Abstract

Mutations that drive the stabilization of hypoxia inducible factor 2α (HIF2α) and downstream pseudohypoxic signaling are known to predispose to the development of pheochromocytomas and paragangliomas (PPGLs). However, any role of HIF2α in predisposition to metastatic disease remains unclear. To assess such a role we combined gene-manipulations in pheochromocytoma cell lines with retrospective analyses of patient data and gene expression profiling in tumor specimens. Among 425 patients with PPGLs identified with mutations in tumor-susceptibility genes, those with tumors due to activation of pseudohypoxic pathways had a higher frequency of metastatic disease than those with tumors due to activation of kinase-signaling pathways, even without inclusion of patients with mutations in SDHB (18.6% vs 4.3% in, \( P < 0.0001 \)). Three out of 271 patients with metastases due to activation of pseudohypoxic pathways had SDHB mutations, whereas none of 154 patients due to activation of kinase-signaling pathways had SDHB mutations (11.1% vs 0.0% in, \( P = 0.0005 \)).

Key Words:
- EPAS1
- pseudopodia
- invasion-metastasis cascade
- epithelial–mesenchymal transition
- liver metastases
of nine (33%) patients with gain-of-function mutations in HIF2α had metastatic disease. In cell line studies, elevated expression of HIF2α enhanced cell proliferation and led to increased migration and invasion capacity. Moreover, HIF2α expression in HIF2α-deficient cells resulted in increased cell motility, diffuse cluster formation and emergence of pseudopodia indicating changes in cell adhesion and cytoskeletal remodeling. In a mouse liver metastasis model, Hif2α enhanced the metastatic load. Transcriptomics data revealed alterations in focal adhesion and extracellular matrix–receptor interactions in HIF2α-mutated PPGLs. Our translational findings demonstrate that HIF2α supports pro-metastatic behavior in PPGLs, though other factors remain critical for subsequent transition to metastasis. We identified LAMB1 and COL4A2 as new potential therapeutic targets for HIF2α-driven PPGLs. Identified HIF2α downstream targets might open a new therapeutic window for aggressive HIF2α-expressing tumors.

Introduction

Pheochromocytomas and paragangliomas (PPGLs) are genetically heterogeneous tumors arising from the adrenal medulla or extra-adrenal paraganglia, respectively. A strong connection between genotype and phenotype has been recognized (Fishbein et al. 2017, Fliedner et al. 2018, Crona et al. 2019). PPGLs with activation of pseudohypoxic pathways (cluster-1) comprise those with mutations in genes encoding the von Hippel–Lindau (VHL) tumor suppressor, succinate dehydrogenase subunits (SDHx), prolyl hydroxylase domain (PHD), fumarate hydratase (FH), malate dehydrogenase 2 (MDH2), mitochondrial 2-oxoglutarate/malate carrier (SLC25A11), isocitrate dehydrogenases (IDH1/IDH2/IDH3B), glutamic-oxaloacetic transaminase 2 (GOT2), dihydrolipoamide S-succinyltransferase (DLST), and hypoxia-inducible factor 2α (HIF2α) also known as EPAS1 (Cascón et al. 2019). Among this group, SDHB-mutated paragangliomas are especially prone to metastatic disease (Gimenez-Roqueplo et al. 2003). Higher metastatic risk is also associated with molecular alterations involving fusion genes, mutations of somatic modifier genes and a hypermetabolic phenotype, whereas lower risk is associated with mutations of genes leading to activation of kinase-signaling pathways (cluster-2) in epinephrine producing PPGLs (Fishbein et al. 2017).

There are also suggestions that stabilization of HIF2α in cluster-1 PPGLs might additionally contribute to disease aggressiveness (Richter et al. 2013, Toleda et al. 2013). Higher expression of HIF2α is in particular a characteristic feature of cluster-1 compared to cluster-2 PPGLs (Favier et al. 2002, Eisenhofer et al. 2004, López-Jiménez et al. 2010, Qin et al. 2014). This lends prominence to the actions of cluster-1 mutations to stabilize HIF2α at the protein level. Subsequent work establishing gain-of-function mutations in HIF2α as a cause of PPGLs cemented the role of HIF2α in PPGL tumorigenesis, but to date it is not established whether cluster-1 mutations are associated with heightened risk of malignancy. Nevertheless, in a small cohort of patients with PPGLs due to mutations in HIF2α, metastatic disease was present in nearly a third of cases (Dättr et al. 2016). Agents that target HIF2α have also been proposed as a therapy for patients with metastatic PPGLs (Toleda 2017).

At the molecular level, both HIF2α and HIF1α interact with HIFβ (also known as ARNT) resulting in a C-terminal transactivation of target genes with hypoxia responsive elements (HRE). Alternative ARNT-independent mechanisms of action of HIF2α and HIF1α on other signal transduction and transcriptional systems involve interactions with MYC/MAX, NOTCH or WNT pathways (Dang et al. 2008, Qin et al. 2014). Presumably through any or all of these actions, mutations of genes that lead to stabilization of HIFs are critical drivers of tumor pathogenesis and may additionally induce malignant processes such as epithelial–mesenchymal transition (EMT) (Kluckova & Tennant 2018). In a variety of tumors, such as neuroblastoma, head and neck, renal and colon cancers, HIF2α expression has been linked to poor prognosis (Holmquist-Mengelbier et al. 2006, Moreno Roig et al. 2018).

During metastasis, tumor cells pass multiple steps to reach distant organs (invasion-metastasis cascade). Initiation of metastasis requires the acquisition of a motile and invasive phenotype (e.g. via EMT), induced, for example, by an insufficient oxygen supply (hypoxia). This might cause intravasation into enclosed blood or lymph vessels followed by circulation of tumor cells to distant tissues. If cells are able to attach to the endothelium of the blood vessels, they arrest or start with extravasation. By interacting with the microenvironment, tumor cells start
to proliferate and induce angiogenesis. During all these processes, HIF signaling plays an important role (Lu & Kang 2010). Hypoxia induces the expression of SNAIL and TWIST1 that promote tumor cell EMT (Imai et al. 2003, Yang et al. 2008) and further increases the motility of these cells by elevating the expression of lysyl oxidase and met proto-oncogene (Pennacchietti et al. 2003, Erler et al. 2006). Through upregulation of several proteases such as matrix metalloproteinases (MMPs) and cathepsin D, hypoxia directly promotes tumor cell invasion (Krishnamachary et al. 2003). Moreover, hypoxia-induced expression of vascular endothelial growth factor A (VEGFA) and MMPs facilitate intravasation into the blood stream (Lu & Kang 2010). All these processes are related to hypoxia with HIF1α as the predominant driver. However, under pseudohypoxic conditions, HIF2α seems to be the main driver (Favier et al. 2002, Piets et al. 2010, Morin et al. 2020), but the precise mechanism of HIF2α in tumor progression and metastasis is largely unknown.

For the present study, we hypothesized that HIF2α supports pro-metastatic cell behavior in pseudohypoxic cluster-1 PPGLs, thereby setting the stage for higher metastatic risk in these patients. For this it was first crucial to establish whether mutations in pseudohypoxic genes other than SDHB are generally independent risk factors for developing metastatic disease. To gain improved understanding of potential underlying mechanisms for increased metastatic risk, we then examined for effects of HIF2α on PPGL progression and metastasis by combining gene-manipulation studies in different pheochromocytoma cell lines and mouse models with retrospective patient studies and gene expression profiling in tumor specimens.

Materials and methods

Patient cohort

A total of 793 patients diagnosed with PPGLs were included in the analysis (Supplementary Patient information, see section on supplementary materials given at the end of this article). Patients were recruited at seven centers: University Hospital Carl Gustav Carus Dresden, Germany; University Medical Centre Schleswig-Holstein Lübeck, Germany; University Hospital of Munich, Germany; University Hospital of Würzburg, Germany; Radboud University Medical Centre, Nijmegen, the Netherlands; Institute of Cardiology, Warsaw, Poland; and National Institutes of Health, Bethesda, USA. Patients were part of the Prospective Monoamine-producing Tumor Study (https://pmt-study.pressor.org/) and/or the Registry and Repository of biological samples of the European Network for the Study of Adrenal Tumours (ENS@T) with ethics approval at each participating institution. Informed consent was obtained from all patients. Patients underwent biochemical and genetic testing as defined in the PMT study protocol (Eisenhofer et al. 2018). The study flow is illustrated in Supplementary Fig. 1.

In vitro cell studies

Mouse pheochromocytoma cells, MPC 4/30/PRR and MTT cells (Powers et al. 2000, Martinova et al. 2009), with stable expression of Hif2α were cultivated as previously described (Bechmann et al. 2019). For human hPheo1 cells (Ghayee et al. 2013) provided by Hans Ghayee (University of Florida College of Medicine and Malcom Randall VA Medical Center, Gainesville, FL, USA), RPMI-1640 containing 10% fetal calf serum and 2 mM Glutamax was used. Cells were cultured at 37°C, 5% CO2 and 95% humidity. MycoAlert Mycoplasma Detection Kit was used to ensure cells were mycoplasma free. After trypsinization (trypsin/EDTA; 0.05%/0.02%), cells were diluted with medium and counted using C-CHIPs (Neubauer improved). To simulate hypoxic conditions, cells were cultivated under reduced oxygen partial pressure (≤1% oxygen) in an incubator with an oxygen sensor (Sanyo InCuSafe O2/CO2 Incubator, Model MCO-5M).

HIF2α silencing

HIF2α was stably silenced by transduction with lentiviral particles containing a shRNA against human HIF2α/EPAS1. Experimental details for the generation of hPheo1(-)EPAS-1 and hPheo1 ctrl cells are provided in the Supplementary Data.

Proliferation assay

Cells were seeded in six-well plates (2.25 × 10³ cells/well) and allowed to attach. Cells were cultivated for 48, 72 or 144 h under normoxia or hypoxia. After incubation, cells were washed with PBS, trypsinized, resuspended in medium and counted using C-CHIPs. Each well was counted in duplicate.

Clonogenic survival assay

Clonogenic survival assays were performed as previously described (Bechmann et al. 2017). Briefly, cells...
(MPC/MTT cells: 1000 cells/well; hPheo1 cells: 500 cells/well) were seeded in six-well plates, cultivated for 6 (hPheo1) or 11 (MPC/MTT) days under normoxia or hypoxia, fixed, stained with crystal violet and colonies (≥50 cells per colony) were counted manually using a stereo-microscope (Stemi 2000-c, Carl Zeiss Microscopy).

Migration and invasion assay

The capacity of the different cell lines to migrate through 8 µm pores was examined using TC-Inserts (Sarstedt) as previously described (Bechmann et al. 2018). In brief, cells were cultivated for 24 h in the absence of serum (0.2% BSA). Cells were then seeded in the upper compartment of the TC-Insert and the lower compartments of the 24-well plate were filled with complete medium as chemoattractant. After 24 h incubation under normoxic or hypoxic condition, cells in the lower compartment were stained with BD™ calcein AM Fluorescent Dye (BD Biosciences), trypsinized and the fluorescence was measured at 485/528 nm by VICTOR3 1420 Multilabel-Counter (Perkin Elmer). For invasion assays, TC-Inserts were coated with Matrigel (Matrigel (BD Bioscience)/DMEM+Glutamax, 1/3, v/v), and the experimental procedure was performed analogous to the migration assay.

Adhesion assay

Cells (4 × 10³) were plated in six-well plates. After adhesion, cells were incubated for 24 h under normoxic or hypoxic conditions. 24-well plates were coated with collagen A or laminin, washed with PBS and unspecific binding sites were blocked with PBS containing 2% BSA for 1 h at 37°C. Cells were washed with PBS, detached and suspended in serum-free medium containing 0.2% BSA. Cells were seeded at 2 × 10⁵ per well into the laminin- or collagen-coated wells and allowed to adhere (MPC-mCherry: 45 min; MTT/hPheo1: 30 min). Non-adherent cells were washed away with PBS. The remaining cells were fixed as previously described (Bechmann et al. 2018), washed with tap water, dried on air and lysed using PBS containing 0.5% Triton-X-100. The cell suspension was transferred into a 96-well plate and absorption was measured at 550 nm (reference 650 nm) by VICTOR3 1420 Multilabel-Counter. PBS containing Triton-X-100 was used as blank.

Spheroid culture

Spheroids were generated using the methyl cellulose method and analyzed as previously described (Bechmann et al. 2019). For the generation of the hPheo1 spheroids, 1000 cells per spheroid were seeded.

RNA isolation and qRT-PCR

RNA from cell pellets or PPGL tissue was isolated using NucleoSpin RNA Plus (Macherey-Nagel GmbH, Düren, Germany) in accordance with the manufacturer’s instructions. Reverse transcription (RT) and qRT-PCR were performed as previously described (Bechmann et al. 2018). Primer sequences are listed in Supplementary Table 1.

Live-cell imaging and image segmentation procedure

Cells were seeded on collagen-coated Ibidi 4-well Ph− µ-Slides (Gräfelfing, Germany) in the required medium containing 25 mM HEPES. Cluster formation and pseudopodia extension were observed under a Zeiss AxioObserver Z1 (Jena, Germany) with a heating station at 37°C. Mosaic images were taken using a 10x phase contrast objective with a resolution of 1.03 µm/pixel. Time series were recorded at time interval of dt = 10 min for 24 h resulting in 145 images per series. For each well 2 × 2 field of views (tiles) were stitched before further processing using Zeiss ZEN software (version 2.6 (blue edition)) to obtain images of 3240 × 1800 pixels. The image segmentation procedure is detailed in the Supplementary Data. Briefly, Bayesian inference for the automated adjustment of an image segmentation pipeline were used to perform image segmentation of the cell covered area (Moskopp et al. 2019). The Trainable Weka Segmentation Fiji plugin was used to perform image segmentation of pseudopodia (Arganda-Carreras et al. 2017).

In vivo animal studies

Animal experiments were carried out at the Helmholtz-Zentrum Dresden-Rossendorf according to the guidelines of German Regulations for Animal Welfare and have been approved by the Local Animal Ethics Committee for Animal Experiments (Landesdirektion Dresden, Germany). The protocols were approved by the local Ethical Committee for Animal Experiments (AZ:24-9168.11-4/2007-2, AZ:24-9168.11-4/2012-1, AZ:24-9168.21-4/2004-1 and AZ:25-5131/474/43). Crl:SKH1-EliteHpr and NMRI-nude mice were obtained from Charles River. The subcutaneous (s.c.) tumor model, the liver metastasis model and small animal imaging are described in the Supplementary Data.
Microarray analysis and data mining

Microarray analysis and data mining are explained in the Supplementary Data.

Statistical analysis

If not indicated otherwise, descriptive data are expressed as means ± S.E.M. with statistical analyses taking into consideration numbers (n) of biological and technical replicates within independent experiments. Statistical analyses were carried out by one-way ANOVA with post hoc Bonferroni tests using SigmaPlot 12.5 (Systat Software GmbH, Erkrath, Germany).

Results

Activation of pseudohypoxic pathways is associated with higher prevalence of metastatic disease

Among 425 patients with identified mutations in tumor susceptibility genes, we determined the frequency of metastatic disease in three sub-populations (Supplementary Fig. 1, Study A): (1) 183 patients with mutations of cluster-1 susceptibility genes, excluding SDHB; (2) 115 patients with mutations in cluster-2 susceptibility genes; and (3) 127 patients with SDHB mutations. Patients with mutations in SDHB were considered a distinct subset because of their high prevalence of metastatic disease (Fig. 1A) (Gimenez-Roqueplo et al. 2003). Indeed, among this cohort 76% of patients with SDHB mutations (median follow-up time: 8.5 years) developed metastatic disease characterized by metastases in bones, liver, lung or lymph nodes (Fig. 1B). Activation of pseudohypoxic pathways due to mutations in one of the other cluster-1 genes was associated with a 4.3-fold higher frequency (median follow-up time: 7.0 years) of metastatic disease compared to patients with cluster-2 PPGLs (18.6 vs 4.3%, P < 0.0001). Among the patients with cluster-1 PPGLs, nine carried gain-of-function mutations in HIF2α including three (33%) with metastatic involvement. Patients with metastatic disease were first diagnosed with PPGL at a similar age independent of the underlying cluster-1 mutation (Fig. 1C). Initial tumor volume was also comparable between these groups. A non-adrenergic biochemical phenotype, associated with tumors that do not produce appreciable epinephrine and excluding...
patients with known SDHB mutations, was furthermore associated with a 2.7-fold higher frequency (29.1 vs 10.4%, $P < 0.0001$) of metastatic disease compared to patients with an adrenergic biochemical phenotype characterized by epinephrine production (Fig. 1D, E and Supplementary Fig. 1, Study B).

**Knockdown of HIF2α diminishes pro-metastatic behavior of human progenitor pheochromocytoma cells**

To examine the effect of HIF2α on pheochromocytoma cell progression and the pro-metastatic phenotype, we knocked down HIF2α in human progenitor pheochromocytoma cells (hPheo1) by lentiviral transduction containing a construct of shRNA against human HIF2α. A stable cell population with HIF2α knockdown of 83.5% (hPheo1(-)EPAS1) was obtained and compared to hPheo1 ctrl cells (Fig. 2A and Supplementary Fig. 2). Knockdown of HIF2α slightly diminished the cellular growth, while cultivation under hypoxic conditions ($\leq 1\%$ O₂) led to significant growth retardation in both cell lines (Fig. 2B). The ability to form colonies was not affected by the expression of HIF2α (Fig. 2C). Cultivation under hypoxic conditions resulted in increased colony formation in both lines. Moreover, we generated 3D-tumor cell spheroids as a more complex model characterized by an oxygen and nutrient gradient leading to the formation of a necrotic core surrounded by a hypoxic area and an outer layer of proliferating cells (Bechmann *et al.* 2019).

**Figure 2**

Stable knockdown of HIF2 α diminished the pro-metastatic phenotype of human pheochromocytoma cells (hPheo1). (A) Knockdown efficiency of HIF2α on gene ($n = 8$) and protein ($n = 3$) level. (B) Growth pattern of hPheo1 ctrl and hPheo1(-)EPAS1 cells under normoxic and hypoxic conditions. Four independent experiments ($n = 8$). (C) Colony formation was not affected by the knockdown of HIF2α, but cultivation under hypoxia ($\leq 1\%$ O₂) resulted in an increased formation of colonies ($n = 12$). (D) Reduced expression of HIF2α diminished spheroid growth. Four independent experiments ($n = 12$). Scale bar: 200 μm. Decreased expression of HIF2α as well as hypoxic conditions diminished cell (E) migration and (F) invasion ($n = 15–24$). Knockdown of HIF2α diminished the ability to attach to (G) laminin, while adhesion to (H) collagen was not affected ($n = 6–8$). ANOVA and Bonferroni post-hoc test comparison vs hPheo1 ctrl *$P < 0.05$, **$P < 0.001$ vs normoxia #$P < 0.05$, ##$P < 0.001$. 

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Reduction of HIF2α expression diminished spheroid growth of hPheo1(-)EPAS1 cells (Fig. 2D).

Boyden chamber assays were performed to investigate migration and invasion capacity of the cells in a HIF-dependent manner. Knockdown of HIF2α reduced the ability of cells to migrate through a porous membrane, which requires an active remodulation of the cytoskeleton. Cultivation under hypoxic conditions furthermore diminished migration capacity of cells (Fig. 2E). Knockdown of HIF2α decreased the invasion through matrigel, supporting our hypothesis that HIF2α supports pro-metastatic features of tumor cells (Fig. 2F). Hypoxic conditions reduced the invasion capacity of both cell lines even further indicating an additional effect of stabilization of HIF1α on the pro-metastatic features of these cells. After intravasation into the blood or lymphatic system, tumor cells must attach to the cellular barrier for extravasation. In keeping with our hypothesis, knockdown of HIF2α in the hPheo1(-)EPAS1 cells significantly diminished their ability to attach to the extracellular matrix proteins, laminin (Fig. 2G), while the adhesion to collagen (Fig. 2H) was not affected in this model system. Hypoxia had no effect on adhesion ability.

Expression of HIF2α increases the pro-metastatic phenotype of mouse pheochromocytoma cells

Findings from the hPheo1 cell line were validated using the mouse pheochromocytoma cell line, MPC. A codon-optimized murine Hif2α was introduced into MPC-mCherry cells naturally lacking Hif2α expression (Seifert et al. 2019). The cells were named MPC-mCherry H2A and the counterpart cell line MPC-mCherry control. In comparison to the knockdown of HIF2α in hPheo1 cells, expression of Hif2α resulted in an increased proliferation capacity (Fig. 3A and B). HIF2α expression enhanced the number of proliferating cells (Fig. 3A) and increased colony formation (Fig. 3B). Cultivation under hypoxic conditions only enhanced the colony formation of the MPC-mCherry H2A cells indicating that this effect was solely related to an enhanced stabilization of HIF2α, while stabilization of HIF1α had no effect. These results support our previous findings in MPC-mCherry cells that expression of HIF2α accelerated spheroid growth (Seifert et al. 2019). Migration and invasion capacity were significantly elevated by expression of Hif2α (Fig. 3C and D). Hypoxia increased the invasion and migration capacity of the MPC-mCherry control cells indicating a Hif1α-driven effect, while the MPC-mCherry H2A cells were only mildly affected underlying the predominant Hif2α-driven effect in these cells. The ability to attach to collagen and laminin showed a trend toward increased attachment in cells with expression of Hif2α (Fig. 3E and F). Treatment with 10 µM PT-2385, a HIF2α inhibitor of the first generation, was not sufficient to reduce the effects induced by the expression of Hif2α in these cells (Supplementary Fig. 8). We further validated these effects in MTT cells, generated via tail vein injection of MPC and subsequent re-selection of cells out of metastatic liver lesions (Martinova et al. 2009). These cells showed a significantly increased pro-metastatic behavior compared to the MPC cells (Supplementary Fig. 3). Expression of Hif2α diminished the proliferative properties and led to diverse effects on the pro-metastatic behavior of these cells (Supplementary Figs 4 and 5). Treatment with PT-2385 had no effect on the number of proliferating cells as well as on the migration and invasion capacity of these cells independent of their Hif2α expression (Supplementary Fig. 9).

Expression of HIF2α enhances metastatic load in a mouse liver metastasis model

Expression of Hif2α had no impact on progression of s.c. MPC and MPC-mCherry tumors in NMRI-nude mice (Fig. 3G and H). We therefore studied the impact of Hif2α on metastatic behavior of MPC and MPC-mCherry cells in SKH1 mice exclusively developing liver metastases after i.v. injection of tumor cells reflecting later metastatic events, such as dissemination of tumor cells in the blood stream and extravasation (Ulrich et al. 2018). In both cell lines, Hif2α expression was associated with higher metastatic burden in the liver as visualized using MRI and fluorescence imaging (FLI), respectively (Fig. 3I). Quantification of MR images showed a 1.9-fold increase in numbers of liver metastases (n) and a 7.9-fold increase in total metastatic load volume (V total) in the MPC.Hif2α metastases model compared to controls (Fig. 3I and K). These results demonstrate that Hif2α expression enhances the capability of MPC cells to colonize the liver and, at the same time accelerate metastatic growth.

Expression of HIF2α increases cell motility and leads to enhanced formation of pseudopodia in pheochromocytoma cells

Migration of single cells out of an intact tumor cell mass reflects an early step in the invasion-metastasis cascade; to investigate the underlying mechanism, we hypothesized that elevated expression of Hif2α enhances
the motility of single cells leading to increased migration. A major characteristic of MPC cells is the formation of cell clusters during monolayer culture. Cluster formation and reorganization was monitored for 24 h. Within the first 12 h MPC-mCherry control cells formed clear distinguishable cell clusters, while MPC-mCherry H2A cells seemed less tightly connected (Fig. 4A, B, C, D, E and F). In contrast to MPC-mCherry control cells, MPC-mCherry H2A cells build wide networks with frayed edges (Fig. 4G, H, I, J, K and L). Within these networks, single cells move between different clusters (Supplementary Video 1). Analysis of the cell covered area over time showed that the cluster formation saturated after 5 h in MPC-mCherry control cells, while the cell covered area of the MPC-mCherry H2A cells continuously increased (Fig. 4M). MPC-mCherry H2A cells showed higher values for changes in cell covered area compared to MPC-mCherry control cells, which indicate a higher instability of cell clusters and furthermore reflect the enhanced motility of MPC-mCherry H2A cells (Fig. 4N).

Actin-dependent emergence of cell pseudopodia is a critical element of mesenchymal cell migration and is involved in extravasation by penetrating the endothelial basement membrane (Al-Mehdi et al. 2000, Shankar et al. 2010). MPC-mCherry control cells only
sporadically form pseudopodia between their compact clusters, while MPC-mCherry H2A cells build a dynamic meshwork of pseudopodia (Fig. 5A, B, C, D, E, F, G, H, I, J, K and L). The pseudopodia meshwork is used for locomotion (Supplementary Video 1) and material transfer (Supplementary Video 2). MPC-mCherry control cells build an almost constant number of single pseudopodia 6 h after seeding, while MPC-mCherry H2A cells continue to form and elongate new pseudopodia (Fig. 5M). Moreover, the number of intersected or branched pseudopodia continued to increase during the observation period in MPC-mCherry H2A cells, while MPC-mCherry control cells form pseudopodia less often; the observed enhanced cell dynamics of MPC-mCherry H2A cells are in good accordance with the increased aggressiveness of cells with elevated HIF2α expression (Fig. 5N). Similar results were observed for the more aggressive MTT cells expressing HIF2α (Supplementary Figs 6 and 7). Our data demonstrate for the first time the contribution of HIF2α on tumor cell progression and the pro-metastatic behavior of pheochromocytoma cells in vitro.

**Gene pathway analysis shows HIF2α-related alteration of focal adhesion and extracellular matrix (ECM) receptor interactions**

To better understand the underlying molecular mechanisms that contribute to impacts of HIF2α on pro-metastatic...
Hif2α supports pro-metastatic behavior

N Bechmann et al.

Endocrine-Related Cancer

behavior, gene expression profiles were examined in seven PPGLs with mutations in HIF2α, the ideal HIF2α model, and 24 PPGLs with mutations in one of the cluster-2 susceptibility genes (NF1, RET or MAX; extracted from (López-Jiménez et al. 2010, Qin et al. 2014)). We identified 309 significantly upregulated genes (fold-change >1 and FDR <0.01) and 234 significantly downregulated genes (fold-change <1 and FDR < 0.01) in HIF2α mutated tumors compared to cluster-2 PPGLs. Gene pathway analysis indicated deregulation of focal adhesion and extracellular matrix receptor interactions as well as other pathways, such as the cAMP pathway (Fig. 6A and Supplementary Figs 10 and 11). Pathway networks illustrate the close link between the focal adhesion and extracellular matrix–receptor interactions pathways (Fig. 6B). Regulated genes within these pathways include integrin subunit alpha 7 (ITGA7), laminin subunit beta 1 (LAMB1), collagen type 4A2 (COL4A2) and catenin B1 (CTNNB1) as upregulated genes in HIF2α mutated PPLGs, while reelin (RELN) and cyclin D2 (CCND2) were downregulated (Fig. 6C). We confirmed the regulation of these genes using a second PPGL data set extracted from The Cancer Genome Atlas (TCGA) (Fig. 6D, E and Supplementary Fig. 12) and for selected genes using qRT-PCR on PPGL tumor tissue (Fig. 6F).

Moreover, the RPPA PPGL TCGA cohort showed also a significant upregulation of caveolin 1 (CAV1) protein levels in tumors bearing a mutation in HIF2α compared to PPGLs with an activation of kinase signaling pathways.

Figure 5
Hif2α-dependent formation of pseudopodia. (A, B and C) Representative sections of MPC-mCherry control cells (blue frame) recorded 0, 12, and 24 h after seeding using phase contrast microscopy. MPC-mCherry control cells form sporadic pseudopodia between their compact clusters, while (D, E and F) MPC-mCherry H2A cells (orange frame) build a dynamic meshwork of pseudopodia. Scale bar: 100 µm. (G, H, I, J, K and L) Individual pseudopodia were identified using the trainable weka segmentation algorithm and our own software. Pseudopodia were classified as either solitary pseudopodia (red) or intersected as well as branched pseudopodia (yellow). (M) The count of single pseudopodia shows that MPC-mCherry control cells had an almost constant count of single pseudopodia 6 h after seeding, while MPC-mCherry H2A cells continued to form and elongate new pseudopodia. (N) Furthermore, the number of intersected or branched pseudopodia continued to increase during the observation period in MPC-mCherry H2A cells. MPC-mCherry control cells showed intersected or branched pseudopodia less often. Four experiments (n = 4).
Figure 6
Pathway analysis indicates an impairment of focal adhesion and extracellular matrix (ECM)-receptor interactions in patients with activating mutations of HIF2α compared to patients with cluster-2 PPGLs. Microarray data including tumor tissue of seven patients with HIF2α mutations and 24 PPGL tumor samples bearing a mutation in one of the cluster-2 susceptibility genes (NFI, RET or MAX). (A) List of the most highly regulated KEGG pathways based on pathway enrichment performed in DAVID. q-values are Benjamini-adjusted p-values. (B) Pathway network showing the interconnection of the affected pathways. Lines connecting the pathways indicate overlaps and is based on the overlap coefficient, which is calculated as the overlap of two gene sets divided by the size of the smaller set. The size of the circle displays the number of genes that are regulated in the given pathway, the larger the number the larger the circle. A more red coloration of the circles marks a more significant regulation of the genes altered in the pathway. Subnetwork connecting pathways regulated in HIF2α-mutated tumors indicate a separation of both of the pathways. (C) Differential expression of genes involved in the KEGG pathways ‘Focal adhesion’ or ‘ECM receptor interaction’ in HIF2α-mutated tumors compared to cluster-2 tumors. A second PPGL data set extracted from The Cancer Genome Atlas (TCGA) confirmed the (D) up- or (E) downregulation of genes involved in the KEGG pathways ‘Focal adhesion’ or ‘ECM receptor interaction’. Box and whisker Tukey plots indicating the expression of a selected gene set in the PPGL TCGA cohort in kinase signaling (n = 68) and in HIF2α (n = 8) tumors (other genes included in Fig. 6C are shown in Supplementary Fig. 12). Two-tailed Mann–Whitney test was applied to test for significant differences. (F) Expression of genes involved in ‘Focal adhesion’ or ‘ECM receptor interaction’ pathway in tumor specimens bearing either a mutation in HIF2α (n = 5) or in one of the cluster-2 (n = 9) genes confirmed by qRT-PCR. Expression relative to Actin. ANOVA and Bonferroni post-hoc test comparison vs control cells. **P < 0.01. ITGA7, integrin subunit alpha 7; LAMB1, laminin subunit beta 1; RELN, reelin; CCND2, cyclin D2; CTNNB1, catenin beta 1; COL4A2, collagen type IV alpha 2 chain.

Discussion
Using a large cohort of patients with PPGLs, we established for the first time that even after exclusion of patients with mutations in SDHB, those with tumors due to activation of pseudohypoxic pathways are at higher risk of metastatic disease than other patients. In our cohort, 33% of the patients with HIF2α mutations were diagnosed with metastatic disease, confirming the relatively high frequency (29%) of metastatic disease in these patients reported previously (Därr et al. 2016). Our results further show that the common denominator of pseudohypoxic
PPGLs, HIF2α, has an impact on the pro-metastatic behavior in cluster-1 PPGLs by influencing several steps of the invasion metastasis cascade (Fig. 7).

EMT is considered a critical initial step of the invasion-metastasis cascade that leads to the development of an invasive phenotype. This trans differentiation process involves a variety of changes in the expression of cell adhesion and extracellular matrix interaction molecules (Lamouille et al. 2014). Loriot et al. identified a pathway associated with activation of EMT that distinguishes SDHB-mutated metastatic PPGLs from all other types of PPGLs (Loriot et al. 2012). Due to the non-epithelial state of chromaffin cells, they further clarified this to be a neuroendocrine-to-mesenchymal transition (Loriot et al. 2015), as later confirmed by Calsina et al. (2019). In SDHB-mutated tumors, both HIF2α and TET-mediated hypermethylation synergistically drive the EMT-like phenotype (Morin et al. 2020). Thus, although HIF2α may be pro-metastatic other factors are undoubtedly required to drive invasive behavior in cluster-1 PPGLs.

Our gene pathway analysis indicated that PPGLs with mutations in HIF2α are associated with alterations in genes of the focal adhesion and extracellular matrix-receptor interaction pathway, such as ITGA7, LAMB1 and COL4A2, compared to PPGLs with mutations in cluster-2 genes.

Poomthavorn et al. demonstrated in neuroblastoma cells that hypoxic conditions led to a different expression pattern of several genes (including ITGA7 and COL4A2) correlating with the induction of EMT (Poomthavorn et al. 2009). Other studies confirmed the importance of ITGA7 during EMT and showed a downregulation of ITGA7 in tumor tissue compared to normal tissue (Bhandari et al. 2018, Guan et al. 2020). High ITGA7 expression correlates with poor prognosis in glioma patients and targeted blocking of ITGA7 in a glioma mouse model led to a delay in tumor growth and invasion (Haas et al. 2017). In a genome-wide gene knockdown analysis of human lung carcinoma cells, LAMB1 was furthermore identified as one of 56 candidate genes that contribute to the cellular response to hypoxia (Yoshino et al. 2012). LAMB1 encodes the β1 chain of laminin-8, whose expression correlates with tumor recurrence and survival in glioma patients. A post-transcriptional regulation of LAMB1 via miR-124-5p showed promise to inhibit the growth of gliomas in vitro and in vivo (Chen et al. 2014). Our data indicate that HIF2α might be a regulator of these genes and increased stabilization of HIF2α may be directly involved in the induction of EMT. Targeting the products of these HIF2α-induced genes might provide a strategy to treat HIF2α-dependent tumors, including metastatic cluster-1 PPGLs.

**Figure 7** Contribution of HIF2α in invasion-metastasis cascade. Initiation of metastasis involves the acquisition of a motile and invasive tumor cell phenotype (epithelial-mesenchymal transition, EMT). Our data indicated for the first time that HIF2α increases motility and migration capacity of pheochromocytoma cells by inducing EMT (A). Moreover, HIF2α enhanced the invasion capacity of these cells and led to an increased formation of pseudopodia (B), which might promote the intravasation to enclosed blood or lymph vessels in vivo. Thereafter tumor cells circulate within the blood stream and if cells are able to attach (E) to the endothelial cell layer of the blood vessels, they arrest or initiate extravasation (B). Elevated stabilization of HIF2α led to an increased adhesion capacity (C). Both events are promoted by an increased expression of HIF2α and thereby allow metastatic colonization. Data from Anderson et al. (2019).
Elevated expression of HIF2α contributes to the acquisition of a motile and invasive phenotype in pheochromocytoma cells that might allow for migration out of the intact tumor cell cluster. An important step of the subsequent intravasation into the blood or lymph stream is the formation of pseudopodia initiating the penetration of the endothelial basement membrane (Al-Mehdi et al. 2000, Shankar et al. 2010). Expression of Hif2α enhanced the formation of pseudopodia and led to unstable cell clusters in MPC and MTT cells. The more aggressive phenotype of the MTT cells might explain why the expression of Hif2α had no additional effect on the proliferating properties of these cells. Increased invasion capacity, cell motility, and pseudopodia formation after expressing Hif2α nevertheless implies an additional effect of Hif2α on the pro-metastatic behavior of these cells predominantly characterized by a higher cellular flexibility. In all three examined cell lines, cultivation under hypoxia resulted in exaggerated effects, which in the presence of HIF2α can be attributed to increased stabilization of HIF2α. At the same time, hypoxia also stabilizes HIF1α, which simultaneously contributes to altered cellular behavior under hypoxic conditions.

Further studies involving use of pharmacological blockers of HIF2α is an obvious next step. PT-2385 and PT-2399 are two selective HIF2α inhibitors developed as anticancer drugs. In a clinical phase I trial (NCT02293980) in clear cell renal cell carcinoma, a tumor entity characterized by inactivation of VHL and thereby constitutive HIF2α activity, treatment with PT-2385, achieved a complete response, a partial response or at least stable disease in 2, 12 and 52% of patients, respectively (Courtney et al. 2018). However, some VHL-mutant ccRCC cell lines are resistant to PT-2385 and PT-2399 (Chen et al. 2016). In the present study, PT-2385 also lacked efficacy to diminish HIF2α-dependent effects. This is in line with the observations of others showing that treatment with PT-2385 does not inhibit target gene expression and proliferation in neuroblastoma patient-derived xenograft cells (Persson et al. 2020). Absence of efficiency of PT-2385 possibly relates to the site of action of the drug to interrupt complex formation of HIF2α with ARNT, thereby diminishing the transcriptional activation of HIF target genes. However, as shown by Gordan et al., both HIF1α and HIF2α can act by ARNT-independent mechanisms involving interaction of HIFs with the Myc/Max to produce HIF-dependent regulation of Myc target genes (Gordan et al. 2007a,b). Such mechanisms have been proposed in PPGL cell line models (Qin et al. 2014) and therefore may explain the lack of efficiency of PT-2385 in Hif2α-dependent models.

To investigate the effect of Hif2α expression on later metastatic events, such as dissemination of tumor cells in the blood stream, extravasations and interactions of tumor cells with target tissues, we used the i.v. injection route for the MPC cells (Ullrich et al. 2018). The formation of this metastasized allograft model arising from circulating MPC cells does not depend on pre-metastatic selection, EMT and intravasation (Labelle & Hynes 2012). Expression of Hif2α was associated with higher metastatic burden in the liver, while progression of s.c. MPC tumors were not affected. This suggests that the pro-metastatic phenotype induced by increased expression of Hif2α is characterized by molecular changes that modulate cellular interaction with the target tissue and induce a more invasive/motile phenotype rather than an increased proliferation rate.

Together, these data emphasize the importance of an inhibitory approach to address HIF2α signaling as a therapeutic strategy in PPGLs, where an elevated expression of HIF2α correlates with tumor progression (Richter et al. 2013, Toledo 2017). As mentioned above, the efficiency of the available HIF2α inhibitors is limited, so that alternative indirect strategies should be considered. Mohlin et al. showed in aggressive neuroblastomas with an immature phenotype that the expression of HIF2α could also be reduced indirectly by treatment with phosphatidylinositol-4,5-bisphosphate 3-kinase (P13K) and mammalian target of rapamycin (mTORC) 2 inhibitors (Mohlin et al. 2015). We evaluated this approach previously using the P13Kα inhibitor BYL719 in combination with the mTORC1 inhibitor Everolimus as synergistic approach to reduced pheochromocytoma cell growth (Fankhauser et al. 2019). These data and the successful identification of novel HIF2α downstream targets will open up new therapeutic approaches for aggressive tumors with elevated HIF2α-expression.

Our findings indicate that HIF2α affects multiple steps of the invasion-metastasis cascade in PPGLs mainly by generating a pro-metastatic cellular phenotype characterized by increased cell flexibility and alterations in focal adhesion and extracellular matrix–receptor interaction pathways. The effects described here are also relevant for other HIF2α-dependent tumors, such as neuroblastomas (Pålman & Mohlin 2018) and ccRCCs (Courtney et al. 2018), which further illustrate the diverse contributions of HIF2α in the metastasis-invasion cascade.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-20-0205.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

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