2. Proliferation and hypoxia in 3D tissue-discs as a function of oxygen and supra-physiological insulin supplementation. HCT116 3D tissue-discs were grown to 200-300 μm thick under normal conditions and then grown for an additional 24 hours under 5, 20 or 95% oxygen ± 10 μg/ml insulin. Tissue cryosections were immunostained to show S-phase cells (BrdUrd) and hypoxia (pimonidazole). Scale bars indicate 150 μm. See supplemental data 1 for a comparison with LR3-IGF1 supplementation.
Figure 3. Analysis of the effect of oxygen and insulin supplementation on proliferation and hypoxia in 3D tissue-discs. A-F | Average BrdUrd & pimonidazole staining as a function of depth into tissue from the bottom side of discs as depicted in FIG 2. Light grey lines show results for the individual tissue sections and black (BrdUrd) and grey (pimonidazole) lines show group averages. Total tissue area analyzed from each cryosection was approximately ten times the area shown in the images from FIG 2.

G | Average BrdUrd staining intensity over first 150 μm from the bottom edge and H | average disc thickness. Bars show average ± s.d. (n=6-9). *, P<0.01.

Figure 4. Time-course of stimulation following oxygen & LR3-IGF1 supplementation in 3D tissue-discs. Comparison of 5% versus 95% oxygen ± LR3-IGF1 in A | HCT116 or B | HT29 3D tissue-discs. Peak induction occurs 12-24 hours following stimulation at which point quiescent areas 100-150 μm from tissue surface reached proliferation levels seen on tissue edge. Data show the fraction by area of each tissue section that stained positive for the S-phase marker BrdUrd (error bars show s.d., n=6). *, P<0.01.

Figure 5. Re-sensitization of 3D tissue-discs to anti-proliferative chemotherapy. Effect of LR3-IGF1 and oxygen supplementation on overall cell kill by chemotherapy in HT29 3D tissue-discs. Discs grown under normal conditions (MEM with 10 % FBS & 5% O₂) were stimulated with 100 μg/ml LR3-IGF1 and/or 95% oxygen for 12 hours and then exposed to a panel of conventional anti-proliferative drugs for 6 hours. Control discs were treated with under normal conditions or with oxygen and LR3 stimulation only at time of drug exposure. 24 h following drug exposure, discs were dissociated and plated for colony formation. Data points show averages ± s.d., n=4. *, P<0.01.

Figure 6. S-phase recruitment in tumor xenografts. A | HCT116 tumor cryosections from control versus LR3-IGF1 stimulated mice (1 mg/kg LR3-IGF1 every 4 hours for 12 hours). B | Overall tissue S-phase fraction and average hypoxia staining intensity (data bars show groups averages ± s.d. n=6). C | S-phase cell and hypoxia distribution in relation to tumor vasculature from control, LR3-IGF1 & oxygen treated.
mice. Each line represents an individual tumor, red (S-phase) & green (hypoxia) lines show group averages. *, $P<0.01$.

**Figure 7. Tumor response following induction of proliferation with LR3-IGF1 prior to chemotherapy.**

**A** | HCT116 tumor growth following 30 mg/kg paclitaxel (q7x2), arrows show drug treatment &

**B** | Time to 3-fold increase in tumor volume following 30 mg/kg paclitaxel or 120 mg/kg gemcitabine ± LR3-IGF1 in HCT116 & HT29 xenografts. 1 mg/kg LR3-IGF1 was administered 0, 4, 8 & 12 hours prior to each weekly anti-proliferative drug treatment. Data show averages ± s.e. n=6. *, $P<0.05$. 


Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
A - Proliferation mapping in tumor xenografts

B - Whole tumor averages

C - Proliferation and Hypoxia distribution profiles

Figure 6
A - HCT116 xenograft growth delay

B - Time to 3-fold increase

Figure 7
Targeting quiescent tumor cells via oxygen and IGF-1 supplementation

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