Title: **Hypoxia induces transcriptional and translational downregulation of the type I interferon (IFN) pathway in multiple cancer cell types**

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AUTHOR CONTRIBUTION

AM and ALH designed the experiments, analysed the data and wrote the manuscript. AM and EA performed the experiments. EB performed the animal experiments and stained the samples. SB performed the single cell experiments and APC analysed them. DD and RB analysed ATAC-Seq data, and JR provided reagents and advice.

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Abstract:

Hypoxia is a common phenomenon in solid tumors and is strongly linked to hallmarks of cancer. Recent evidence has shown that hypoxia promotes local immune suppression. Type I IFN supports cytotoxic T lymphocytes by stimulating the maturation of dendritic cells (DC) and enhancing their capacity to process and present antigens. However, little is known about the relationship between hypoxia and the type I interferon (IFN) pathway, which comprises the sensing of double-stranded RNA and DNA (dsRNA/dsDNA) followed by IFNα/β secretion and transcriptional activation of IFN-stimulated genes (ISG). In this study, we determined the effects of hypoxia on the type I IFN pathway in breast cancer and the mechanisms involved. In cancer cell lines and xenograft models, mRNA and protein expression of the type I IFN pathway were downregulated under hypoxic conditions. This pathway was suppressed at each level of signaling, from the dsRNA sensors RIG-I and MDA5, the adaptor MAVS, transcription factors IRF3, IRF7, and STAT1, and several ISG including RIG-I, IRF7, STAT1, and ADAR-p150. Importantly, IFN secretion was reduced under hypoxic conditions. HIF1α- and HIF2α-mediated regulation of gene expression did not explain most of the effects. However, ATAC-seq data revealed in hypoxia that peaks with STAT1 and IRF3 motifs had decreased accessibility. Collectively, these results indicate that hypoxia leads to an overall downregulation of the type I IFN pathway due to repressed transcription and lower chromatin accessibility in a HIF1/2α-independent manner, which could contribute to immunosuppression in hypoxic tumors.

Statement of significance: Findings characterize a new mechanism of immunosuppression by hypoxia via downregulation of the type I IFN pathway and its autocrine/paracrine effects on tumor growth.

INTRODUCTION:

Oxygen can only diffuse 100-180μm from the nearest capillary and consequently, poorly vascularized tumours or those that grow quickly suffer from hypoxia, low pH and nutrient starvation. Hypoxia is closely linked to the hallmarks of cancer as it induces the switch to glycolytic metabolism, enhances resistance to apoptosis, induces unlimited replication potential and genomic instability, reduces immuno-surveillance, and induces angiogenesis and migration to less hypoxic areas (1).

This hypoxia response is driven by a family of dimeric hypoxia-inducible transcription factors (HIF-1, HIF-2, HIF-3) which are composed of an oxygen-sensitive α-subunit (HIF-1α, HIF-2α, HIF-3α) and a constitutively expressed β-subunit (HIF-1β) (2). Under well oxygenated conditions, HIF-1/2α are bound by the von Hippel-Lindau (VHL) protein which targets them for proteasomal degradation (3). VHL binding is dependent upon hydroxylation of specific proline residues by prolyl hydroxylase PHD2, which uses O_2 as a substrate, and as a consequence its activity is inhibited under hypoxia (4).

Hypoxia induces an immunosuppressive microenvironment as it inhibits the ability of macrophages to phagocytose dead cells, present antigens to T cells, inhibits the anti-tumour effects of macrophages (5), and favours their differentiation to M2 type which is more immunosuppressive (6). T-cell-mediated immune functions are also affected by low oxygen levels, resulting in impairment of cytokine expression and less T-cell activation (7) although there are controversial results about the hypoxia effect on T cell function (8). Moreover, the cytotoxic potential of natural killer (NK) cells is reduced under hypoxia resulting in a decreased anti-tumour response that allows metastasis (9).

Type I interferons (IFNs) comprise multiple IFNα's (encoded by 13 genes), IFNβ (encoded by one gene) and other less studied IFNs (IFNε, IFNκ, IFNω) that can act in an autocrine or paracrine manner. They are produced by most cell types and their

transcription is upregulated after the activation of pattern recognition receptors (PRR) which sense viral/bacterial dsRNA (through RIG-I, MDA5, or TLRs) and dsDNA/unmethylated DNA (through cGAS, DDX41, IFI16, or TLRs). Type I IFNs bind to the heterodimeric IFNα/β receptor composed of IFNAR1 and IFNAR2, triggering the JAK-STAT pathway and activating the transcription of IFN-stimulated genes (ISGs) (10) (Supplementary figure 1). Type I IFNs are involved in all three phases of the cancer immunoediting process by which the immune system eliminates the malignant cells, moulds the immunogenic phenotypes of developing tumours and selects those clones with reduced immunogenicity (11). Type I IFNs support cytotoxic T lymphocytes in a paracrine manner by stimulating the maturation of dendritic cells (DCs) and enhancing their capacity to process and present antigens (12). Endogenous IFNα/β is required for the lymphocyte-dependent rejection of highly immunogenic sarcomas in immunocompetent hosts (11), and they are crucial for the innate immune response to transformed cells as *Ifnar1*$^{-/-}$ CD8^{+} dendritic cells are deficient in antigen cross-presentation, and fail to reject highly immunogenic tumours (13). Moreover, type I IFNs boost immune effector functions by enhancing perforin 1 and granzyme B expression (14), and promoting survival of memory T cells.

Type I IFN is involved in the success of current anticancer treatments both directly (tumour cell inhibition) and indirectly (anti-tumour immune responses to the nucleic acids and proteins released by dying cells, recently named immunogenic cell death, ICD) (15) (10). In mouse models, IFNAR1 neutralization using antibodies blocked the therapeutic effect of antibodies against human epidermal growth factor receptor 2 (HER2) or epidermal growth factor receptor (EGFR) (16, 17). Moreover, radiotherapy, via DNA damage, induces intratumoural production of IFNβ which act in a paracrine way to enhance the cross-priming capacity of tumour-infiltrating dendritic cells (18).

Chemotherapeutic agents such as anthracyclines, bleomycin or oxaliplatin induce ICD, and the activation of an IFN-dependent program is required for successful therapy (19, 20).

Tumour hypoxia creates resistance to many cancer treatments as oxygen is essential for ROS formation to kill cells during ionizing radiation (21, 22), and HIF1α upregulation mediates resistance to chemotherapy by upregulating anti-apoptotic and pro-survival genes (23).

Thus we investigated the regulation of the type I IFN pathway in breast cancer cells under hypoxia at basal levels and upon activation of IFN signalling using the dsRNA mimic polyinosinic:polycytidylic acid (poly I:C), and the possible autocrine/paracrine effect in monocytes. We found there was significant HIF-independent downregulation of IFN signalling which lowered the production/secretion of type I IFN and influenced immune cells by a paracrine method, thus potentially affecting all the before mentioned therapies and contribute to treatment resistance.

MATERIAL AND METHODS

Cell culture and transfection

All cell lines used are listed in supplementary table 1. They were cultured in DMEM low glucose medium (1g/l) supplemented with 10% FBS no longer than 20 passages except for the THP-1 cells which were grown in RPMI-1640 supplemented with 10% FBS and 2mM glutamine and fibroblasts (CC-2511, Lonza) grown in FGM™-2 Fibroblast Growth Medium-2 BulletKit™ (CC-3131, Lonza). They were mycoplasma tested every 3 months and authenticated during the course of this project. Cells were subjected to 1% or 0.1% hypoxia for the periods specified in each experiment using an InVivO$_2$ chamber (Baker). Transfection of poly I:C (P1530, Sigma) was performed in Optimem reduced serum (11058021, Thermo Fisher Scientific) medium for 6h unless it is otherwise indicated. Lipofectamine 2000 (11668019, Thermo Fisher Scientific) was used following the manufacturer's instructions.

For the THP-1 stimulation experiments, MCF7 cells were transfected with 100ng/mL poly I:C for 1h. Cells were washed with PBS twice and 3mL DMEM was added. Then, cells were placed into normoxia or 0.1% hypoxia for 24h. Afterwards, media was collected, centrifuged at 300g for 5min and stored at -80º until use. Control wells were proceeded the same way without the addition of poly I:C. 1x10^6 THP1 cells were incubated with MCF7's conditioned media for 2h in normoxia. Cells pellets were obtained and RNA extraction was performed using TRI Reagent protocol.

Western blot and RT-qPCR

Methods detailed in Supplementary materials (Antibodies listed in Supplementary table S2 and primers in Supplementary table S3).

IFN bioassay

HEK293-3C11 cells stably transduced with an ISRE-Luc reporter construct (24) were used to detect possible IFN present in the supernatant. $2x10^4$ MCF7 cells/well were transfected in triplicate with different concentrations of poly I:C and washed with PBS after 1h, cells were incubated for 24h in normoxia or 0.1% hypoxia and 24h after, the supernatant was transferred into $2x10^4$ 3C11 cells kept in normoxia. After 24h incubation, 3C11 cells were lysed and measured using OneGlo luciferase assay (E6120, Promega) in a FluorOPTIMA luminometer.

Single Cell sequencing and analysis

Single-cell RNA-seq libraries were prepared as per the Smart-seq2 protocol by Picelli et al (25) with minor technical adaptations detailed in Supplementary material.

scRNA-seq data were deposited in Gene Expression Omnibus under SuperSeries accession number GSE134038 and detailed in Supplementary materials.

Xenograft growth and IHC/IF

Procedures were carried out after the approval by the institutional review board at the University of Oxford and under a Home Office license following the Animals (Scientific Procedures) Act 1986. Xenograft experiments were performed as described in (26) and detailed in Supplementary materials.

ATAC-seq and analysis

Samples were prepared and analysed as previously described (27) with minor modifications detailed in Supplementary materials. ATAC-seq data is available in the Gene Expression Omnibus (GSE133327).

RESULTS

Hypoxia causes downregulation of the type I IFN pathway in unstimulated cells both at mRNA and protein level

MCF7 cells were subjected to normoxia, 1% hypoxia or 0.1% hypoxia for 48h. Oxygen deficiency led to a decrease of both protein (figure 1A, Supplementary figure 2) and RNA (figure 1B) of members of the type I IFN pathway. The effect was seen in the pathway from dsRNA sensors (RIG-I [encoded by *DDX58*], MDA5 [encoded by *IFIH1*]), adaptor MAVS, transcription factors triggering IFNα/β production (IRF3, IRF7) and transcription factors involved in ISGs' activation (STAT1, STAT2), to the downregulation of downstream ISGs (exemplified by ADAR-p150). This inhibitory effect was oxygen concentration-dependent and 0.1% hypoxia caused a greater downregulation of the pathway than 1% hypoxia at mRNA level (figure 1B), however there was a relatively high biological variation, so 10 biological replicates were used to capture it. At protein level, there was a significant downregulation for all proteins tested in hypoxia except for MAVS and the constitutively expressed isoform of ADAR (ADAR-p110). There was no difference between 0.1% and 1% hypoxia, except for IRF3, which recovered normoxia levels (figure 1A, Supplementary figure 2).

This finding was confirmed in other breast cancer types including oestrogen receptor positive (ER+; T47D), HER2+ (BT474) and triple negative (TN; MDA-MB-453, MDA-MB-231, HCC1187, MDA-MB-468) cells. In general, a downregulation of the type I pathway was observed in all cell lines in hypoxia, although there was heterogeneity in the specific genes involved. However, TN cell lines seemed to be more resistant to the effect of hypoxia, showing a lower or no downregulation of some transcripts such as *DDX58*, *IRF3* or *MX1* (figure 1C).

<u>Single cell transcriptomic analysis of the effects of hypoxia on the type I IFN pathway</u>

Next, we performed a single cell RNA-Seq experiment using MCF7 cells subjected to 0.1% hypoxia for 72h. We found that 14/20 genes in the type I IFN pathway (such as

DDX58, IRF3 or *MX1*) are significantly less expressed in hypoxia (figure 2A), including several genes associated with the DNA sensing branch (*IFI16, TMEM173* [encodes STING], *DDX41* and *MB21D1* [encodes cGAS]) in MCF7 cells exposed to hypoxia. However, some did not show differences as they had very low levels to start with such as *IFIT1, IFIT3, MX2*. Next, we investigated the expression of a number of well described ISGs (28) revealing a clear subpopulation of cells in which ISGs were downregulated under hypoxia, e.g. *STAT1, STAT2* or *ISG15*. In contrast, HIF1α targets such those involved in glycolysis, were upregulated as expected (*PDK1, PGK1, P4HA1, NDRG1, CA9;* figure 2B).

Reoxygenation after hypoxia leads to recovery of the type I IFN pathway

A time course in 0.1% hypoxia up to 48h showed that members of the type I IFN pathway were downregulated at protein level after 48h in hypoxia (figure 3A), whereas at RNA level, there was a significant decrease from 16h onwards in *IFIH1, MAVS, STAT1* and *ADAR* (figure 3B).

MCF7 cells were incubated in normoxia or 0.1% hypoxia for 48h and subjected to reoxygenation for 8h, 16h or 24h. At protein level (figure 3C), hypoxia caused the downregulation of the pathway but reoxygenation up to 24h did not reverse this effect except for RIG-I. However, at RNA level (figure 3D), reoxygenation caused a gradual upregulation of type I IFN pathway levels. Thus RNA expression responded more rapidly to O_2 tension than protein.

Hypoxia downregulates the dsRNA-mimetic activation of type I IFN pathway at protein level

MCF7 cells were incubated in normoxia or 0.1% hypoxia for 48h and transfected with poly I:C (mimetic of dsRNA) in the last 6h of the incubation to determine if the

downregulation caused by hypoxia would also reduce the response to a strong stimulus. Poly I:C activated the type I IFN pathway as shown at protein level using pIRF3-Ser386 and pSTAT1-Tyr701 or pSTAT1-Ser727; however hypoxia led to lower activation (lower pIRF3 and pSTAT1) and to lower expression of RIG-I, MDA5, MAVS and IRF7 (figure 4A). These findings suggested that production/secretion of IFNs would be lower in hypoxia than in normoxia.

To investigate this, the paracrine stimulation of IFN-stimulated response element (ISRE) by potential IFNs present in the supernatant from normoxic or hypoxic cells was measured using indicator cells (HEK293-3C11, hereafter 3C11) (24). 3C11 cells were incubated with supernatant from MCF7 cells previously treated with different concentrations of poly I:C and normoxia or hypoxia, as described in the Supplementary Materials section. Hypoxic supernatants showed lower activation of ISRE confirming the lower production/secretion of IFNs in these conditions (figure 4B).

The paracrine effect on monocytes was evaluated using conditioned media from MCF7 cells growing in normoxia or 0.1% hypoxia for 24h and stimulated with 100ng/ml polyI:C. The expression of ADAR-p150 as an example of ISG was evaluated in the monocytes using the conditioned medium from MCF7 cells for 2h. Conditioned media significantly induced *ADAR-p150* expression compared to unstimulated conditioned media, and hypoxic supernatant induced significantly less *ADAR-p150* expression than normoxic media (figure 4C). Although the reduction is by 25%, this was over a short time, whereas *in vivo* it would be chronic.

A time course in hypoxia was performed using MCF7 cells cultured for 6h, 24h and 48h in normoxia or 0.1% hypoxia and transfected with poly I:C in the last 6h of incubation. pIRF3 and pSTAT1 were induced upon poly I:C transfection and their expression was downregulated when the cells were exposed to 48h hypoxia but not at shorter incubations.

RIG-I, MDA5 and MAVS were downregulated at 24h, suggesting that hypoxia diminishes the sensitivity of this pathway, particularly affecting late signalling in the amplification phase (figure 4D).

The reponse to hypoxia was analysed in a panel of breast cancer cell lines which were incubated in normoxia or 0.1% hypoxia for 48h and transfected with poly I:C during the last 6h. Again, the activation of the pathway was lower in hypoxia and the expression of MDA5, RIG-I, STAT1 and IRF7 was also decreased, showing a general effect in breast cancer cell lines (figure 4E).

We assessed cell lines from other cancer types or normal tissues e.g. HepG2 hepatocarcinoma, HKC8, a non-transformed proximal renal tubular cell line and fibroblasts. The pSTAT1-Tyr701 response to poly I:C was reduced in all cell lines in hypoxia (supplementary figure 3). Interestingly, pIRF3 expression in HepG2 was higher in hypoxia than in normoxia, whilst IRF3 expression which was lower (supplementary figure 3A). This different response may contribute to the higher viral replication under hypoxia in hepatocarcinoma (29). In HKC8 cells and fibroblasts (supplementary figures 3B and 3C), IRF3 expression was higher in hypoxia or did not change but pIRF3 was reduced. Thus, hypoxia causes a general downregulation of the type I IFN pathway in cancer, and similarly in normal cell lines.

Type I IFN downregulation is partially dependent on HIF1α

To determine the role of HIF1α in the results observed under hypoxic conditions, we used MCF7-HIF1α-KO and MCF7-WT cells. Interestingly, MCF7-HIF1α KO cells in normoxia showed higher RIG-I and STAT1 levels than MCF7-WT cells although in hypoxia, their levels were reduced, indicating a HIF1α-independent mechanism to further regulate them.

Poly I:C induced IRF3, pIRF3, IRF7 and pSTAT1 in normoxia and this induction was higher in MCF7-HIF1α-KO cells, apart from pSTAT1, which was still induced but to lower level than the MCF7-WT cells. Again hypoxia downregulated the expression of all these proteins in both cell lines (figure 5A).

Higher IRF3, pIRF3 and IRF7 induction in MCF7-HIF1α-KO than in MCF7-WT suggested a possible increase in IFN production. Using the supernatant from these cells to treat 3C11 cells, as described above, did not activate ISRE at basal level. However, after poly I:C treatment, ISRE was activated and hypoxic MCF7-WT supernatant caused a significantly lower ISRE stimulation, which was not observed in hypoxic MCF7-HIF1α-KO supernatants (Supplementary figure 4A).

IFNAR1 and *IFNAR2* expression was determined and they were significantly downregulated in MCF7-WT cells in hypoxia. Interestingly, MCF7-HIF1α-KO cells showed in normoxia significantly lower expression of both compared with MCF7-WT and hypoxia did not further decrease their mRNA levels (Supplementary figure 4B).

Some ISGs associated with chronic anti-viral response such MDA5, MX1 or STAT1 can be transcribed by unphosphorylated STAT1, and this effect is driven by higher IRF9 expression (30). MCF7-HIF1α-KO cells showed higher IRF9 protein levels again downregulated in hypoxia (figure 5B) associated with significantly higher ISG expression even in hypoxia compared to MCF7-WT cells (figure 5C). Interestingly, *IRF9* mRNA was downregulated by HIF1α deficiency suggesting that HIF1α could stabilize IRF9 protein (Supplementary figure 4C).

These data suggest that HIF1α upregulation in hypoxia is partly responsible for the type I IFN pathway downregulation observed but other HIF1α-independent mechanisms cause a further decrease.

Type I IFN downregulation and role of HIF2α

The converse experiment was performed with renal cancer cells, which showed vHL loss or mutations leading to stabilization of HIF1α/HIF2α in normoxia. RCC4-EV (empty vector, mutant vHL), RCC4-vHL (transfected with wild type vHL and, as a result, displaying lower levels of HIF1α/HIF2α in normoxia), 786-0 parental (vHL mutation and HIF1α deficient) and 786-0-HIF2α-KO (deficient in both HIF1α and HIF2α) were used.

In RCC4 cells, poly I:C strongly induced MDA5, RIG-I, IRF3 and pIRF3 in normoxia and this was reduced in hypoxia Much higher STAT1 and pSTAT1 was detected in RCC4-vHL cell line in normoxia and both decreased in hypoxia, clearly linking suppression of STAT1 and pSTAT1 to HIF1α/2α (Supplementary figure 5A).

In 786-0 cells (Supplementary figure 5B), poly I:C did not affect RIG-I or MDA5 levels but it induced pIRF3 and pSTAT1 expression in both cell lines. As observed in other HIF1α-KO cell lines, STAT1 level was highly expressed in both cell lines. However, hypoxia led to downregulation of MDA5, IRF3, pIRF3, STAT1, pSTAT1 and ADAR expression.

In general and despite of the differences observed in these models, there is a partial role of HIF1/2α in regulating some members of the pathway, but more importantly, it is clear that there is a HIF1/2α-independent downregulation of the type I IFN pathway.

Type I IFN pathway is also downregulated in hypoxic areas *in vivo*

To confirm that hypoxic areas *in vivo* also showed lower expression of type I IFN pathway, xenograft experiments were conducted using MCF7 and MDA-MB-231 cells. Avastin, a VEGF antagonist, was used to create tumour hypoxia by reducing angiogenesis, as described previously (31-33). Immunohistochemistry was performed using antibodies against IRF3, IRF7 and ADAR in 5 control and 5 Avastin-treated mice

for each cell line. Avastin treatment decreased the total number of blood vessels and the percentage of CA9+ or pimonidazole+ cells was significantly greater in Avastin-treated xenografts compared to PBS, thus confirming hypoxia. Interestingly, IRF3, IRF7 and ADAR were significantly downregulated in Avastin-treated mice in the case of MCF7 xenografts (figure 6A), and IRF3 and IRF7 also showed significantly lower expression in MDA-MB-231 xenografts (figure 6B). These data was supported by colocalization experiments (supplementary figure 6A and 6B for MCF7 and MDA-MB-231 xenografts, respectively).

ATAC-Seq data shows lower chromatin accessibility on STAT1 and IRF3 containing promoters

To investigate how hypoxia may downregulate the type I IFN pathway independently of HIF1α/HIF2α we performed ATAC-seq on MCF7 cells cultured for 48h in normoxia or 0.1% hypoxia. We found 5,577 peaks (7.4%) with differential accessibility (figure 7A). Peaks showing increased accessibility during hypoxia (n=2,439) were enriched for promoters, whereas those with decreased accessibility (n=3,138) tended to be intergenic or intronic (figure 7B), showing a switch in regulatory networks driving gene expression. Consistent with HIF1/HIF2 driving an active hypoxia response, motif analysis showed enrichment for HIF1 sites in distal peaks with increased accessibility (figure 7C). Conversely, peaks with differential decreased accessibility showed higher levels of STAT1, IRF3, FOXA1 and GATA3 motifs, than unchanged peaks. FOXA1 and GATA3 motifs were present more frequently in distal peaks with decreased accessibility and less frequently present in peaks with differential increase accessibility. IRF3 motifs were significantly less represented in peaks with increased accessibility both at promoters and distal sites. STAT1 motifs only showed significantly less presence in increased accessible

peaks at promoters. The observed loss of accessibility at FOXA1 and GATA3 sites is consistent with their roles downstream in ER signaling and the degradation of ER under low O_2 conditions (34). Therefore, hypoxia appears to drive global changes in chromatin accessibility, partially through the shutdown of both type I IFN and ER signalling responsive promoters and enhancers, although globally most decreases are in intergenic regions and introns.

DISCUSSION

We describe a new mechanism of moderation of the autocrine/paracrine effect of the type I IFN pathway in tumour cells by hypoxia which can contribute to hypoxic-induced immunosuppression. This occurred at oxygen tensions commonly found in tumours and in more severe hypoxia present in many cases (1% vs 0.1%). The type I IFN pathway is downregulated at RNA level in less than 16h under hypoxia whereas protein level is downregulated after a 48h exposure. Reoxygenation gradually reverses the levels and it takes 24h to reach normoxic levels again. The observation that RNA recovers more quickly than protein after reoxygenation points to *de novo* transcription or stabilisation occurring during the reoxygenation phase, and RNA changes leading to the protein expression changes rather than protein degradation.

Furthermore, apart from the downregulation induced at basal/unstimulated level by hypoxia, we also observed lower activation of the type I IFN pathway and less production/secretion of interferons under hypoxia in all cell lines tested when they were activated with poly I:C. This suggests that endogenous activators of the pathway in a hypoxic tumour microenvironment would lead to lower stimulation and consequently to lower immune response. This hypothesis is supported by lower presence of T-cells and NK cells in hypoxic areas in lung and dermal tumours (35) and in prostatic tumours (36). In both studies the immune infiltration increased when the hypoxic areas were reduced

using respiratory hyperoxia (35) or the hypoxia-activated prodrug TH-302 (36), respectively.

We observed that the type I IFN downregulation in hypoxia is independent of HIF1α and HIF2α, although some members (RIG-I, and mainly STAT1) are regulated by HIF1α as observed in MCF7-HIF1α-KO cells. Previous work reported that RIG-I harbours hypoxic response elements in its 3'UTR and RIG-I expression was upregulated during hypoxia in human myotubes (37). However, a different study in tumour cells supported our finding that RIG-I and MDA5 are downregulated under hypoxia and they required functional IFNAR1 to respond to stimulus (38). Recently, type I IFN signalling was shown to be inhibited by high lactate levels generated during the metabolic switch to glycolysis occurring in hypoxia (39). As lactate production requires HIF1α, this result could explain the partial effect we observed in HIF1α-KO cells. However, the inhibitory effect seen in hypoxia needed 16h to be significant and it could be a consequence of the HIF1α-independent general transcription inhibition occurring during hypoxia that leads to 42% and 65% decrease in total RNA transcription after 24h and 48h exposure to 0.2% hypoxia, respectively (40).

We investigated each step in the type I IFN pathway, from the sensors (RIG-I, MDA5), transcription factors (IRF3, IRF7, STAT1), ISGs (MX1, ADAR) and interferon release, and each stage was reduced under hypoxic conditions, although there was heterogeneity and TNBC cell lines were more resistant to the hypoxic effect. In this context, it was previously reported that estrogen receptor induced IFN production (41) and STAT1 regulates ER at mRNA and protein level generating tamoxifen resistance (42). However, TNBC showed higher levels of IFN (43) and this could help TNBC to be more aggressive and metastasize and respond to immunotherapy (44).We therefore studied the chromatin accessibility in hypoxia related to these genes by ATAC-Seq. Overall we found that 48h

exposure to hypoxia led to global changes rather than changes in specific pathways and only 7% of the type I IFN pathway were associated with differentially accessible peaks. Interestingly, hypoxia caused decreased accessibility at STAT1 and IRF3 containing promoters, suggesting lower transcription from those genes but the downregulation and lower activation observed in hypoxia cannot be explained only by this. However, a recent paper showed that 1h exposure to hypoxia caused a rapid and HIF1α-independent induction of histone methylation, and the locations of H3K4me3 and H3K36me3 predict the transcriptional response hours later (45). These methylation markers are normally related to active transcription and they were significantly less present in type I IFN genes after hypoxia, potentially indicating that the expression of genes in this pathway are repressed by histone modifications that occurred in hypoxia due to decreased enzyme activity of oxygen-requiring JmjC-containing enzymes, and particularly KDM5A, that would affect transcription and translation of this pathway later on (45).

Here, we propose a model in which hypoxia downregulates every single step in the type I IFN pathway. However, there is a partial effect of HIF1α as its deletion increased the levels of MDA5, RIG-I, IRF3, IRF7, STAT1 and IFN secretion upon activation in normoxia by upregulating IRF9 or stabilising it at protein level despite the downregulation caused by hypoxia, and not by increasing phosphorylation of STAT1. Conversely, hypoxia is still able to decrease the IFN response when HIF1α is deleted potentially by specifically decreasing the transcription of this pathway (figure 7D).

Supporting our work, ISG15 (a typical ISG increased by IFNαβ) was reported to decrease in hypoxia and interact and modify HIF1α via ISGylation, thus reducing its levels and affecting its biological impact on EMT and cancer stemness acquisition (46).

All together, these data suggest that the relevance of this hypoxic downregulation of the IFN response is the potential effect on enhancing tumour growth in a paracrine manner

decreasing the cytotoxicity of T lymphocytes and the capacity of dendritic cells (DCs) to process and present antigens (12). As a result, this downregulation could contribute to the radio- and chemo-resistance observed in hypoxic areas as these therapies rely on intact type I IFN signalling (15). This provides a further rational for targeting the hypoxic tumour population in combination with check point inhibitor therapy (47) and selection of hypoxic patients for study of IFN response inducers is warranted.

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Figure legends:

Figure 1. Type I interferon (IFN) pathway expression in breast cancer cells. A) Western-blot showing protein levels of different genes involved in the pathway in MCF7 cells

cultured in normoxia, 0.1% and 1% hypoxia for 48h. B) qPCR data showing mRNA expression of genes involved in the pathway in the same experiment (n=10). C) mRNA expression of genes in the type IFN pathway in different breast cancer cell lines cultured in normoxia or 0.1% hypoxia for 24h (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 2. Single-cell analysis of type I IFN pathway in MCF7. A) Violin plots showing expression (log counts) of type IFN genes in normoxia (orange) and 0.1% hypoxia (blue) after 72h. B) Heatmap correlating mRNA expression type I IFN genes previously published with hypoxia target genes.

Figure 3. Type I IFN over time and during reoxygenation in MCF7 cells. A) Western-blot showing protein levels of different genes involved in the pathway in MCF7 cells cultured in normoxia for 48h or 0.1% hypoxia for 4h, 8h, 16h, 24h and 48h. B) qPCR data showing mRNA expression of genes involved in the pathway in the same experiment (n=3). C) Western-blot showing protein changes in MCF7 when exposed to normoxia or 0.1% hypoxia for 48h and after reoxygenation for 8h, 16h and 24h. D) qPCR data showing mRNA expression changes for the same experiment (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4. Type I IFN activation by poly I:C in breast cancer cells. A) Western-blot showing protein levels of MCF7 cells cultured in normoxia or 0.1% hypoxia for 48h and transfected with poly I:C in the last 6h. B) IFN bioassay showing Interferon Stimulated Response Element (ISRE) activation via luciferase activity in HEK293 cells using supernatants of MCF7 cells previously treated with normoxia or 0.1% hypoxia for 24h and transfected with different concentrations of poly I:C (n=3). C) ADAR-p150 expression in THP-1 cells after treatment with normoxic or hypoxic conditioned supernatant from MCF7 cells. D) Time course of 0.1% hypoxia treatment for 6h, 24h and

48h and transfected with 20ug/ml poly I:C in the last 6h of the treatment. E) Western-blot showing protein changes in different breast cancer cells when exposed to normoxia or 0.1% hypoxia for 48h and transfected with 20ug/ml poly I:C in the last 6h of the treatment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ in normoxia and normoxia vs hypoxia, # $p<0.05$, ## $p<0.01$, ### $p<0.001$ in hypoxia samples.

Figure 5. HIF1α-independency of type I IFN downregulation in hypoxia. A) Western-blot showing protein changes in different MCF7-WT vs MCF7-HIF1α-KO cells when exposed to normoxia or 0.1% hypoxia for 48h and transfected with 0.5ug/ml poly I:C in the last 6h of the treatment B) IRF9 protein levels in MCF7-WT and MCF7-HIF1α-KO cells in the same as experiment as in A). C) mRNA expression in MCF7-WT and MCF7-HIF1α-KO cells cultured in normoxia or 0.1% hypoxia for 48h. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ n=3

Figure 6. Downregulation of the type I IFN staining in hypoxic tumours *in vivo*. A) IRF3, IRF7, ADAR, CA9 and PIMO staining in MCF7 xenografts from control and Avastin-treated mice and their respective quantification. B) IRF3, IRF7, ADARCA9 and PIMO staining in MDA-MB-231 xenografts from control and Avastin-treated mice and their respective quantification. n=5 per group, ** $p<0.01$, *** $p<0.001$.

Figure 7. ATAC-seq reveals dynamics of chromatin accessibility during hypoxia. A) MA plot of differentially accessible peaks during hypoxia with peaks significantly more (red) or less (blue) accessible during hypoxia. B) Annotation of all ATAC-seq peaks, and peaks with significant changes in accessibility. C) Percent of ATAC-seq peaks containing HIF1, STAT1, IRF3, FOXA1 and GATA3 transcription factor binding motifs. Peaks are divided into transcription start site (TSS) associated and TSS-distal peaks (all other categories in B). The number of peaks that are unchanged, decreased and increased in A)

635 are shown graphically and following the same colour-code in C). Number of differentially
636 accessible peaks in each class is shown in the HIF-1 graph and are identical for the others.
637 P-values are for a Fisher's exact test with Bonferroni multiple test correction. D)
638 Schematic representation of the hypoxia-dependent and HIF1α-independent mechanism
639 by which hypoxia downregulates the type I IFN pathway.

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