Author's response to reviews

Title: Interstitial fluid pressure, vascularity and metastasis in ectopic, orthotopic and spontaneous tumours.

Authors:

Sarah Jane Lunt (slunt@uhnres.utoronto.ca)
Tuula M.K. Kalliomaki (tkalliom@uhnres.utoronto.ca)
Allison Brown (allison.brown@rmp.uhn.on.ca)
Victor X Yang (victor.yang@utoronto.ca)
Michael Milosevic (mike.milosevic@rmp.uhn.on.ca)
Richard P Hill (hill@uhnres.utoronto.ca)

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Author's response to reviews: see over
23rd August 2007

Dear Dr Saltman,

We are re-submitting a revised version of MS# 1765358339136524 entitled "Interstitial fluid pressure, vascularity and metastasis in ectopic, orthotopic and spontaneous tumours" in response to the reviewers comments received on 20th July 2007.

We have made substantial modifications to the manuscript in order to address the reviewers comments. In brief, the background, discussion and conclusion sections have been rewritten more stringently to clearly express the relevance and interpretation of the data. Also, further analyses have been carried out to examine the intra-tumoural heterogeneity apparent in various phenotypic parameters (blood and lymphatic vessel density, hypoxia and proliferation) in relation to tumour IFP. A complete response to the reviewers comments is provided in the rebuttal below.

All of the authors have approved the final version of the manuscript and the work described has not been published previously. All the animal studies have been performed in compliance with the guidelines laid down by the Canadian Council for Animal Care.

We hope that you will find the manuscript suitable for publication in BMC Cancer.

Sincerely,

Richard P. Hill

Richard P. Hill, Ph.D.
Senior Scientist OCI/PMH
Professor Dept. of Medical Biophysics, Univ. of Toronto
610 University Avenue, Rm 10-113
Toronto, Ontario, Canada, M5G 2M9
Phone: 416-946-2979
Fax: 416-946-2984
Email: hill@uhnres.utoronto.ca
Reviewer: A major criticism of the manuscript is that it is hard to get bottom line of the study. The conclusion section covers more than a page. No specific mechanistic insights are provided by the present collection of data. The general conclusion that tumor IFP varies with tumor type and implantation site is not novel and this conclusion can be drawn from published data. Also, the presented data show that IFP varied remarkably little between various tumor types (means between 6 and 9 mmHg with modest spreading within each cohort of the different tumor types) with the exception of SiHa tumors grown orthotopically with a mean IFP of 14 mmHg. The largest differences were seen when tumors were grown i.m. compared with growth at the other sites, however, no experimentally validated conclusions can explain this difference. The finding of a potential correlation between blood perfusion and IFP is of interest but is not experimentally followed up. The data on a lack of relation between metastases and IFP is also interesting.

Response: We have modified the final conclusion section of the manuscript to address the reviewer’s concern about the “bottom line” of the study. However, there is, to our knowledge, no data in the literature where tumour IFP in multiple tumour models growing at different sites has been directly addressed. Certainly, different studies have used different tumour types but the majority of the studies are in s/c models and most of the other studies have used a single different site. Only one study compared the orthotopic with ectopic site in an osteosarcoma line (Brekken et al, 2000). Our study specifically addressed the importance of site in different tumour types including a spontaneously arising mammary gland tumour model, and in a cervical carcinoma model with a clinically relevant metastatic profile. Furthermore, we have examined the relationship between IFP and metastases in all models; these data are of clinical interest and there is limited data available in the literature.

With regard to the comment that there is little variation in IFP between various tumour types; the median IFP values vary between 6 and 30 mmHg. Excluding the i/m data, the medians are similar, varying between 6 and 15 mmHg but the range in each cohort is large, generally between 2-20 mmHg. Thus, there is substantial heterogeneity in IFP values in individual tumours regardless of the site of transplantation.

Reviewer: The text is at places hard to follow and vague. Examples are listed below.
Abstract, conclusion (line 4). What is meant by ‘...irrespective of previous molecular interactions was further...’ Previous to what? Which molecular interactions?

Response: We have removed this comment from the Abstract.

Reviewer: Abstract, last sentence. What is meant by ‘...future studies may be more effective using tumors grown orthotopically,...’. Effective in what sense? Whether orthotopic or e.g. subcutaneous (s.c.) models should be used has to be determined by taking into account the aim of the experiment, availability of technology and ethical considerations (animal welfare). It is obvious that in general terms orthotopic growth more closely mimic a clinical tumor, but for specific studies may not be the most effective choice. Also, in the models investigated the
differences in IFP between cohorts of tumors grown sub-cutaneously or orthotopically were similar.

**Response:** We agree that orthotopic models are not always practical, and that there can be technical and ethical limitations. We have removed the concluding sentence as part of the reduction in the conclusion section.

**Reviewer:** Introduction, first sentence. It is stated that the elevated tumor IFP largely is due the abnormal vasculature generated via angiogenesis. This statement raises two questions. First, which mechanism other than angiogenesis forms a vasculature in tumors? Second, in the further text other mechanisms for the elevated tumor IFP are given. Thus, in the Discussion (page 17, line 11) it is stated that the stroma (collagen and fibroblasts) together with infiltrating macrophages act to elevate IFP. On page 18, line 10 it is stated that the elevated tumor IFP most probably is a result of the combination of genetics, the mechanics at the recipient site and host-tumor physiologic interactions. Finally, in the Conclusion (page 20, line 11 from bottom) it is stated that the present data suggest 'that factors influencing tumor IFP are complex and in need of further study'.

**Response:** We have modified the first paragraph of the background (page 4) to address the stated concerns. The paragraph has been expanded so that the various different potential mechanisms related to elevated IFP are addressed at the start before being raised later in the text. With regard to the statement that “factors influencing tumor IFP are complex and in need of further study”, we believe this to be true. The mechanisms discussed in the new introductory paragraph and the discussion are those that have been described in the literature; however, there are many factors that remain unclear. Accurate measurement of vascular resistance, hydraulic conductivity and other factors that may contribute to the IFP value within an individual tumour is difficult and prediction of IFP values in an individual tumour is not possible. Furthermore, there is no data on whether tumour IFP alters gene expression in any way or whether it is a purely mechanical problem. We believe that the development of tumour IFP and its potential impact on progression and therapeutic outcome still requires extensive study.

**Reviewer:** Page 18, line 10. The sentence is vague. Which genetics? Do tumor cells have physiologic interactions with the host?

**Response:** We have rewritten that section of the discussion and although we refer to genetics in the discussion, the sentence (page 18, lines 6-9) reads “Systematic differences in tumour IFP as a function of both tumour type and implantation site were observed, consistent with an effect of predetermined genetic factors and interactions between the tumour and host microenvironments in regulating IFP”, specifically referring to the tumour cells and host to avoid ambiguity. With regard to “physiologic interactions with the host” we agree with the reviewer that the use of the term physiologic is incorrect and have removed it.

**Reviewer:** Conclusion, page 19 onwards. The text rambles and it is hard to understand which are the major conclusion(s) of the study. In the Discussion it should be stated which of the mechanistic possibilities for an increased IFP that is favored by the present data and discuss this in the context of previous reports.

**Response:** We have modified the conclusion to make it more concise and to state more clearly the purpose of the study and the interpretation of these data. In particular we have highlighted the role of tumour vasculature as the driving factor of elevated tumour IFP in the discussion.
Reviewer: Key experiments are not well described.
Fig 5. Lymph vessel density (LYVE-1), hypoxia (EF-5) and PECAM distributions are shown in low-power fields from ME180 xenografts. Sections were compared by pixel-counting on whole tissue segments, which show low variations between the different tumor groups and locations. Data on spatial distributions would be of more interesting and needed in order to draw the conclusion of that there is no correlation between IFP and the studied parameters. Better data as to these relations would be needed.

Response: We felt that a global analysis of these parameters would be more relevant in relation to our IFP values which are, in a sense, ‘global’ values representing the average of multiple IFP measurements within each individual tumour (3 per tumour). The IFP value is the result of what is happening in the tumour as a whole, the microvascular pressure and vascular resistance, the composition of the extracellular matrix and the hydraulic conductivity. However, we have reanalysed these data in the cervix and i/m ME180 tumours with varying IFP values, looking at 20 (or 10 for CD31) random 20x fields per tumour. These data are presented in Additional File 4, and show that although there was variability between fields, as would be expected, this variability was not related to tumour IFP.

Reviewer: Fig 6. What is meant by, 'the maximal perfused area for each tumor of the 5 planes analyzed'. Is the plane with the highest perfused area selected or what? Is it suggested that the modest difference (Fig 6D) could in part explain the rather large difference in IFP between ME180 tumors xenograft i.m. or orthotopically. Is there no difference in perfusion between tumors grown s.c. and orthotopically? A potential relation between perfusion and IFP is interesting and these data should be expanded. Three samples in each experimental group is too small.

Response: We agree with the reviewer that it would be desirable to have a larger data set and have expanded upon the cervix data set by adding analysis of 3 more tumours. We have also removed the discussion referring to the maximal perfused area (since both Reviewers 1 and 3 express legitimate concerns about this analysis) and now just show the mean perfused area for each tumour. However, these observations do warrant substantial further studies at various different sites of transplantation, and these are planned but will be too extensive to be included in this manuscript. They will form the basis of another manuscript on this issue.

Reviewer: I suppose that metastases were assessed in animals bearing primary tumors in which IFP had been determined. Perhaps it would better to plot IFP versus metastases for each animal rather than dichotomize in high and low pressure.

Response: We have re-plotted the metastases data so that these data are now presented as IFP versus metastases. There is no correlation between tumour IFP and metastatic disease.

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Reviewer 2; Kristian Pietras

Reviewer: The most interesting sets of experiments presented are the attempts to correlate tumor IFP with growth and phenotypic characteristics, such as proliferation, hypoxia and vascular density (Fig. 5). However, the authors have chosen to compare the various characteristics in
tumors grown at different sites, intramuscularly (high IFP) or orthotopically (low IFP). As the authors point out in the discussion (p19-20), the large difference in absolute values of tumor IFP between the different sites is most likely due to the mechanical properties of the implantation site. Thus, a more relevant comparison would be to correlate the phenotypic characteristics with the IFP of tumors from the same implantation site.

**Response:** As discussed, we believe that the mechanical properties of the implantation site are likely to impact on the IFP value. However, it is not clear that this would be the sole explanation and we felt that there may be site specific differences in blood and lymphatic vascularity that play a key role in regulating tumour IFP. As such, we examined these parameters in relation to site; figure 5F. However, we agree that a comparison in relation to the IFP values of tumours from the same implantation site is relevant, and these data are also shown in figure 5, G-J. The data is shown for cervix and i/m tumours, split according to whether the tumour had an above or below median IFP value.

**Reviewer:** The finding that donor IFP did not predict for IFP after transplantation of tumors into a recipient mouse is interesting and reflects an apparent and significant degree of stochastic determination of the tumor IFP. However, the interpretation of the data presented in Fig. 2c is confounded by the inclusion of transplantations of donor tumors grown intramuscularly to an orthotopic site in the recipient mouse. Since the authors already demonstrated that tumors at these sites display disparate IFP values, the comparison is flawed. Instead, the data from orthotopic-to-orthotopic site transplantations should be extended to allow stand-alone conclusions.

**Response:** We agree with the reviewer that there is a concern in the context of figure 2C that our standard procedure for transplantation into orthotopic sites is via pieces of i-m grown tumours. However, all of the MMTV-PyMT data shows comparison between orthotopic to orthotopic site; the details of these transplantations are outlined in the methods, page 8, paragraph 1. In neither case was there a correlation between donor and recipient IFP, and further, mice bearing two MMTV-PyMT orthotopically transplanted mammary tumours showed disparities in tumour IFP measurements irrespective of whether they were initiated from different or the same donors. These data are consistent and reflect an apparent and significant degree of stochastic determination of the tumour IFP.

**Reviewer:** In the attempt to correlate tumor IFP with metastatic incidence, the authors compare groups of tumors with high IFP (above median) or low IFP (below median) (Fig. 4a-e), but find no correlations. It may be that the division of groups based on above or below median tumor IFP is too coarse. The authors should present a similar analysis based on more divergent groups, e.g. lower vs upper quartile.

**Response:** We originally chose to examine metastases presentation according to whether the tumour IFP was above or below the median IFP value based on the clinical data in cervical carcinoma patients (Milosevic et al, 2004). However, in response to the comments of reviewer 1 we have re-plotted the data as metastases against tumour IFP; there is no significant correlation for any of the models. As suggested, we have also examined the data in terms of lower vs. upper quartile for the KHT-C and ME180 models (there were insufficient numbers to do so for the PyMT growing i/m); again there were no significant differences.
**Reviewer: Minor Essential Revisions**

On p18, the authors have stated that “…heterogeneity in tumour IFP values is probably largely attributable to differences in vascular resistance and permeability…”. While differences in vascular resistance and permeability are possible, and maybe even likely, explanations for heterogeneous tumor IFP values, the authors do not address this possibility experimentally, and thus the statement is pure speculation. The authors should substantiate the statement with experimental data, or rephrase.

**Response:** We have rephrased the statement to read “We speculate that heterogeneity in IFP values among individual tumours of the same type growing at the same site reflect the stochastic nature of unregulated angiogenesis in tumours and the random nature of the resultant vasculature” (page 19, lines 20-23). We have also expanded our discussion to add support to this statement.

**Reviewer: Discretionary Revisions**

The authors discuss “angiogenic and vascular normalisation properties” (p18) as a possible cause for the observed variability in tumor IFP. In line with this, and with the studies on vascular density and perfusion, the authors should present correlations between pericyte coverage (i.e. degree of vascular “normality”) and tumor IFP.

**Response:** We agree that this would be interesting data and thank the reviewer for this suggestion; we will examine this issue our in future studies.

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**Reviewer 3; Olivier Feron**

**Reviewer:** The description and the interpretation of the Figure 6 in the Results/Discussion sections are confusing. The number of mice (n=3?) looks too low to claim any biologically relevant difference in % perfused area and should incite the authors to be cautious in interpreting these results. I would suggest to suppress this figure.

**SJL:** The reviewer is correct; the numbers are low and we have added analyses of a few more tumours to this section. Nevertheless the significance of any differences in individual planes of the images remains uncertain so we have also removed the data looking at maximal perfused area to avoid confusion.

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**Additional Change**

Due to a recent study that causes uncertainty as to the exact origin of the CaSki cells used in this study, we have decided to remove this data from our manuscript. The data with these tumour cells was similar to the data with the other tumour models so we do not believe that removal of this data significantly influences the conclusions of our work.

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For the reviewers convenience, a copy of the manuscript with the changes highlighted in red is copied below.
Interstitial fluid pressure, vascularity and metastasis in ectopic, orthotopic and spontaneous tumours

Sarah Jane Lunt$^{1,3}$, Tuula M.K. Kalliomaki$^{1,3}$, Allison Brown$^{2,4}$, Victor X Yang$^{1,5}$, Michael Milosevic$^{2,4}$ and Richard P. Hill$^{1,3,4}$
Ontario Cancer Institute$^1$ and Radiation Medicine Program$^2$, Princess Margaret Hospital, Departments of Medical Biophysics$^3$, Radiation Oncology$^4$, and Division of Neurosurgery$^5$, University of Toronto, University of Toronto, 610 University Ave, Toronto, Ontario, Canada, M5G 2M9

Address for correspondence

Richard P. Hill, Ph.D.
Ontario Cancer Institute/Princess Margaret Hospital
Rm 10-113.
610 University Ave
Toronto, Ontario
Canada M5G 2M9

Phone: 416-946-2979
Fax: 416-946-2984
Email: hill@uhnres.utoronto.ca

Email addresses:

SJJ: slunt@uhnres.utoronto.ca
TMKK: tkalliom@uhnres.utoronto.ca
AB: allison.brown@rmpuhn.on.ca
VXY: victor.xd.yang@gmail.com
MM: mike.milosevic@rmpuhn.on.ca
Abstract

Background: High tumour interstitial fluid pressure (IFP) has been adversely linked to poor drug uptake in patients, and to treatment response following radiotherapy in cervix cancer patients. In this study we measured compared IFP values in an array of murine and xenograft models, spontaneously arising or transplanted either intramuscularly (i/m) or orthotopically and analysed their relationship to tumour vascularity and metastatic spread.

Methods: KHT-C murine fibrosarcoma, ME180CaSki, Me180 and SiHa human cervix carcinoma were grown either intramuscularly (i/m), sub-cutaneously or orthotopically. The Polyoma middle-T (MMTV-PyMT) transgenic spontaneous mammary tumour was studied either as spontaneous tumours or following orthotopically or i/m transplantation. One model was included for comparison. IFP was measured in all tumours using the wick-in-needle method. Spontaneous metastasis formation in the lungs or lymph nodes was assessed in all models. An immunohistochemical analysis of tumour hypoxia, vascular density, lymphatic vascular density and proliferation was carried out in ME180Me180 tumours grown both i/m and orthotopically. Blood flow was also assessed in the ME180Me180 model using high-frequency micro-ultrasound functional imaging.

Results: Tumour IFP was heterogeneous in all the models irrespective of growth site: KHT-C i/m: 2-42 mmHg, s/c: 1-14 mmHg, ME180CaSki: i/m 6-63 mmHg, s/c: 1-20 mmHg, cervix 1-18 mmHg, Me180: i/m 5-68 mmHg, cervix 4-21 mmHg, SiHa: i/m 20-56 mmHg, cervix 2-26 mmHg, MMTV-PyMT: i/m: 13-45 mmHg, spontaneous 2-20 mmHg and transplanted 2-22 mmHg. Additionally, there was significant variation between individual tumours growing in the same mouse, and there was no correlation between donor and recipient tumour IFP values. Metastatic dissemination to the lungs or lymph nodes demonstrated no correlation with tumour IFP. Furthermore, immunohistochemical analysis of tumour hypoxia, proliferation, and lymphatic or blood vessel density showed no demonstrate any relationship with tumour IFP. Speckle variance analysis of ultrasound images...
showed no differences in vascular perfusion between ME180 tumours grown i/m versus orthotopically despite differences, suggesting that the functionality of the vasculature is a key factor in IFP, the inter-model heterogeneity.

Conclusions: Our studies across a range of different tumour models showed substantial heterogeneity in tumour IFP, suggesting differences in the vascular development and interstitial fluid dynamics in the individual tumours. The results demonstrate a strong existence of a stochastic aspect to tumour IFP development, notably irrespective of previous molecular interactions was further enforced by the variation apparent between in different tumours within the same animal and the lack of correlation between donor and recipient tumours. Given the marked variability in IFP as a function of tumour type and implantation site, future studies may be more effective using tumours grown orthotopically, which are more relevant to both host-tumour-specific interactions and clinical interpretation.
Background

Solid tumours show interstitial fluid pressures (IFP) that are elevated above that of normal tissues. Tumour growth and development is supported by both tissues, largely as a result of the pre-existing host vasculature and by neoabnormal-vasculature generated through the process of via angiogenesis. Tumour angiogenesis generates abnormal vessels [1-3] that. The vessels formed through this process demonstrate several anomalies including an incomplete or absent endothelial cell layer and basement membrane which makes them hyper-permeable [4]. These vessels exhibit a high resistance to capillary blood flow and a low resistance to transcapillary flow, resulting in a net efflux of fluid into the surrounding interstitial space where a lack of functional lymphatics allows it to accumulate, distending the elastic extracellular matrix and increasing the interstitial pressure [5, 6]. AnMathematical models suggest that an equilibrium is established where the capillary and interstitial pressures are equivalent resulting in reduced fluid movement through the interstitium [6]. In addition, the tumour interstitium itself is thought to be abnormal, comprising a dense network of collagen fibres, as well as increased fibroblasts, macrophages and other cells involved in inflammation, which further contribute to elevated IFP values [3]. It is clear from these previous studies that high IFP in tumours arises because of the complex interplay between the abnormal vasculature and the abnormal interstitium. However, the pathophysiologic mechanisms underlying widely varying IFP values in human and experimental tumours of the same and differing types, and the influence of growth site and the host, is less well understood.

Elevated tumour IFP plays a role in the pathophysiological microenvironment that characterises solid tumours contributing to disease progression and therapeutic resistance [3, 6-9]. The mechanisms involved by which these effects are achieved remain to be fully elucidated but several experimental animal studies have shown an improved uptake of therapeutic agents in response to a reduction in tumour IFP suggesting that high tumour IFP acts as a barrier to drug
delivery [10-17]. Furthermore, there are clinical data showing that tumour IFP correlates with response to treatment [18, 19], with strong evidence for high IFP as an adverse prognostic indicator in cervix cancer patients treated with radiotherapy [7, 9]. Patients in the latter study were significantly more likely to develop distant recurrence if they presented with a tumour IFP value above the group median (19 mmHg), which suggests a role for IFP in metastatic spread. A similar relationship has also been observed between tumour IFP values and metastasis in experimental melanoma xenografts [20].

These data, coupled with the breadth of data demonstrating elevated IFP in a wide range of human tumours [7, 18, 19, 21-25], designate high tumour IFP as an important therapeutic problem. Further preclinical investigation is needed to understand the mechanisms underlying the adverse prognostic effect of high IFP and the implications for treatment. However, little is known about the most appropriate experimental model for these studies in relation to clinical tumour behaviour. To date most studies have focused on tumour models grown sub-cutaneously [13, 26-28] although evidence suggests that orthotopic models may be more clinically relevant [29, 30]. Although IFP has been measured in a variety of different tumour models, to our knowledge no previous study has focussed on the influence of tumour growth site on the development of the pathophysiological tumour microenvironment, or more specifically, tumour IFP. As such, the purpose of this study was to assess IFP in a number panel of different murine (KHT-C) and xenograft (ME180, CaSki, Me180, SiHa) tumour models growing both ectopically and orthotopically, and to examine features of the tumours (lymphatic and blood vascular density, hypoxia, perfusion) that might relate to the IFP levels and to disease progression. The murine and xenograft models were selected on the basis of previous studies within our lab [31, 32] and the polyoma middle-T (MMTV-PyMT) transgenic spontaneous
mammary tumour model was included to allow comparison with the transplanted models [33].

IFP has only previously been assessed in one other spontaneously arising tumour model [34].

The orthotopic human cervix cancer xenograft models were included because of the direct relevance of this model to our clinical program [7,9]; to date almost 300 patients with cervix cancer have undergone measurement of tumour IFP and other microenvironmental parameters prior to treatment. IFP was examined for each of the different tumour models and growth sites and related to tumour size, metastatic dissemination, tumour hypoxia, proliferation and vascular and lymphatic density.
Methods

Mice and tumour cell lines

Experiments were performed using MMTV-polyoma middle-T transgenic mice (MMTV-PyMT;[33]) bred in-house, the previously described KHT-C murine fibrosarcoma cell line [35][34] and the ME180CaSki, Me180 and SiHa human cervical carcinoma cell lines stably transfected to constitutively express the fluorescent marker DsRed [36][35]. All cell lines were maintained on an alternative in vitro in vivo growth cycle. In vitro cells were maintained as monolayers in plastic tissue culture flasks using α-MEM medium (Life Technologies, Inc., Burlington, Canada) supplemented with 10% fetal bovine serum (Wisent, Quebec, Canada). The cervical carcinoma cell lines were maintained under G-418 selection (400µg/ml). Cells between their 2nd and 5th in vitro passage were removed from the flasks during exponential growth using 0.05% trypsin for transplantation into mice. KHT-C cells were transplanted into syngeneic 8-12 week old C3H/HeJ male mice (Jackson Laboratory, Bar Harbour, ME). ME180CaSki, Me180 and SiHa cells were transplanted into 8-12 week old female CB-17/SCID mice obtained from an in-house breeding program. PyMT cells were transplanted into female FVB (wild-type, w.t.) or SCID mice. Tumours were initiated either intramuscularly (i/m) in the left gastrocnemius muscle, or sub-cutaneously (s/c) on the flank by injection of 2.5x10^5 or 5x10^5 cells respectively in a 50µl volume of α-MEM media. Tumours growing i/m were monitored by measuring the external leg diameter of the mouse. Tumours growing and by callipers for s/c were measured directly.

Orthotopic cervical and mammary gland tumours were initiated from donor tumours using protocols described below. Animals were housed at the Ontario Cancer Institute animal facility and had access to food and water ad libitum. All experiments were performed under protocols approved according to the regulations of the Canadian Council on Animal Care.
Orthotopic implantation in the cervix or mammary gland

The method for orthotopic implantation of tumour fragments into the cervix has been described in detail previously [36][35]. In brief, donor tumours grown i/m (or orthotopically) were excised under sterile conditions and small fragments (1.5-2 mm in diameter) were sutured into the site of a small incision in the uterus at the level of the cervix. Once tumours were palpable, IFP measurements were taken and tumours, lumbar lymph nodes and lungs were imaged/removed for further analysis.

Spontaneously arising donor tumours in MMTV-PyMT transgenic mice were excised under sterile conditions and divided into small fragments of approximately 2 mm in diameter. A fine incision was made in the skin to expose the 4th mammary gland. A small incision was made in the fat pad of the 4th mammary glands and a tumour fragment was sutured in place using a single 8-0 silk suture. The skin was closed using stainless steel wound clips. The left and right 4th mammary glands were implanted with a donor tumour fragment from either the same or a different donor tumour (Additional File 2). This allowed the effect of donor tumour variability on the subsequent development of the recipient tumour microenvironment to be assessed without the confounding factor of inter-animal variability. All surgical procedures were carried out under anaesthesia (2% isofluorane). Buprenorphine (0.1 mg/kg) was administered s/c following surgery to alleviate pain.

Tumour growth was monitored using callipers to measure the width and length of the tumour; once a size of 50-80 mm² was attained IFP measurements were initiated. Mice were sacrificed once a tumour reached a size of 200-250 mm². Half of each tumour was fixed in 10% neutral buffered formalin and half was snap frozen in OCT for histological analyses. The lungs were excised for examination of metastases.

All surgical procedures were carried out under anaesthesia (2% isofluorane). Buprenorphine (0.1 mg/kg) was administered s/c following surgery to alleviate pain.
Pressure treatment in vitro

**CaSki or KHT-C tumour cells were exposed to elevated pressures (20 or 50 mmHg) for various times in vitro in 10 cm tissue culture dishes seeded with a sub-confluent cell monolayer in a modular incubator chamber (Billups-Rothenberg, Del Mar, Canada). Pressure levels used were based on the average IFP values apparent when in each tumour type grown i/m. Pressure was controlled through adjustments of a dual scale low pressure gauge (0-15KPa; Cole Parmer, Quebec, Canada) on the outlet port. Control cells were gassed using the same system, but without the addition of a pressure gauge on the outlet port, allowing the gas to flush through freely at atmospheric pressure. The metastatic potential of pressure treated tumour cells was assessed using an experimental lung metastases assay [37][36].**

IFP measurements

**Interstitial fluid pressure was measured using a wick-in-needle technique [38].**

**Interstitial fluid pressure was measured using a wick-in-needle technique (Fadnes et al 1977).** Measurements were made using a 23-gauge needle with side port connected to a pressure transducer (Model P23XL, Viggo-Spectramed, Oxnard, CA) and an electronic data acquisition and recording system (Model MP100, World Precision Instruments, Sarasota, FL) through 470 mm of PE22 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ, USA). A “wick” was placed in the distal portion of the needle, and the entire system was flushed with a heparin sulphate/saline solution (1:10) [39][37]. IFP measurements were taken at three to four different locations in the tumour, and the mean value of these readings was taken to represent the tumour IFP.

Assessment of macroscopic and microscopic metastases
Following sacrifice of KHT-C and MMTV-PyMT tumour bearing animals, the lungs were removed and fixed overnight in Bouin’s solution (BDH Inc., Toronto, Canada). A dissecting microscope was used to count the number of visible metastases in each of the five lobes and the total number of lesions counted per lung reported (KHT-C and MMTV-PyMT). In the case of too many metastatic lesions to count, the wet weight of the lungs was taken as representative of metastatic burden. For the orthotopic ME180 tumours that had been transfected to express DsRed, lymph node metastases were visualised by fluorescence and counted as previously described [36].

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Microscopic lung metastases were detected immunohistochemically (ME180 and SiHa) for the CaSki, Me180 and SiHa tumour bearing animals, lung metastatic burden was estimated from DsRed fluorescence using a Leica MZ FLIII fluorescent stereomicroscope as described previously [31]. Metastases are reported as total volume in pixels. Lymph node metastases were visualised by fluorescence counted for the Me180 tumour model.

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Micro-metastases were detected immunohistochemically following fixation of lungs in Bouin’s solution (BDH Inc., Toronto, Canada). All five lobes were paraffin embedded and four 4µm sections 150 µm apart were cut from each lobe. The number of visible micro-metastases in each of the five lobes was then counted using a light microscope. Two or more clumped tumour cells were scored as a lesion. Micro-metastases are reported as the total number of lesions counted per lung.

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Histological Analyses

Analyses were carried out using immunohistochemistry for tumour hypoxia (EF5), vascular density (CD31), lymphatic vessel density (LYVE-1), and proliferation (Ki67). Tumour bearing animals were injected with the hypoxia marker EF5 [2-(2-nitro-1H-imidazole- 1-yl)-N-(2,2,3,3,3-
pentafluoropropyl) acetamide; obtained from Dr. Cameron Koch, University of Pennsylvania, at 10 mg/kg 2.5 hours prior to tumour excision. Once excised, half of the tumour was fixed in 10% neutral buffered formalin, and the remaining half placed in optimal cutting temperature (OCT) embedding medium (Tissue-Tek, Sakura, USA), and snap frozen in liquid nitrogen. Paraffin-embedded tissue was used for all markers with the exception of CD31.

For each marker, two sections were cut 100 µm apart due to address the issue of intra-tumoural heterogeneity (4µm sections for paraffin-embedded tissue, 5µm sections for frozen tissue). The slides were then processed according to standard immunohistochemical protocols. The primary antibodies used were: for EF5, the biotinylated antibody ELK3-51 (1/500; a gift from Dr. Cameron Koch, University of Pennsylvania); for CD31 the rat anti-mouse CD31, clone MEC 13.3 (1/500; Pharmingen, Canada); for Ki67 mouse anti-human Ki67 clone MIB-1 (1/100; DAKO, Canada); for LYVE-1, rabbit anti-mouse LYVE-1 (1/200 Abcam, Canada). For all markers, apart from EF5, primary incubation was followed by a 30 minute incubation with a biotinylated secondary (Vector Labs, Canada) and horseradish peroxidase conjugated ultrastreptavidin labelling reagent (ID labs, Canada). Nova Red (Vector) with Mayer’s hematoxylin counterstain was then used for chromogenic detection.

The stained sections were analysed using the Aperio imaging system (Aperio Technologies, California). Entire sections were scanned using the ScanScope CS and the total area of positive staining quantified using a positive pixel algorithm designed for brown/blue immunohistochemical stains. The same settings were used for each stain across all images, and the area of positive staining was calculated by dividing the total number of positive pixels (weak, medium and strong staining) by the total number of pixels in the image (positive + negative pixels) to yield the overall percentage of positive pixels. To assess intra-tumour heterogeneity for each stain, ten (CD31) or 20 (EF5, Ki67 and LYVE1) high-magnification (20X) fields were
randomly selected from each tumour section and analysed independently using the same methodology. Values are presented as percent positive pixels.

**Blood flow detection using high-frequency micro-ultrasound functional imaging.**

Real-time ultrasound biomicroscopic imaging of anaesthetised (2% isofluorane) mice was carried out using speckle variance analysis of a high frequency ultrasound (system: Vevo660, VisualSonics, Inc., Toronto, ON, Canada) images as previously described [40][38]. The ultrasound transducer transmits at a central frequency of 40 MHz with a focal length of 6 mm. The lateral and axial resolutions were 68 and 38 µm, respectively. Ultrasound gel (Aquasonic 100, Parker Laboratories, Fairfield, NJ) was used as a coupling agent on the skin. A minimum of five brightness mode (B mode) two-dimensional image planes were acquired per tumour, each with and cinelop of (300 frames) for each tumour were acquired at a frame rate of 17/s, frames per second for all time points. Speckle pattern and intensity during real-time B mode imaging of stationary tissue remain constant, and the temporal variance of speckle intensity increases with tissue motion. Consequently, a speckle-variance flow-processing algorithm devised by Yang [41][39] was used to calculate changes in speckle intensity between sequential frames as an indication of functional blood flow as described previously [40][38]. This technique gives a relative indication of the number of perfused vessels in each tissue plane, and has been validated previously against Hoescht perfusion [40][38]. Each of the 5 B mode image planes three mice per-group were analysed for 3 i/m at each time point and six cervix ME180 tumours five cinelops per-mouse were acquired in each tumour.

**Statistical Analysis**
Experiments with three or more groups were analysed for statistical significance using the Kruskal-Wallis statistic, and individual comparisons within these groups were carried out using Dunn’s test. Experiments with two groups were analysed for statistical significance using the Mann-Whitney statistical test. Correlation was assessed using the correlation coefficient derived from linear-regression analysis.
Results

IFP varies between tumours of both the same and different origins

Tumour IFP was measured in a number panel of different murine and xenograft tumour models grown both ectopically and orthotopically. Substantial inter-tumour (between individual tumours) heterogeneity was apparent for all tumour models irrespective of site (Figure 1 and Additional File 1). Interestingly, ME180, CaSki, Me180 and SiHa tumours grown orthotopically in the cervix or MMTV-PyMT tumours in the mammary fat pad consistently demonstrated IFP values lower than those grown ectopically i/m (p<0.05). Similarly, KHT-C and CaSki tumours growing s/c had lower IFP values as compared to tumours growing in the i/m site, demonstrating that in these models tumour IFP is higher when growing intra-muscularly. The least intra-tumoural variability in the i/m site was seen in the KHT-C tumour model, possibly attributable to the lower IFP values. In the cervical carcinoma xenograft models growing orthotopically, the CaSki and Me180 tumours were comparable, but IFP was significantly higher in the SiHa model as compared to the ME180 model.

The inter-tumour heterogeneity apparent in tumours growing at the same site in different animals was also observed in individual tumours growing within the same animal (Figure 2). Multiple spontaneously arising tumours in the mammary glands of an individual transgenic MMTV-PyMT mouse demonstrated a wide range of IFP values (5-20 mmHg, Figure 2A). Similarly, when mammary tumours derived from the same donors were directly transplanted orthotopically into both the left and right 4th mammary gland of recipient mice, the subsequent tumours showed divergent IFP values (Figure 2B). However, the range of IFP values seen in
spontaneously arising mammary gland tumours was comparable to the range of values seen in tumours transplanted orthotopically (Additional File 1).

To investigate whether transplant into different host mice impacted IFP levels, mammary gland tumours were transplanted into the mammary glands of either SCID (immune deficient) or FVB (syngeneic) recipient mice. Tumour IFP values were found to be similar irrespective of the mouse background (Figure 2E), suggesting that the tumour cell genetics may have more impact on the range of IFP values for a specific tumour type than the host genetics in experimental models.

Donor tumour IFP is not predictive of recipient tumour IFP

To ascertain whether the pre-existing tumour microenvironment and molecular interactions can impact on tumour development, donor tumour IFP values were measured prior to transplantation into the relevant orthotopic site in the ME180, CaSki, Me180, SiHa and MMTV-PyMT tumours. IFP values in the transplanted tumours showed no correlation with those in the donor tumours (Figure 2C). Although, although it should be noted for the donor ME180 and SiHa cervix tumour models the donor tumours were grown i/m prior to orthotopic transplantation, which might have confounded this comparison, the donor PyMT- ME180 and SiHa cervix tumour models the donor tumours were grown i/m prior to orthotopically, and there was no correlation between donor and recipient IFP values in the mammary fat pad either case. Furthermore, the mice bearing two MMTV-PyMT orthotopically transplanted mammary tumours showed disparities in tumour IFP measurements irrespective of whether they were initiated from different or the same donors (Additional File 2 & Figure 2D).
All these results are consistent with no relationship between IFP values in the donor and transplanted tumour irrespective of transplantation site.

**Correlation between tumour size and IFP is tumour specific**

Previous studies have noted a tumour specific correlation between tumour size and IFP [7, 26, 28, 42-45]. Although, the mean tumour size at the time of the IFP measurements was similar for all of the tumour models at all sites (Figure 3A), the use of tumours with different sizes permitted an analysis of the relationship between tumour size and IFP in a subset of tumours within most of the models (Figure 3B-F). The mean tumour size at the time of the IFP measurements was similar for all of the tumour models at all sites (Additional File 3A), with the exception of the orthotopic CaSki models where the tumours were significantly larger. There was a significant correlation between tumour size and IFP was only seen for KHT-C tumours growing i/m (p ≤ 0.02; Figure 3B) and in CaSki tumours growing orthotopically in the cervix (p ≤ 0.002; Figure 3C and A, and Additional File 3). Interestingly, when growing i/m, CaSki tumours did not show a significant correlation between size and IFP (Figure 3B), although the range of tumour sizes was narrower in this instance.

**High tumour IFP does not correlate with increased metastatic efficiency**

Cervical carcinoma patients with a high tumour IFP are treated with radiotherapy have shown an increased risk of incidence of distant recurrence after treatment with radiotherapy spread. [7, 9]. Furthermore, Rofstad et al (2002) [20] showed experimentally that oxic melanoma tumours with a high IFP showed a significantly increased metastatic ability. However, the relationship between tumour IFP and metastatic disease has not been widely studied.
Consequently, we examined metastases formation presentation in each of the tumour models (apart from the SiHa model which did not metastasise from either the orthotopic or i/m site) but found no correlation between a high tumour IFP and enhanced metastatic potential (Figure 4A-G). For the 4A-E). It was not possible to determine whether there was any relationship between IFP and metastases presentation in the MMTV-PyMT model growing orthotopically or in the transgenic animals, due to the presence of multiple tumours with divergent IFP values in each animal, metastases data are plotted according to the mean IFP value of all the tumours within that animal.

Elevated tumour IFP is a consequence of complex pathophysiologic interactions between in the tumour vasculature and the interstitium. Factors that influence IFP may also influence micro-environment and there are other aspects of the tumour microenvironment, including the development of heterogeneity in tumour hypoxia, which have previously been shown to enhance metastatic ability [31, 32, 37, 46-50] and could potentially confound the in vivo analysis. Thus, in an attempt to elucidate the role of elevated pressure per se, KHT-C and CaSki tumour cells were also grown under conditions of high pressure in vitro prior to analysis of metastatic ability with an experimental metastases assay in vivo. No evidence for a correlation between exposure to elevated pressure and metastatic ability was observed for the either KHT-C or CaSki tumour lines (Additional File 3,4), consistent with the spontaneous metastases data. The other models were not investigated for this study, as they do not form a sufficient number of lung metastases following i/v injection.

Model specific differences in vascular density, tumour hypoxia and tumour proliferation in relation to tumour IFP
Differences in IFP from one tumour to the next may be due to differences in the underlying tumour microvascular and lymphatic architecture. Consequently, an immunohistochemical analysis was carried out using CD31 (vascular density) and LYVE-1 (lymphatic vessel density) staining. In addition, proliferation was assessed using the proliferation marker Ki67, as it has been suggested that genes involved in cellular proliferation may be upregulated in tumours with higher IFP values [51, 52][49, 50]. Tumour hypoxia, a common feature of the pathophysiological tumour microenvironment, was also examined using the hypoxic marker EF5.

The ME180 model growing either i/m or orthotopically was chosen for detailed characterisation, because this model is the most appropriate for an analysis of metastatic progression to the regional lymph nodes, the most clinically relevant metastatic site, as well as the lungs. In addition, previous studies have demonstrated that exposure of ME180 orthotopic tumour bearing mice to cyclic hypoxia enhances metastatic spread to the lymph nodes [31]. Two tumour sections taken 100 µm apart showed a good agreement for all of the markers (Figure 5E). The analysis of these sections indicated no significant differences in tumour hypoxia, proliferation, vascular area or lymphatic vascular area between ME180 tumours growing in the i/m site versus orthotopically. Furthermore, there were no significant differences between tumours with a high (above median) or low (below median) IFP for either site (Figure 5F-J). To assess intra-tumour variability, a random high field (20x) analysis was carried out for each parameter. The average values for all fields were analogous to the values obtained through whole image analysis. There were no significant differences between the tumours growing i/m versus orthotopically (Additional File 4A). Furthermore, although there was substantial
heterogeneity between fields within any one tumour section, the degree of intra-tumour heterogeneity was not related to tumour IFP (Additional File 4B-I).

Similar, more limited, analyses were carried out in the MMTV-PyMT model and the CaSki cervical carcinoma models, both growing in different sites. There were no significant differences in lymphatic or blood vascular area or hypoxic fraction for MMTV-PYMT or CaSki tumours across the different sites. Proliferation was seen to vary; however, there was no relationship to tumour IFP (Additional File 5A-D).

**Imaging** Me180 xenografts growing orthotopically in the cervix demonstrate regions of greater perfusion in ME180 than Me180 tumours growing orthotopically or in the i/m site.

High-frequency micro-ultrasound functional imaging and speckle variance analysis was used to detect blood flow and examine perfusion in Me180 Me180 tumours growing both orthotopically and i/m. Composite images of positive speckle signal associated with blood flow, overlaid on top of the greyscale B mode ultrasound anatomical image of the tumour, are shown for each site in Figures 6A & 6B. Analysis of cineloops from five different randomly selected image planes in regions throughout each tumour indicated that the mean perfused area for each tumour did not differ significantly according to site (Figure 6C, p=0.06) although there was a trend towards greater perfusion in the orthotopic tumours. However, the maximal perfused area for each tumour, taken as the plane with the highest perfused area of the 5 planes analysed, was significantly higher in the tumours growing orthotopically (Figure 6D, p=0.03; mean IFP 7 ± 3 mmHg) as compared to those growing i/m (mean IFP 34 ± 7 mmHg), suggesting that although on average they have similar levels of
perfusion, there are areas which are perfused to a significantly greater extent in Me180 tumours growing in the cervix.
Discussion

There is increasing interest in the growing literature on the adverse effects of elevated tumour IFP on drug delivery and treatment response. The aim of this study was to characterise this relatively unexplored parameter of the pathophysiological tumour microenvironment. The aim of this study was to characterise IFP values in a range of tumour models growing in different sites as a foundation for future experiments and to explore various phenotypic properties such as blood and lymphatic vascular density and tumour hypoxia that may impact on the development of tumour IFP. Systematic differences in tumour IFP as a function of both tumour type and implantation site were observed, consistent with an effect of predetermined genetic factors and interactions between the tumour and host microenvironments in regulating IFP. However, this explained only a portion of the total observed variability in IFP, possibly reflecting stochastic development and remodelling of the tumour vasculature as important determinants of tumour IFP.

IFP levels in individual tumours are influenced primarily by three fundamental pathophysiologic parameters: the trans-capillary and interstitial hydraulic conductivities and the capillary pressure [5, 53, 54]. In general, tumours are characterized by abnormal, highly permeable vessels and a relatively impermeable interstitium [6]. Fluid that leaks from the vessels accumulates in the interstitium and causes the pressure to rise. However, there is probably wide variability in each of these parameters among individual tumours, which contributes to heterogeneity in IFP values. In tumours where the trans-capillary conductivity is substantially less than interstitial conductivity, the IFP is low and much less than the capillary pressure. At the other extreme, where trans-capillary conductivity is high and interstitial conductivity is low, IFP becomes almost equal to the capillary pressure. This is thought to occur in many pre-clinical
tumour models [55] and possibly also in human malignancies. Variation in IFP among tumours then mainly reflects differences in the underlying capillary pressure and vascular blood flow resistance. Tumours with high flow resistance due to unregulated angiogenesis, high cell density or tumour growth in a confined space with vascular compression would be expected to have both high capillary and high interstitial pressure values. The important role of the vasculature in determining IFP is supported by studies of anti-vascular drugs that have shown reductions in tumour IFP with vascular regression or normalization [11, 39, 56].

In our study, interestingly, tumours grown orthotopically consistently demonstrated IFP values lower than those grown in the i/m ectopic site (Figure 1). This is in contrast to results described by Brekken et al. [30] who demonstrated higher IFP values in a human osteosarcoma line grown orthotopically as compared to s/c. This difference highlights the interaction between the tumour and the mechanical interplay of the surrounding normal tissue and the influence on IFP. For example, tumours growing in environment exerting a positive effect on the tumour IFP, as bone would be expected to have high blood flow resistance and high capillary pressure because of progressive vascular compression as cell mass increases in a confined, noncompliant space. In addition, there may be fewer normal lymphatic vessels in close proximity to the tumour, effectively reducing interstitial conductivity and driving up IFP until it equals the capillary pressure. We also provides a far more rigid, less elastic environment than that of the s/c region. In this instance less interstitial fluid would be required in order to elevate tumour IFP, driven primarily by the vascular pressure, which will also vary according to physiological parameters. In this study, tumours grown i/m demonstrated that tumours grown i/m have higher IFP values than those in tumours grown s/c, in the cervix, or in the mammary fat pads, probably reflecting similar pathophysiologic mechanisms. Overall, our results suggest
that three locations with little surrounding mechanical forces. More importantly, site-specific differences in the vascular development and remodelling, and consequently flow vascular pressure and resistance, as well as site-driven differences in the extracellular matrix, lead to systematic differences in IFP values. We speculate that heterogeneity in IFP values among individual tumours of the same type growing at the same site reflect the stochastic nature of unregulated angiogenesis in tumours and the random nature of the resultant vasculature, and its interactions (impacting on the elasticity of the interstitium) are also likely to impact tumour IFP.

In further support of these concepts, the MMTV-PyMT transgenic mammary gland tumours growing orthotopically in the mammary fat pad showed a range and median IFP values that were comparable to the spontaneously arising tumours. Also, there were no differences when tumours were transplanted into FVB or SCID hosts. However, when grown i/m, the IFP in the MMTV-PYMT tumours was significantly increased. Since the data showed no relationship between donor IFP and recipient IFP this suggests a site-specific effect. Tumours are characterised by an interstitium comprising a dense network of collagen fibres, as well as increased fibroblasts and infiltration of macrophages and other cells involved in an inflammatory response, which together act to elevate IFP [3].

Consistent with reports in the literature, we observed that tumour IFP can increase with tumour size, but this is tumour-specific. Tumour size and IFP were found to correlate with one another in only two models, both growing in their orthotopic site. Despite a relationship between the ranges of IFP values seen in spontaneous versus orthotopically transplanted MMTV-PyMT mammary-gland tumours, there was no correlation between donor tumour IFP and recipient tumour IFP. Similar findings in the cervical tumour models suggest individual tumour
development irrespective of any tumour microenvironment-induced previous molecular interactions.

There is substantial inter-tumour and inter-model heterogeneity in tumour IFP, a fact that is comparable to the high degree of heterogeneity apparent in other microenvironmental parameters, in particular tumour hypoxia. The observed heterogeneity in tumour IFP values is probably largely attributable to differences in vascular resistance and permeability, reflecting the stochastic development of angiogenesis and vascular recruitment. Both tumour angiogenesis and tumour interstitial hydraulic conductivity may reflect the genetics of the different tumour cells, as the data indicates that the different tumour models show different median IFP values. Some proteins, along with their receptor tyrosine kinases, have existed in the original donor tumour and also been linked with the regulation of tumour IFP, in particular VEGF-A [51] and PDGF-B [13, 52]. In view of the angiogenic and vascular normalisation properties of these proteins, it is likely any effects on IFP are achieved through vascular effects, although they have also been implicated in the “reactive stroma” [3]. Overall tumour IFP is probably a result of the combination of genetics, the mechanics of the recipient site, and host-tumour physiologic interactions.

It is interesting that although the tumour vasculature plays a vital role in the development of both tumour IFP and tumour hypoxia, there was no correlation between these two parameters, an effect that has also been observed both experimentally [26, 57, 53] and in the clinic [7]. In addition, there was no relationship between vascular area and IFP, with similar values being observed in all models. Furthermore, in our high frequency ultrasound speckle variance analysis of blood flow in a small number of tumours, vascular perfusion was also comparable between ME180 tumours growing in the cervix versus i/m, seen in all models. However, in our speckle
variance analysis of high frequency ultrasound images of a small number of tumours there was
evidence for differences in vascular perfusion between Me180 tumours growing in the cervix
versus i/m, despite showing comparable vascular density. The Me180 tumours growing in the
cervix, which consistently demonstrate considerably lower IFP values as compared to Me180
tumours growing i/m, showed regions that were perfused to a significantly greater extent. This
suggests that the mass of tumour cells, the interstitial physiology and resultant interstitial
hydraulic conductivity, as well as the elevated IFP in the i/m tumours brings about a level of
vascular compression thereby reducing tumour perfusion. The overall perfused area and vascular
density were comparable between both the i/m and orthotopic sites, yet the maximal perfused
areas differed; this implies the presence of relatively under-perfused areas in the orthotopic
tumours, possibly reflective of differences in intermittent blood flow.

There is some evidence in the literature that high primary tumour IFP is associated with a
higher incidence of metastatic disease in tumours is associated with a high IFP [7, 20, 23],
although Rofstad et al. (2002) suggest that it is neither necessary nor sufficient. In the present
study we found no indication of a relationship between high tumour IFP and metastatic disease
in any of the models in any of the sites. A controlled in vitro experiment designed to test the
influence of pressure on the metastatic ability of cancer cells without the potentially confounding
influence of other microenvironmental factors also showed no effect. These data are consistent
with clinical data where, although a high pre-treatment IFP in cervical carcinoma patients was
associated with a high risk of distant. Additionally, tumour models that showed higher median IFP
values did not demonstrate enhanced metastatic potential. For example, the SiHa tumour model
showed a significantly higher median IFP as compared to the Me180 and CaSki tumour models
and yet it was the only cervical carcinoma model that presented with no metastatic disease to
either the lymph nodes or the lungs. Indeed, the CaSki model which is arguably the most metastatic shows the lowest IFP values. Similarly, the KHT-C model, which is highly metastatic, demonstrated a significantly lower median IFP growing i/m than any of the other models.

**Conclusions**

In conclusion, we have carried out an analysis of tumour IFP across a selection of different tumour models growing in different sites. We present evidence for inherent inter-tumour heterogeneity, lack of correlation between tumour donors and recipients as well as between different tumours growing within the same animal, data that are suggestive of a strong stochastic element reflecting the underlying vasculature in the development of tumour IFP. However, there is also a specific effect on tumour IFP, as well as an apparent genetic component in view of the divergent medians and ranges of IFP values seen in the different models. Furthermore, any correlation between tumour size and IFP is model specific, and there was no correlation between IFP and tumour hypoxia, tumour proliferation, or metastases presentation in these models. These data show a good agreement with the clinical data where, although a high pre-treatment IFP in cervical carcinoma patients associated with a high risk of recurrence with metastases following treatment with radiotherapy, there was no correlation between IFP and metastatic disease at the time of diagnosis [7]. It is possible that tumour IFP may be more a marker of treatment response than have a direct causative effect on metastatic spread. The data suggest that factors influencing tumour IFP are complex, and in need of further study. The high degree of inter-tumour heterogeneity apparent in these models, plus the knowledge gained with regard to the “baseline” in relation to IFP, provides the opportunity for further studies examining the role of IFP in treatment response, and whether change in IFP can be
predictive of therapeutic efficacy, or of alterations within the microenvironment that may impact efficacy, studies with a strong translational element in view of the increasing importance of tumour IFP in therapeutic response. Given the marked variability in IFP as a function of tumour type and implantation site, future studies may be more effective using tumours grown orthotopically, which are more relevant to both host-tumour specific interactions and clinical interpretation.

Conclusions

The data presented provide, for the first time, a specific analysis of tumour IFP across a selection of different tumour models encompassing human, murine transplanted and spontaneous tumours, growing in different sites. We demonstrate inherent inter-tumour heterogeneity, and a lack of correlation both between tumour donors and recipients and between different tumours growing within the same animal. Our findings indicate systematic differences in IFP as a function of tumour type consistent with predetermined inherent genetic differences that influence vascular development and the composition and organization of the interstitium. There are also systematic differences as a function of growth site, presumably reflecting the interaction between the tumour and the surrounding host normal tissue. Nevertheless, the heterogeneity of IFP in individual tumours growing under similar conditions suggests that IFP is probably influenced to a large extent by the stochastic nature of vascular development and remodelling during tumour growth. In this context it is surprising that an analysis of various phenotypic parameters failed to show any correlation between IFP and tumour hypoxia, tumour proliferation, blood or lymphatic vascular density. Whether the lack of correlation in these parameters is indicative of no
involvement in the development of tumour IFP remains uncertain, however, since the parameters are interdependent and may act together to elevate IFP. It is clear that factors influencing tumour IFP are complex and that further study is required to reveal the mechanisms that elevate IFP and its importance in therapeutic response either as a standalone factor or in combination with other factors.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SJL participated in the design of the study, performed the animal experiments, analysed the IFP data and histology slides, and wrote the manuscript. TMKK was responsible for the breeding of the MMTV-PyMT transgenic mice, aided in the development of the mammary tumour surgical transplant technique, and contributed to the manuscript. AB, aided by VXY, was responsible for the analysis of the Ultrasound speckle variance data, and contributed to the manuscript. MM and RPH participated in the design of the study and were involved in writing the manuscript.

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References


**Figures**
**Figure 1 - Comparison of IFP values between different growth sites and different models.**

Mean IFP is shown for each tumour line growing orthotopically and ectopically; KHT-C i/m (n=104) and s/c (n=12) tumours, MMTV-PyMT i/m (n=15), transgenic spontaneous mammary gland (n=27), and transplanted orthotopic mammary gland (n=62) tumours, ME180CaSk i/m (n=69), orthotopic cervix (n=67) and s/c tumours (n=6), Me180 i/m (n=26) and orthotopic cervix (n=28) tumours, SiHa i/m (n=9) and orthotopic cervix (n=10) tumours. The error bars represent the standard error of the mean. An asterisk indicates that a group is significantly (p≤0.05) different to the other groups.

**Figure 2 - Inter-tumour variability within the same animal.**

A) The mean IFP value (mmHg) for each individual mammary gland tumour arising in four different MMTV-PyMT transgenic mice is shown. The bar represents the median IFP value (mmHg) for each mouse. There was substantial variation between different tumours within the same mouse. B) The mean IFP value (mmHg) for each of two orthotopic MMTV-PyMT tumours, one transplanted into the left and one into the right 4th mammary gland, is shown. The tumours from the same donor animal are connected by a solid line. Again, there was obvious variation in the IFP values of different tumours growing within the same animal. C) Recipient tumour IFP (mmHg; y-axis) plotted as a function of the mean donor IFP (mmHg; x-axis). The line of identity is shown. There was no correlation between donor and recipient IFP. D) The difference in the mean IFP values of two different tumours growing in the left and the right mammary glands is shown, grouped according to whether the two tumours were initiated from the same or different donor tumours. The median IFP for each group is indicated; there was no significant difference (Mann Whitney test p≥0.05).

E) The mean tumour IFP is shown for each individual MMTV-PyMT mammary gland tumour.
transplanted into either SCID or FVB mouse backgrounds. The bar indicates the median IFP values. The IFP values were comparable for both mouse backgrounds.

**Figure 3 - Tumour size and IFP values.** Graph A shows the mean tumour size at the time of IFP measurement for each model. Error bars represent standard error of the mean, and a significant difference is indicated by an asterix. There were no significant differences in mean tumour size between models, with the exception of SiHa tumours growing in the cervix, which were significantly larger than SiHa or KHT-C tumours growing i/m. Graphs B-F show the mean IFP (mmHg, y-axis) of individual tumours plotted against the tumour size (g or mm²; x-axis). The R² value is shown on each graph. B) KHT-C i/m tumours, C) CaSki i/m tumours, D) ME180 cervix tumours, E) SiHa cervix tumours, F) MMTV-PyMT transplanted orthotopic mammary gland tumours, G) MMTV-PyMT i/m tumours. There was only a correlation between tumour size and IFP in KHT-C tumour model growing i/m. CaSki cervix tumours.

**Figure 4 - Metastases presentation as a function of tumour IFP.** The number of macro- or microscopic lung lesions or involved lymph nodes counted (A-D, F), or the total area of lung metastases measured using DsRed fluorescence (E) is shown for different tumour models. The data is presented as number of metastatic lesions/involved lymph nodes (y-axis) of each point shows the metastases presentation for an individual tumours plotted against mean animal, grouped according to whether the primary tumour IFP (mmHg; x-axis). The R² value (mmHg) was above (high IFP) or below (low IFP) the median IFP (A-E). The median is shown on for each graph. There was no significant correlation for any of the models. A) KHT-C i/m, B) KHT-C s/c tumours, C) MMTV-PyMT i/m tumours, D) MMTV-PyMT MG tumours (total
mean IFP shown), E) ME180 i/m tumours, F) ME180 Me180 cervix & i/m tumours (lung metastases), G) ME180. D) Me180 cervix tumours (lymph node metastases). E) CaSki cervix and i/m tumours.

Figure 5 - Immunohistochemical analyses of tumour hypoxia, proliferation and vascular area. A representative image for each marker is shown (10x magnification; ME180 Me180 tumour growing i/m) A) EF-5, B) Ki67, C) CD31, D) LYVE-1. E) Sections were taken on two levels, 100 µm apart to assess intra-tumoural heterogeneity. The correlations between levels 1 (L1) and 2 (L2) are shown. Each point represents an individual tumour (includes data from PyMT and CaSki tumours; see Additional File 5), and the legend indicates the tumour model and site of growth. The line of identity is shown. Quantitative analysis of two sections from each tumour; bars represent the median percentage of positive staining [(positive pixels/total number of pixels (positive + negative)) x 100] and error bars show the range. F) Each marker according to whether the tumour was grown in the cervix (n=9) (n=10) or i/m (n=9) (n=10). G-J) Each marker in both growth sites split according to whether the tumour IFP was above (high; n=5) or below (low; n=5) the median IFP; F) EF-5, G) Ki67, H) CD31 and I) LYVE-1.

Figure 6 - High-frequency micro-ultrasound functional imaging. Representative composite images of ME180 Me180 tumours growing A) in the cervix and B) i/m are shown. Positive speckle signal associated with blood flow is overlaid on top of the greyscale B mode ultrasound image. C) The tumour mean (from the 5 frames) percentage positive signal (blood flow) and D) the highest percentage positive signal (greatest percent perfused area) detected for each tumour are shown for ME180 Me180 tumours growing either in the cervix or i/m. Each point represents
an individual tumour. The median is indicated on the graph. An asterix indicates significance (p≤0.05).

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**Additional files**

**Additional File 1 – Table showing tumour model IFP ranges, and mean and median values.**

Table showing the range of mean tumour IFP values (mean of 3 measurements for each tumour) for each tumour model growing orthotopically and ectopically. The group mean and median IFP value and the number of tumours measured is shown (n=x). The values for tumours growing in their orthotopic site are in bold.

**Additional File 2 - Map of donor sites and corresponding recipient sites for MMTV-PyMT orthotopic mammary gland implantation.** Donor tumour fragments were taken from either the 2nd or 4th mammary gland. The tumour fragments were implanted into the 4th mammary glands of recipient mice in one of three options: **Option 1)** Both the right and left 4th mammary glands were implanted with donor fragments from the 2nd mammary gland; **Option 2)** The 2nd mammary gland donor was implanted in one side of the recipient animal, and the 4th mammary gland donor in the other; **Option 3)** Both the right and left 4th mammary glands were implanted with donor fragments from the 4th mammary gland.

**Additional File 3**

**Additional File 3 – Tumour size and IFP values.** Graph A shows the mean tumour size at the time of IFP measurement for each model. Error bars represent standard error of the mean, and a significant difference is indicated by an asterix. There were no significant
differences in mean tumour size between models with the exception of the CaSki cervix tumour model. Graphs B-E show the mean IFP (mmHg, y-axis) of individual tumours plotted against the tumour size (g or mm², x-axis). The R² value is shown on each graph. B) Me180 cervix tumours, C) SiHa cervix tumours, D) MMTV-PyMT transplanted orthotopic mammary gland tumours, E) MMTV-PyMT i/m tumours. There was no correlation between tumour size and IFP in these models.

**Additional File 4 - Metastatic potential of tumour cells exposed to elevated pressure in vitro.** Tumour cells were exposed to elevated pressures in vitro for 24, 48 or 96 hours prior to intra-venous injection in vivo. The lung wet weight (g) as an indication of tumour burden (A), or the number of macroscopic lung lesions counted (B-C) (B-D) is shown for each animal, grouped according to treatment group. The median is indicated for each group. A) KHT-C cells exposed to 20 mmHg for 24 hours, B) 48 hours or C) 96 hours. D) CaSki cells exposed to 50 mmHg for 24 hours.

**Additional File 4 - Immunohistochemical analyses of intra-tumoural heterogeneity of tumour hypoxia, proliferation and vascular area.**

Analyses were carried out on ME180 tumours growing either in the cervix (n = 9) or i/m (n = 9) (see Figure 5); 10 (CD31) or 20 (EF5, Ki67, LYVE-1) random fields were analysed for each tumour section. A) A value for each tumour was generated as the mean percentage of positive staining [(positive pixels/total number of pixels (positive + negative)) x 100] for all fields analysed. The data are presented as median for all tumours and error bars show the range. The median is shown for each marker according to whether the tumour was grown in the cervix (n=10) or i/m (n=10). B-I) The median % positive staining and range for all 20 (or 10 for CD31)
frames analysed is shown for each tumour (y-axis) plotted against tumour IFP (mmHg, x-axis);

B) EF-5 cervix, C) EF5 i/m, D) Ki67 cervix, E) Ki67 i/m, F) CD31 cervix, G) CD31 i/m, H) LYVE-1 cervix and I) LYVE-1 i/m.

Additional File 5 - Immunohistochemical analyses of tumour hypoxia, proliferation and vascular area. Tumour sections from the PyMT two different tumour model growings in three different sites each were stained for EF5 as a marker of hypoxia, Ki67 as a marker of proliferation, CD31 as a marker of tumour vasculature and LYVE-1 as a marker of lymphatic vasculature. The median percentage of positive staining [(positive pixels/total number of pixels (positive + negative)) x 100] and the range is shown for each tumour model and site of growth; A) EF5 (n=3 for all groups), B) Ki67 (MMTV-PyMT transgenic n=3, MMTV-PyMT orthotopic n=6, MMTV-PyMT i/m n=4, CaSki i/m n=3, CaSki cervix n=3, CaSki s/c n=4), C) CD31 (MMTV-PyMT transgenic n=3, MMTV-PyMT orthotopic n=8, MMTV-PyMT i/m n=3), n=3, CaSki i/m n=3, CaSki cervix n=3, CaSki s/c n=4), D) LYVE-1 (N=3 for all groups). An asterix indicates significance (p≤0.05).