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Circulating tumour cells demonstrate an altered response to hypoxia and an aggressive phenotype

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BACKGROUND: Tumours contain hypoxic regions that select for an aggressive cell phenotype; tumour hypoxia induces metastasis-associated genes. Treatment refractory patients with metastatic cancer show increased numbers of circulating tumour cells (CTCs), which are also associated with disease progression. The aim of this study was to examine the as yet unknown relationship between hypoxia and CTCs.

METHODS: We generated human MDA-MB-231 orthotopic xenografts and, using a new technology, isolated viable human CTCs from murine blood. The CTCs and parental MDA-MB-231 cells were incubated at 21 and 0.2% (hypoxia) oxygen, respectively. Colony formation was assayed and levels of hypoxia- and anoxia-inducible factors were measured. Xenografts generated from CTCs and parental cells were compared.

RESULTS: MDA-MB-231 xenografts used to generate CTCs were hypoxic, expressing hypoxia factors: hypoxia-inducible factor1 alpha (HIF1α) and glucose transporter protein type 1 (GLUT1), and anoxia-induced factors: activating transcription factor 3 and 4 (ATF3 and ATF4). Parental MDA-MB-231 cells induced ATF3 in hypoxia, whereas CTCs expressed it constitutively. Asparagine synthetase (ASNS) expression was also higher in CTCs. Hypoxia induced ATF4 and the HIF1α target gene *apelin* in CTCs, but not in parental cells. Hypoxia induced lower levels of carbonic anhydrase IX (CAIX), GLUT1 and BCL2/adenovirus E1B 19-KD protein-interacting protein 3 (BNIP3) proteins in CTCs than in parental cells, supporting an altered hypoxia response. In chronic hypoxia, CTCs demonstrated greater colony formation than parental cells. Xenografts generated from CTCs were larger and heavier, and metastasised faster than MDA-MB-231 xenografts.

CONCLUSION: CTCs show an altered hypoxia response and an enhanced aggressive phenotype *in vitro* and *in vivo*.

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Metastasis is a multi-step process leading to the spread of neoplastic cells to distant sites. Circulating tumour cells (CTCs) have been detected in the peripheral blood of cancer patients and provide an index of disease aggression in patients with distant metastasis (Dawood *et al*, 2008). During the metastatic process, CTCs are generated from primary tumour, and subsequently invade and colonise distant organs (Gupta and Massague, 2006). Tumours are heterogeneous and contain regions of hypoxia and anoxia, which may select for cells with more aggressive phenotypes and diminished apoptotic potential (Graeber *et al*, 1996; Zhang and Hill, 2004). The tumour hypoxic microenvironment contributes to increased metastases by regulating the number of genes that are implicated in the metastatic process. Clinical evidence also suggests that patients with tumours that contain more extensive hypoxic and anoxic areas have higher rates of metastases (Brizel *et al*, 1996). Therefore, tumour hypoxia and anoxia may be important in modulating and determining the metastatic ability of CTCs, as well as their progression once secondary tumour metastases are established.

Cells respond to hypoxia and anoxia differently. A major response of cells to hypoxia is the induction of hypoxia-inducible factor 1 (HIF1), which is associated with tumour progression and metastasis (Liao *et al*, 2007). Hypoxia-inducible factor 1 is a heterodimeric protein that is composed of a constitutively expressed HIF1β subunit and an oxygen (O_2)-regulated HIF1α subunit. Both the stability and transcriptional activity of HIF1 are negatively regulated by O_2-dependent hydroxylation. Under conditions of higher O_2 supply, including normoxia, which are used in tissue culture studies (O_2 levels of 21%), HIF1α is synthesised and hydroxylated on proline residue 402 and/or 564 by prolyl hydroxylase domain proteins that use O_2 and α-ketoglutarate as substrates. Prolyl hydroxylation is required for the binding

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of the von Hippel–Lindau protein, which then targets HIF1α for degradation by the proteasome (Epstein *et al*, 2001). Hypoxia-inducible factor 1α is also hydroxylated on asparagine residue 803 by Factor Inhibiting HIF-1 (FIH-1), which negatively regulates the transactivation function (Mahon *et al*, 2001). With decreasing O_2 levels, the rate of HIF1α hydroxylation is reduced because of substrate (O_2) deprivation. This results in the accumulation and activation of HIF1α, leading to its dimerisation with HIF1β, forming HIF1. Hypoxia-inducible factor 1 then induces target genes that include several adaptive, metastatic, and pro-apoptotic proteins, such as glycolytic enzymes, glucose transporter protein type 1 (GLUT1), lysyl oxidase (LOX), and BCL2/adenovirus E1B 19KD protein-interactive protein 3 (BNIP3), respectively (Semenza *et al*, 1996; Sowter *et al*, 2001; Erler *et al*, 2006).

The anoxia pathway is distinct from the hypoxia–HIF1α pathway. Recent studies suggest that extremely low levels of O_2, including anoxia, lead to endoplasmic reticulum stress response (ERSR) and activation of a signal transduction pathway known as the unfolded protein response, which is HIF1α independent. Endoplasmic reticulum stress response triggers a complex programme that results in the reduction of mRNA translation. However, it specifically increases the translation of factor activating transcription factor 4 (ATF4) that protects cells from damaging effects of ER stress (Blais *et al*, 2004). This same anoxia pathway can also be activated by nutritional deficiencies such as glucose and/or amino acid deprivation, and can lead to ERSR and translational increase of ATF4 (Harding *et al*, 2000). In addition to an increase in mRNA translation of factors related to the unfolded protein response pathway, such as ATF4, cancer cells also respond to anoxia via other multiple HIF1α-independent pathways, including the mitogen-activated protein kinase pathway and protein stabilisation pathways, which result in the induction of factors such as ATF3 (Ameri *et al*, 2007; Nemetski and Gardner, 2007) and ATF4 (Ameri *et al*, 2004; Koditz *et al*, 2007; Rzymski *et al*, 2008).

Although the response of cells to hypoxia has been studied in detail and the inter-relationships between hypoxia and anoxia pathways are beginning to emerge, the relationship between CTCs and tumour hypoxia has not been investigated. In humans, triple negative (oestrogen receptor negative, progesterone receptor negative, human epithelial growth factor receptor 2, HER2 negative) breast cancer is an aggressive tumour phenotype that commonly metastasises and for which there is no targeted therapy. The aggression of these tumours may be due to a pronounced hypoxic response (Tan *et al*, 2009). Using the human triple-negative breast cancer cell line MDA-MB-231, we generated orthotopic xenografts in mice that produced CTCs (Eliane *et al*, 2008) and resulted in lung metastases. Xenografts were profoundly hypoxic and produced CTCs that could be captured and cultured using a new technology developed by our laboratory. We examined the response of CTCs and parental MDA-MB-231 cells to hypoxia (O_2 levels of 0.2%) and compared the ability of both cell types to develop tumour xenografts *in vivo*.

MATERIALS AND METHODS

Xenograft generation

NOD.CB17–*Prkdc*^{scid}/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME and Sacramento, CA, USA). Animals were housed in micro-isolator cages with autoclaved bedding, and fed autoclaved food and water. All experiments were approved by Stanford's Institutional Animal Care and Use Committee and in accordance with all Administrative Panel on Laboratory Animal Care (APLAC) regulations at Stanford University and were in compliance with the National Institutes of Health Guide for Care and Use of Animals. A total of 20 NOD/SCID (non-obese diabetic–severe combined immunodeficiency) female mice had 8×10^6 MDA-MB-231 breast cancer cells in 100 μl phosphate-buffered saline (PBS; pH 7.4) plus 100 μl of matrigel (BD Biosciences, San Jose, CA, USA) injected into their left second mammary fat pads. Control mice were injected with PBS into the same location. At 55 days after injection, when the average tumour volume was 1.5 cm^3, mice were euthanised for isolation of CTCs. To assess the tumour formation capacity of CTCs, mammary fat pads of 10 NOD–SCID mice were injected with a total of 8×10^6 MDA-MB-231 cells or CTCs in a total volume of 200 μl, consisting of 100 μl PBS and 100 μl matrigel. Tumour size was measured over a period of 3–6 weeks.

Immunohistochemistry

After the mice were euthanised, their organs and tumours were fixed in formalin, routinely processed, embedded in paraffin, and 5-μm sections were placed on glass slides for haematoxylin & eosin staining and immunohistochemistry (IHC). Microwave heat-induced epitope retrieval in citrate buffer was used for IHC as previously described (Higgins and Warnke, 1999). Tumour and lung sections were stained for the intrinsic marker GLUT1 (ab15309, Abcam, Cambridge, MA, USA), ATF3 (sc-188, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ATF4 (ab31390, Abcam), and HIF1α (ab51608, Abcam). At 4 h before being killed, mice were injected through the intraperitoneal route with the hypoxia marker pimonidazole, and tumour and lung sections were stained using Hypoxyprobe-1 (NPI, Belmont, MA, USA).

Isolation of circulating tumour cells

As soon as the mice were euthanised, blood was drawn by means of a cardiac puncture, recently shown to be the method of choice in obtaining CTCs from mouse blood (Eliane *et al*, 2008). Mouse blood (200–900 μl per mouse) was pooled and collected into a 2-ml EDTA tube (BD, Franklin Lakes, NJ, USA). Blood was diluted to 6 ml with PBS and labelled with magnetic beads functionalised with epithelial cell adhesion molecule antibody (Dynabeads, Invitrogen, Carlsbad, CA, USA). Magnetically labelled blood samples were transferred to a six-well plate and brought up to a final volume of 10 ml using PBS. The magnetically labelled samples were then processed by the newly developed MagSweeper that isolates CTCs by multiple rounds of magnetic capture, wash, and release as previously described (Talasaz *et al*, 2009). Briefly, magnetic capturing rods covered by plastic sheaths were robotically swept through wells containing magnetically labelled mouse blood. Circulating tumour cells captured on the covered magnetic rods were transferred and washed in a well containing PBS, and then released into another well containing PBS by removal of the magnetic rod from its sheath. After a second cycle of capture, wash, and release, individual CTCs were microscopically visualised and transferred to a new six-well plate containing Dulbecco's modified Eagle's medium high glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (vol/vol) foetal calf serum, penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}). The purified CTCs in the six-well plates were cultured in a 5% CO_2 incubator at 37°C.

Cell culture and hypoxic conditions

Hypoxia was generated using the anaerobic jar HP0011A (Oxoid, Cambridge, UK and distributed by Remel, Lenexa, KS, USA) and oxygen levels were monitored using the Analox Mini O_2 DII monitor (Amron International, Vista, CA, USA). Cells were maintained in Dulbecco's modified Eagle's medium high glucose (Invitrogen) supplemented with 10% (vol/vol) foetal calf serum, penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}). The

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passage number of CTCs was <7. For hypoxia experiments, 3×10^5 of MDA-MB-231 cells or CTCs were seeded in 5-cm plates and incubated with 70–80% confluency in the humidified jar. A gas mixture of 5% CO_2/95%N_2 was purged into the jar for 20 min until the O_2 monitor indicated <0.1% O_2, after which additional gas was purged into the jar for an extra 20 min. When experiments were terminated after 24 h, the O_2 monitor indicated oxygen levels of 0.2%.

Colony formation assays

A total of 400 cells were seeded in 5-cm Petri dishes and incubated for 10 days in either normoxia or hypoxia ($n = 5$ for each condition). After 10 days, the colonies were rinsed with PBS and stained with Giemsa stain-modified solution (Sigma-Aldrich, St Louis, MO, USA) for 20 min. Stained colonies were rinsed with water, and visually visible colonies, which consisted of approximately 30 cells, were counted.

Immunoblot

The ATF3, ATF4 and GLUT1 antibodies used for western analysis were the same as those used for immunostaining. The HIF1α antibody used for immunoblot analysis was from BD transduction laboratories (610958, BD Biosciences). CAIX (ab15086) and BNIP3 (ab10433) were from Abcam. The apelin antibody (sc-33804) was from Santa Cruz Biotechnology. Cell extracts were generated in a cold room (4°C). The cell lysis buffer used for preparing total cell extracts was a urea-denaturing buffer (6.7 M urea, 10 mM Tris–HCl (pH 6.8), 5 mM dithiothreitol, 1% sodium dodecyl sulphate, and 10% glycerol) supplemented with Complete mini-protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN, USA). Cultured cells were washed rapidly once with ice-cold PBS. A volume of 100–200 μl of urea lysis buffer was added directly to the plates of cells, scraped into an Eppendorf tube, and sonicated on ice by using a Vibra Cell ultrasonic processor (Sonics&Materials, Newton, CT, USA). A detergent-compatible bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA) was used to estimate the protein concentration of extracts according to the manufacturer's protocol. Total cell extracts (30–60 μg per lane) were subjected to reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis by first mixing equal volumes of protein extract and Laemmli sample buffer containing dithiothreitol and β-mercaptoethanol and running them on a 12% readymade gel system (Bio-Rad, Hercules, CA, USA). The resolved proteins were then electroblotted (semidry) onto immune blot polyvinylidene fluoride membrane (Bio-Rad). Bound antibodies were detected using the chemiluminescent substrate ECL + Plus (Amersham Biosciences, Buckinghamshire, UK).

RESULTS

Hypoxic tumour xenograft model

Tumour hypoxia and anoxia have been shown to select for an aggressive subtype of tumour cells. Such cells would metastasise to distant organs through the blood stream. We hypothesised that tumours containing regions of hypoxia and anoxia would give rise to CTCs that, on the basis of their selection by hypoxia, should be more aggressive than non-CTCs, and would show an altered response to hypoxia. To examine this, we first generated a tumour model containing profound regions of hypoxia and anoxia. We used the human breast cancer cell line MDA-MB-231 to make orthotopic xenografts and grew them to an average tumour volume of 1.5 cm^3. Staining for pimonidazole (Figure 1A) and the intrinsic hypoxia marker GLUT1 (Figure 1B; Airley *et al*, 2003) confirmed that all tumours contained hypoxic regions, particularly around necrotic areas. Although GLUT1 expression and pimonidazole uptake were more pronounced in tumour cells within perinecrotic

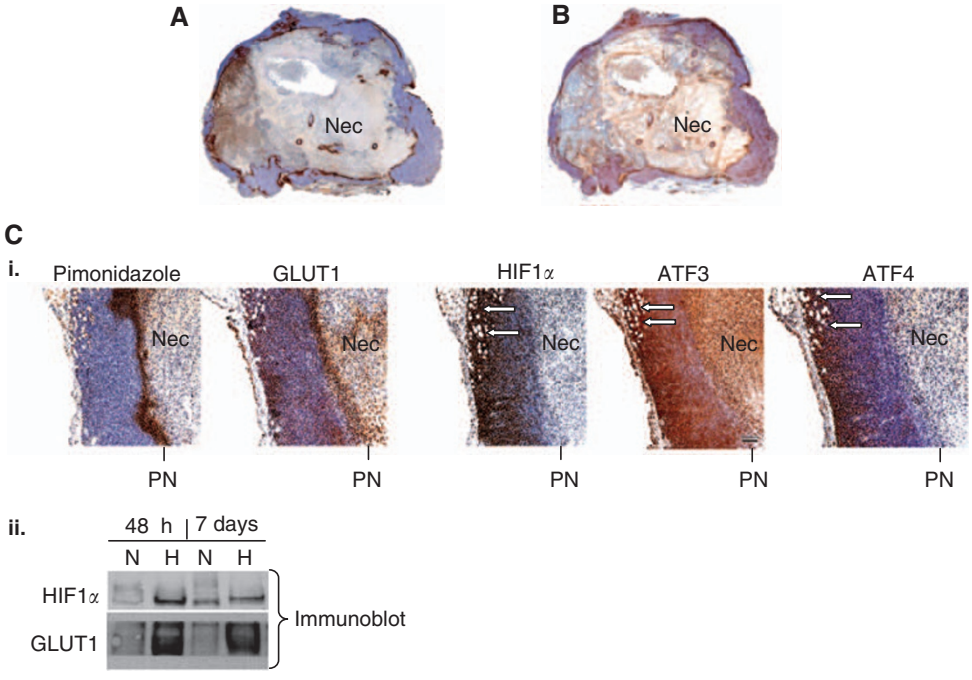

Figure 1 Human breast cancer MDA-MB-231cells grown in mammary fat pad of NOD/SCID (Non-obese diabetic–severe combined immunodeficiency) mice display hypoxic/anoxic regions and express hypoxia and anoxia factors. (**A**) Tumour sections stained for pimonidazole and (**B**) the intrinsic marker GLUT1 (× 10 magnification). (**C**i) HIF1α, ATF3, and ATF4 expression in tumours and at the leading edge at sites of local invasion into surrounding normal tissues (white arrows, × 100 magnification). (**C**ii) Expression of HIF1α and GLUT1 in MDA-MB-231 cells after 7 days of chronic hypoxia *in vitro*. N, normoxia; H, hypoxia; Nec, necrotic region; PN, perinecrotic region.

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regions, the distribution of HIF1α, ATF3, and ATF4 expression rarely coincided with the pimonidazole and GLUT1 staining pattern. (Figure 1Ci).

As *GLUT1* is an HIF1α target gene, one would expect a similar pattern of expression. As tumour cells in perinecrotic regions are more likely to experience chronic hypoxia, we wanted to determine whether MDA-MB-231 cells subjected to chronic hypoxia *in vitro* would also demonstrate disparate induction of HIF1α and GLUT1. Whereas HIF1α and GLUT1 were both induced in acute hypoxia (48 h), chronic hypoxia (7 days) induced only GLUT1 but not HIF1α (Figure 1Cii).

Our xenograft model, based on injection of large numbers of tumour cells (8×10^6) and an extended growth period (55 days), created profound regions of hypoxia and anoxia in the primary tumour. Lung metastases developed in all mice. Hypoxia and anoxia factors expressed in primary tumours were also expressed in pulmonary metastases (Figure 2).

Distinctive expression of anoxia-induced factors in hypoxic CTCs

To determine whether CTCs have an altered hypoxia response, they had to be isolated and grown in culture. We demonstrated that our MDA-MB-231 xenograft model shed CTCs into mouse circulation using a new technology that enabled viable human CTC capture from mouse blood (Talasaz *et al*, 2009). Magnetic beads attached to the epithelial cell adhesion molecule antigen on CTCs appeared as dark clusters of beads on the cell membrane surrounding translucent cells. In contrast, there were no dark cell/magnetic bead clusters visible in the blood of control mice. Instead, only small dark circles representing individual magnetic beads were observed in control mouse blood (Figure 3Ai). Captured CTCs were viable and could be subsequently grown in culture (Figure 3Aii).

It is known that anoxia exerts a selection pressure for aggressive cells with diminished apoptotic potential. Therefore, anoxia-selected cells *in vivo* could enter the blood stream as CTCs and demonstrate a distinct expression and/or induction of anoxia factors. To examine this, we compared anoxia factor expression in CTCs with that of parental MDA-MB-231 cells.

The CTCs demonstrated greater expression and/or induction of the anoxia-induced factors ATF3 (Figure 3B), ATF4 (Figure 3C and D), and the ATF4 target gene, *ASNS* (Figure 3D), than parental MDA-MB-231 cells. Western blot analysis demonstrated that ATF3 protein was expressed constitutively higher in CTCs compared with MDA-MB-231 cells, and hypoxia did not dramatically induce further ATF3 expression as confirmed by densitometry measurements. In contrast, MDA-MB-231 cells induced ATF3 2.4-fold in hypoxia (Figure 3B). Western blot analysis and densitometry measurements demonstrated that the ATF4 protein was not induced in hypoxic MDA-MB 231 cells but was induced 2.4-fold in hypoxic CTCs (Figure 3C). Western blot analysis showed that the ATF4 target gene *ASNS* was also induced to a greater extent in hypoxic CTCs than in hypoxic MDA-MB-231 cells (Figure 3 D). At a higher cell passage number (passage >7), CTCs demonstrated some reversion back to the parental phenotype with respect to ATF3 expression and increased induction in hypoxia (data not shown). In summary, CTCs demonstrated an anoxia phenotype by constitutively expressing and/or inducing anoxia factors at higher levels compared with parental MDA-MB 231 cells under normoxia and/or hypoxia.

Distinctive expression of hypoxia-induced factors in CTCs

In addition to anoxia factor expression, tumour aggression has been related to enhanced expression of hypoxia-induced factors. Therefore, we questioned whether the hypoxic response of CTCs is altered. To examine this, we compared the expression of hypoxia

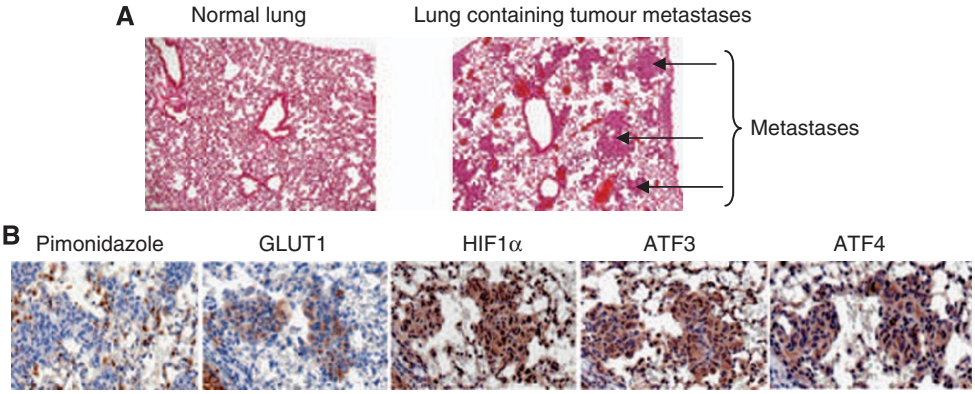

Figure 2 MDA-MB-231 tumours result in pulmonary metastases, which express the hypoxia factors HIF1α and GLUT1, as well as the anoxia factors ATF3 and ATF4. (**A**) Lungs of xenograft-bearing mice demonstrated metastatic foci, as indicated by the solid arrows on haematoxylin & eosin (H&E) slides. Lungs from control mice did not contain any tumours (× 100 magnification). (**B**) Expression of HIF1α, GLUT1, ATF3, and ATF4 in tumour cells in pulmonary metastatic foci.

Figure 3 Circulating tumour cells (CTCs), isolated and cultured from murine blood, demonstrate a distinct response to hypoxia compared with parental MDA-MB-231cells. (**A**i) Freshly captured CTCs are cells with attached magnetic beads as indicated by white arrows. The magnetic beads are individual small dark circles, as can be seen in the control; CTCs covered with magnetic beads appear as dark clusters of beads on the cell membrane with translucent centres, as indicated by white arrows; lack of CTCs in blood samples obtained from six control mice without xenografts. (**A**ii) The magnetically captured CTCs in culture on day 10 after capture and after day 60. (**B**–**F**) Induction of HIF1α in hypoxic MDA-MB-231 cells and CTCs. (**B**) Expression of the anoxia factor ATF3 in CTCs and MDA-MB-231 cells. Graphs demonstrate densitometry measurement of ATF3 expression normalised to actin expression from three experiments. (**C**) Induction of the anoxia-induced factor ATF4 in CTCs compared with MDA-MB-231 cells. Graphs demonstrate densitometry measurements of ATF4 expression normalised to actin expression from three experiments. (**D**) Expression of ATF4 and its target gene *ASNS* in CTCs, compared with parental MDA-MB-231 cells. (**E** and **F**) Differences between CTCs and parental MDA-MB-231 cells with respect to induction of HIF1α target genes. (**E**) Induction of apelin in hypoxic CTCs compared with parental MDA-MB-231. Hypoxic induction of (**E**) GLUT1 and (**F**) CAIX, BNIP3 in CTCs, compared with parental MDA-MB-231 cells. Experiments were performed at least three or four times with three or four different extracts. N, normoxia; H, hypoxia.

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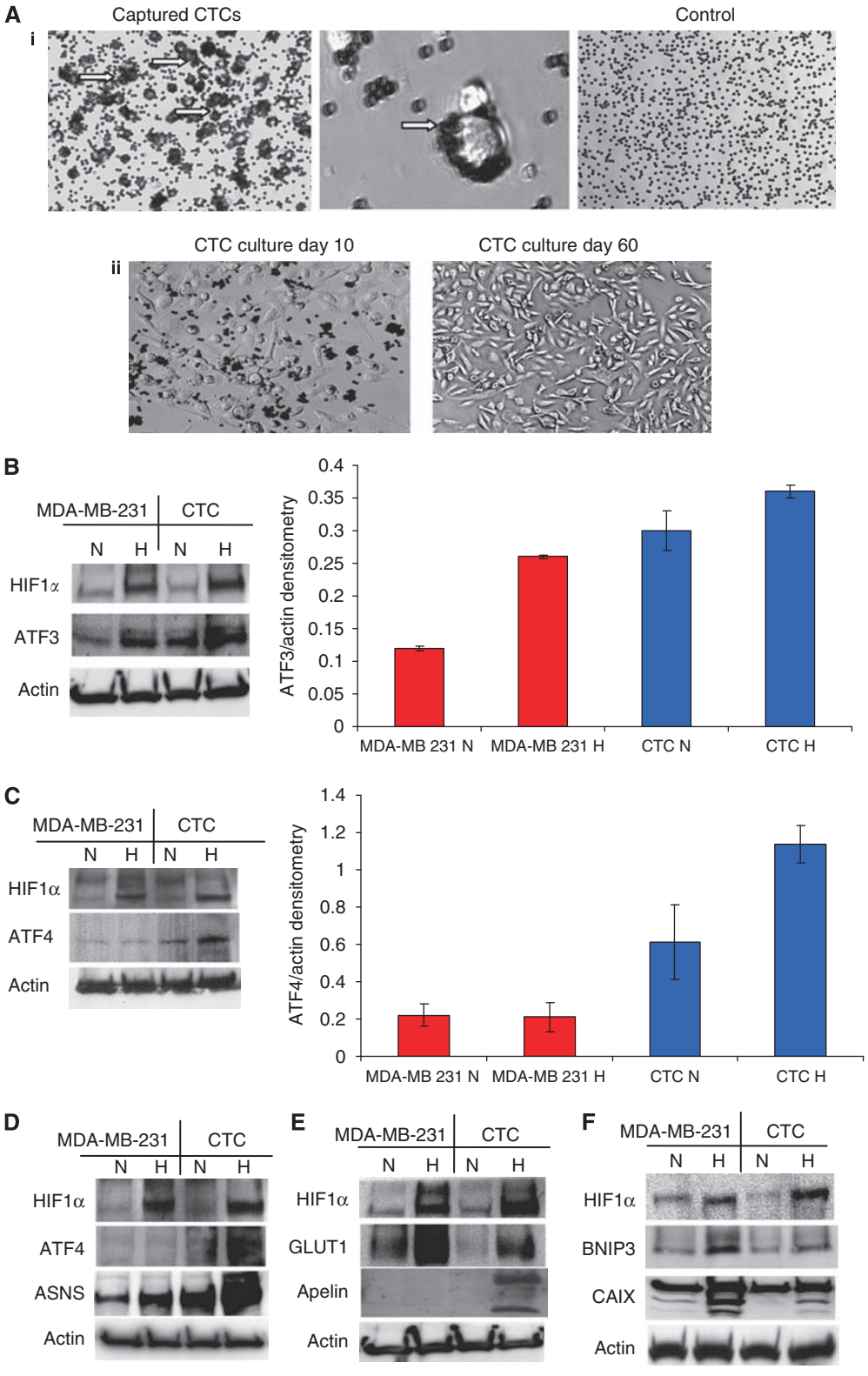
factors in CTCs and parental MDA-MB-231 cells. Although MDA-MB-231 cells and CTCs did not differ in their response to hypoxia with respect to HIF1α induction, CTCs demonstrated a distinct expression of proteins regulated by HIF1α under hypoxic conditions. Apelin was induced at greater levels in CTCs than in parental MDA-MB-231 cells (Figure 3E). In hypoxia, CTCs did induce GLUT1 (Figure 3E), CAIX and BNIP3 proteins (Figure 3F) but the inductions were lower than those in parental MDA-MB-231 cells. In summary, CTCs demonstrated an altered response to hypoxia, showing both pronounced (apelin) and reduced induction (GLUT1, CAIX, and BNIP3) of specific hypoxia/HIF1-regulated genes.



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CTCs grow more aggressively as colonies than MDA-MB-231 cells in chronic hypoxia and create larger tumours *in vivo*, which rapidly result in lung metastasis

The anoxia factors expressed in CTCs are known to be associated with cell proliferation, aggression, and survival. Therefore, we questioned whether CTCs possess an enhanced colony formation capacity in hypoxia. To test this, we performed colony formation assays. A greater number of colonies was observed with CTCs than with parental MDA-MB-231 cells, when grown under conditions of chronic hypoxia for 10 days (Figure 4A). In normoxia, both CTCs and MDA-MB-231 cells resulted in a similar number of colonies after 10 days. Results were similar when different numbers of cells and/or different Petri dish sizes and/or different incubation periods were used for the colony formation assays. Therefore, CTCs had a greater colony formation capacity in chronic hypoxia compared with MDA-MB 231 cells.

The greater clonogenicity under chronic hypoxic conditions suggested that CTCs were more aggressive than their parental MDA-MB-231 cells. To examine tumourigenicity and metastatic potential of CTCs *in vivo*, we generated 10 orthotopic xenografts from cultured CTCs and compared tumour growth with that of 10 parental MDA-MB-231 xenografts. Within 3 weeks, two mice carrying CTC tumour xenografts died. Of the remaining 8 mice that had CTC xenografts and 10 mice that had MDA-MB-231 xenografts, five were euthanised from each tumour group at week 3. Tumour xenografts in the remaining mice (five with MDA-MB-231 xenografts and three with CTC xenografts) continued to grow

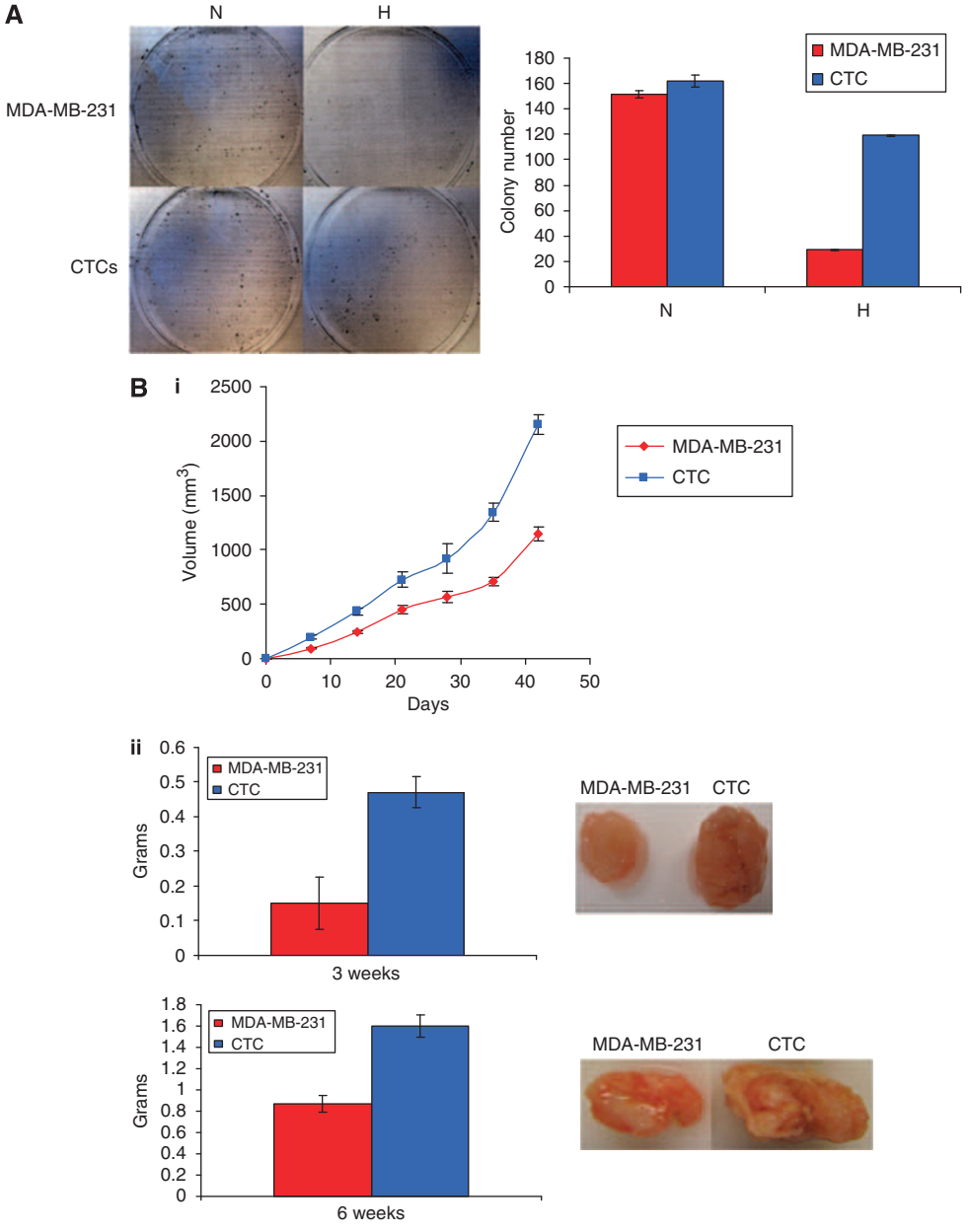

Figure 4 Circulating tumour cells (CTCs) demonstrate greater colony formation capabilities in chronic hypoxia compared with parental MDA-MB-231 cells and grow larger and heavier tumours. (**A**) Colonies grown from CTCs incubated in normoxia and chronic hypoxia compared with colonies grown from parental MDA-MB-231 cells after 10 days (graph shows mean numbers ± s.e.). (**B**i) Comparing sizes of xenogafts generated from CTCs with xenografts generated from MDA-MB-231 cells within 3–6 weeks of growth (graph represents mean volume ± s.e., $n = 5$); (**B**ii) comparing size and weight of xenografts from CTCs with xenografts from MDA-MB-231 cells at 3 and 6 weeks (graphs represent mean weight ± s.e.). N, normoxia; H, hypoxia.

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and were measured from week 3 to week 6, after which the mice were euthanised. As indicated in Figure 4Bi and ii, xenografts generated from CTCs grew larger and heavier than xenografts from MDA-MB-231 cells. Pulmonary micrometastases became evident at week 3 only in mice with xenografts grown from CTCs. By week 6, mice with parental cell xenografts developed pulmonary metastases. In summary, the more aggressive phenotype of CTCs as observed *in vitro* by the expression of anoxia factors and greater colony formation in chronic hypoxia was also reflected *in vivo* by the development of more rapidly growing and metastasising tumours.

DISCUSSION

Although CTCs have been detected in peripheral blood of cancer patients, and tumours contain areas of hypoxia and anoxia, which is prognostically significant in many clinical studies, the response of CTCs to hypoxia has not been addressed. We have established an approach to generate and isolate viable CTCs to study their response to hypoxia. We did this by first generating xenografts that contained profound areas of hypoxia, and produced lung metastases. Our xenograft model showing more pronounced GLUT1 expression and pimonidazole uptake within perinecrotic regions, and ATF3, ATF4, and HIF1α expressions throughout the xenografts are in agreement with previous studies of patient tumours showing that GLUT1 expression was mainly perinecrotic and did not coincide with HIF1α distribution (Sobhanifar *et al*, 2005; Vleugel *et al*, 2005; Tan *et al*, 2009).

Our hypoxic tumour xenograft model shed CTCs into the bloodstream, which we isolated and grew in culture. These CTCs demonstrated an altered response to hypoxia compared with the parental MDA-MB-231 cells from which they were derived. One altered response was ATF4 induction seen only in hypoxic CTCs but not in parental MDA-MB-231 cells. The lack of ATF4 induction in hypoxic MDA-MB-231 cells confirms our previous results in which anoxic conditions rather than hypoxic conditions were shown to be necessary to induce ATF4 (Ameri *et al*, 2004). The ATF4 target gene *ASNS* and the anoxia-induced factor ATF3 were also expressed at greater levels in CTCs. These results support the premise that CTCs have a phenotype different than parental MDA-MB-231 cells. The reason for such an altered phenotype with respect to expression of anoxia factors remains unclear, but may be because of tumour tissues being hypoxic *in vivo* and synchronously exposed to nutrition deprivation/ER stress. In this case, the hypoxia pathway alone will not be sufficient for the survival, but additional adaptation responses to nutrition deprivation, such as ATF4-mediated asparagine synthetase (ASNS) induction, are necessary for survival (Cui *et al*, 2007). Therefore, our results demonstrating that CTCs express the anoxia-induced factors to a much greater extent in hypoxia compared with parental MDA-MB-231 cells evoke the possibility that CTCs may have been selected by a combination of tumour hypoxia and nutrition deprivation/ER stress *in vivo*, and hence, there is greater expression of anoxia-induced and ERSR factors, ATF4 and ASNS, in CTCs.

The mechanism by which CTCs express and/or induce anoxia factors in hypoxic conditions remains to be elucidated. Previous publications have suggested that GLUT1 may modulate the ERSR. For instance, a diminished functional status or lowered expression level of GLUT1 can induce the ERSR arm of the anoxia pathway (Li *et al*, 2007), and hence result in induction of factors related to the unfolded protein response, such as ATF4. Therefore, it will be important to uncover the potential involvement of a dysregulated hypoxia–HIF1 pathway that could result in regulation of anoxia-induced factors in hypoxic conditions.

Phenotype alterations may be because of the tumour microenvironment, which contains regions of hypoxia, anoxia, and nutritional deficiency that exert evolutionary/selective pressure on tumour cells. Moreover, selected cells that detach from the tumour and become CTCs will be subjected to additional mechanical stress from pressure and shear forces of the circulatory system. Cells that survive such microenvironments may display a more aggressive phenotype. Greater expression of factors: ATF3, ATF4, and ASNS suggests an aggressive CTC phenotype associated with cancer progression and metastasis, and implies a role for HIF1-independent pathways in CTCs, which could be considered for future therapeutic interventions. Indeed, our results showed that CTCs were more aggressive *in vitro* under chronic hypoxia and *in vivo*, which may be due to more pronounced anoxia pathways. The *ATF3* is a gene with a known role in metastasis, and has been described to induce genes relevant to epithelial-to-mesenchymal transition (Yin *et al*, 2008), a histological feature of invading cells (Guarino *et al*, 2007). Activating transcription factor 4 is involved in drug resistance (Igarashi *et al*, 2007) and is a major factor in nutrition sensing, regulating the expression of ASNS (Siu *et al*, 2002). Under conditions of hypoxia and/or glucose deprivation, cells can switch to ATF4-mediated ASNS expression as a protective mechanism (Cui *et al*, 2007). Furthermore, ASNS is also known to be associated with drug resistance in leukaemia (Williams, 2007) and oncogenesis triggered by mutated p53 (Scian *et al*, 2004).

In addition to expressing the anoxia-induced factors at greater levels, CTCs also demonstrated an altered regulation of hypoxia–HIF1α target genes. Circulating tumour cells did induce BNIP3, CAIX, and GLUT1, but the induction of these factors was much lower than in parental MDA-MB-231 cells. In contrast, induction of apelin was much higher in CTCs, compared with parental MDA-MB-231 cells. These results further support that CTCs have an altered phenotype. The distinctive expression of hypoxia-induced factors could have implications in the outcome of CTC-mediated metastasis and in secondary tumour survival and growth. The significance of greater induction of apelin could be relevant to tumour growth and metastasis. Apelin is a potent activator of tumour angiogenesis and is associated with earlier onset of tumour development and increased tumour growth *in vivo* (Sorli *et al*, 2007). The consequences of decreased BNIP3 induction in CTCs may determine the metastatic potential, as the expression of BNIP3 has been associated with a less-aggressive phenotype in invasive breast carcinoma (Tan *et al*, 2007), and a reduction in expression of BNIP3 has been suggested to be critical for metastasis (Manka *et al*, 2005).

The mechanism that accounts for the distinct hypoxia/HIF1 response in CTCs is a subject for further investigation. Activating transcription factor 4 may downregulate certain HIF1α genes but upregulate others (Rzymski *et al*, 2007), and during completion of this study, it was shown that ATF4 can modulate the hypoxic induction of HIF1 target genes selectively by inducing CAIX but not GLUT1 in mouse embryonic fibroblasts, as well as in the human colon cancer cell line HCT116 and glioblastoma cell line U373 (van den Beucken *et al*, 2009). In contrast, we did not observe greater induction of CAIX in CTCs in which ATF3 and ATF4 were highly induced by hypoxia. Activating transcription factor 4 (Ameri and Harris, 2008) and ATF3 (Hai *et al*, 1999) are known to be transcriptional activators and repressors, and ATF3 is known to antagonise ATF4. Thus, whether ATF3 antagonises ATF4-mediated induction of CAIX in CTCs needs to be considered. In addition, regulation of CAIX by ATF4 could be cancer-type specific.

Cross-sectional evaluation of CTC tumours did not show smaller zones of necrosis that might be expected if all CTCs are assumed to be resistant to hypoxia. We think that areas of necrosis seen in CTC tumours are due to greater proliferation in hypoxia, with a rapid enlargement of tumour mass outgrowing blood/nutrient supply, and further selection of aggressive cells within a CTC tumour. If such highly metabolic cells consumed residual oxygen and nutrients, they would deprive less aggressive populations of critical nutrients and promote their death. We observed that with higher passage numbers

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of CTCs in culture, there was some reversion back to parental phenotype. If some CTC daughter cell populations growing *in vivo* also reverted to parental cell behaviour during subcutaneous tumour growth, these tumour cells could be less resistant to hypoxia and could affect necrotic zone size.

In summary, we demonstrate that CTCs show an altered phenotype compared with the parental tumour cells from which they were derived. We show that CTCs demonstrate a distinct response to hypoxia *in vitro* and a greater aggression *in vivo*. Our study also shows that the circulating cells captured from blood by the MagSweeper are indeed cancer cells in that they produce tumours in mice that metastasise. Our experimental system may be applied to test drug efficacy in targeting CTCs for the prevention of metastases as suggested by Bondareva *et al* (2009). Our approach and data provide a foundation to investigate selective factors associated with CTC aggressiveness and enable the identification of additional therapeutic targets related to tumour hypoxia and CTCs.

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Conflict of interest

Drs Stefanie Jeffrey and Ashley Powell are co-inventors of the MagSweeper CTC isolation technology used in this study. They can receive royalties from this invention through Stanford, but Dr Jeffrey has donated her royalties to support student programmes at The Jackson Laboratory, a non-profit biomedical research institution.

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