Second messengers in cancer
Jansen, Sepp Reinier

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chapter five.

epac2 regulates hypoxia-induced gene transcription and β-catenin/tcf transcription
Sepp R. Jansen¹,*, Reinoud Gosens¹,², Martina Schmidt¹,²

¹University of Groningen, Department of Molecular Pharmacology, Groningen, The Netherlands
²University of Groningen, University Medical Center Groningen, Groningen Research Institute for Asthma and COPD, GRIAC, Groningen, The Netherlands

* Corresponding author:
Sepp Jansen
University Centre for Pharmacy
Antonius Deusinglaan 1
9713 AV Groningen, The Netherlands
s.r.jansen@umcg.nl
+31 50 363 3320
abstract

Hypoxia occurs when oxygen supply fails to meet demand and results in the stabilization of hypoxia inducible-factor 1α (Hif1α). Hif1α functions as a transcription factor and regulates the expression of genes involved in cellular adaptation to hypoxic conditions. During tumor progression, hypoxia underlies the excretion of pro-angiogenic factors. Hif1α associates with β-catenin under hypoxic conditions and it has been suggested that this underlies the growth arrest observed in hypoxia. Recently, we have demonstrated that the exchange protein directly activated by cyclic AMP 1 (Epac1) is required for β-catenin-mediated transcription. Here, we questioned whether Epac regulates the hypoxic transcriptional response in malignant cells.

By using luciferase reporter gene assays, we show that hypoxia attenuates β-catenin/TCF-mediated transcription and activates Hif1α-mediated transcription. In addition, we demonstrate an increased Epac2, but not Epac1, gene expression in hypoxia. Pharmacological inhibition of Epac2 using ESI-05 abolished the canonical Wnt/β-catenin pathway and β-catenin/TCF-mediated gene transcription, while stimulating expression of the Hif1α-regulated gene vascular endothelial growth factor A (VEGFA). Our data suggest that Epac2 may function to maintain a balance between β-catenin/TCF and β-catenin/Hif1α-mediated transcription.

keywords

Hypoxia, β-catenin, Hif1α, VEGFA, Epac2
Introduction

Cancer cells display biological capabilities that allow the cells to become malignant and survive the constantly changing tumor microenvironment. These capabilities are commonly known as the hallmarks of cancer and include sustained proliferative signaling, evasion from growth suppressors, resistance against cell death, acquisition of replicative immortality, induction of angiogenesis, and activation of invasion and metastasis (Hanahan, Weinberg 2011). With regard to failure of cancer treatment, the last two are of particular interest as they underlie the formation of secondary tumors. For tumor cell dissemination, their escape from the primary tumor site and entrance into the systemic circulation is required. In this regard, the formation of novel vasculature in and around the tumor is of crucial importance as these vessels are not only the first place where tumor cells come in contact with the systemic circulation, but also have a deregulated “leaky” phenotype, allowing migrating tumor cells to intravasate and disseminate (Carmeliet, Jain 2011b, Carmeliet, Jain 2011a).

Cells within the tumor excrete factors that stimulate nearby endothelial cells to migrate in the direction of the tumor, proliferate and form new vessel-like structures. These pro-angiogenic factors are produced and excreted by malignant cells in response to certain cues, the most important being hypoxia, or a low oxygen pressure inside the tumor. Hypoxia occurs when oxygen supply fails to meet oxygen demand and solid malignant tumors commonly contain hypoxic areas (Carmeliet, Jain 2011b, Carmeliet, Jain 2011a).

The cellular response to hypoxia is primarily mediated by transcription factor of the hypoxia-inducible factor (Hif) family, which regulate the expression of genes involved in the adaptation and progression of tumor cells. Each Hif transcription factor is composed of two subunits: the α- and the β-subunit (Goel, Mercurio 2013). While the β-subunit is ubiquitously expressed, the α-subunit is oxygen sensitive. In the presence of oxygen, proline residues on the α-subunit are hydroxylated by oxygen-, iron-, and ascorbate-dependent enzymes known as prolyl-4-hydroxylases (PHDs) (Schofield, Ratcliffe 2004). Hydroxylated Hif1α is recognized by the von Hippel-Lindau protein (pVHL), which subsequently targets it for degradation by ubiquitination. Under hypoxic conditions, PHDs cannot hydroxylate Hif1α resulting in protein stabilization, dimerization with Hif1β to form the Hif transcription factor, and subsequent nuclear translocation. Inside the nucleus, Hif binds to the hypoxia-responsive elements (HREs) and by recruiting transcriptional co-activators regulates the expression of numerous genes involved in cellular adaptation to hypoxia (Semenza 2003).

A key feature is the upregulation of genes that promote angiogenesis/vascularization, most notably vascular endothelial growth factor A (VEGFA) known to be important for endothelial cell activation and proliferation. Interestingly, the metastasis promoting effect of the canonical Wnt pathway effector β-catenin was absent upon Hif1α silencing, indicating an interaction between β-catenin and Hif1α (Santoyo-Ramos et al. 2014, Zhang et al. 2013). Cytoplasmic β-catenin is degraded by a multiprotein destruction complex in the absence of Wnt ligand (Clevers, Nusse 2012). This complex targets β-catenin by phosphorylation for ubiquitination and subsequent degradation. Wnts bind to a family of seven-transmembrane domain receptors, Fzd, disrupt the destruction complex, thereby stabilize cytosolic β-catenin.
and allow it to translocate to the nucleus. Inside the nucleus, β-catenin acts as a transcriptional co-activator by associating with several transcription factors, most notably the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. Hypoxic conditions inhibited the β-catenin/TCF complex formation, and thus β-catenin transcriptional activity (Kaidi, Williams & Paraskeva 2007). This study demonstrated that both transcription factors, Hif1α and TCF, compete for direct binding of β-catenin. The interaction between Hif1α and β-catenin occurs at HREs and as such, β-catenin enhances Hif1α-mediated gene transcription, promoting cellular adaptation to hypoxia.

Recently, we reported that the cyclic AMP regulated guanine exchange factor (GEF) exchange protein directly activated by cyclic AMP 1 (Epac1) was required for nuclear translocation of β-catenin and subsequent β-catenin/TCF target gene transcription in non-small cell lung carcinoma cells (Chapter 4). Here, we aim to answer the question put forward by our observations, whether Epac proteins are involved in promoting β-catenin/TCF or β-catenin/Hif1α-mediated transcription under hypoxic conditions.

**Materials and Methods**

**Cell culture.** HCT116, HepG2, Panc-1, A549, A375 and SK-N-AS cells were obtained from ATCC (Manassas, VA, USA). Res256 medulloblastoma cells were a gift from Prof. Dr. De Bont, Pediatric Oncology group, UMCG Groningen. Cells were maintained in DMEM (1 g/l glucose) supplemented with 10% v/v heat-inactivated FCS and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C. Hypoxic treatment was performed in a hypoxic incubator (1% O₂, 5% CO₂). Cells were washed with HBSS (400 mg/l KCl, 60 mg/l KH₂PO₄, 8 g/l NaCl, 350 mg/l NaHCO₃, 50 mg/l Na₂HPO₄, H₂O, 1 g/l glucose, pH 7.4), dissociated from the plate with trypsin EDTA and seeded in appropriate cell culture plate format. Cells were always maintained subconfluent 24h before and during stimulation, cells were maintained in 0.5% FCS.

**Transfection, TOPFlash and HRE assays.** Cells grown to 60% confluence were transfected in complete growth medium with plasmid DNA (TOPFlash, FOPFlash, HRE firefly luciferase, renilla luciferase (Upstate Biotechnology, Charlottesville, VA), using TransIT-X2 transfection reagent (Mirus Bio, Madison, WI). TOPFlash, FOPFlash or HRE transfected cells were subjected to stimulation for 24h, and luciferase activity was assayed via the Dual Reporter luciferase assay system (Promega, Madison, WI). During transfection with TOPFlash, FOPFlash or HRE plasmids, serum was omitted from the growth medium because it induces nuclear translocation of β-catenin (own observations).

**Isolation of mRNA and real-time PCR analysis.** Total mRNA extraction was performed using a TRizol extraction. cDNA was acquired using reverse transcription by AMV Reverse Transcriptase Kit (Promega, Madison, WI). qPCR was performed with the Illumina Eco Personal qPCR System (Westburg, Leusden, The Netherlands). Cycle parameters (30s each): denaturation at 94°C, annealing at 60°C and extension at 72°C. Target genes were normalized to the geometric mean of reference genes GAPDH and 18S. Primer sequences are listed in table 1.

**VEGFA release.** VEGFA levels were quantified using enzyme-linked immunosorbent assays (ELISA), using the VEGF DuoSet ELISA kit according to the manufacturer’s instructions.
We diluted samples were needed to remain in the range of the standard curve.

Viability assay. Cell viability was determined using mitochondrial reduction of AlamarBlue. Prior to measurement, cells were washed with calcium containing HBSS (400 mg/l KCl, 60 mg/l KH₂PO₄, 8 g/l NaCl, 140 mg/l CaCl₂, 100 mg/l MgCl₂•6H₂O, 100 mg/l MgSO₄•7H₂O, 90 mg/l Na₂HPO₄•7H₂O, 1 g/l glucose, pH 7.4) and then incubated with 5% v/v AlamarBlue (Invitrogen, Carlsbad, CA, USA) followed by fluorescence spectrophotometry. Treated cultures were normalized to control cultures.

Preparation of Wnt3a conditioned medium. Incubation with Wnt3a was performed using Wnt3a conditioned medium and compared to control conditioned medium without Wnt3a. Wnt3a conditioned and control conditioned medium were prepared using mouse L cells, cultured in DMEM supplemented with 10% v/v heat-inactivated FCS and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C. These cells were stably transfected with a vector containing the Wnt3a complementary DNA under the control of the PGK promoter, and G418-resistant clones were selected for production of Wnt3a protein (Willert et al. 2003). Control conditioned medium was from L-cells transfected with the same vector without the Wnt3a insert. After selection, cells were allowed to grow to confluency in the absence of G418. Medium was collected and sterile filtered and mixed 1:1 with another sterile filtered batch of conditioned medium. Before use, the conditioned medium was diluted 20 times in serum-free medium to achieve a final FCS concentration of 0.5% v/v.

Reagents. ESI-05 was a kind gift from Prof. Dr. Xiaodong Cheng (Tsalkova et al. 2012). All other chemicals were of analytical grade.

Statistics. Data represent means ± S.E.M, from n separate experiments. Normality and equal variance were evaluated by Shapiro-Wilk test and f-test. Statistical significance of differences was evaluated by Student’s t-test and 1-way or 2-way ANOVA followed by a Tukey multiple comparison test. Differences were considered to be statistically significant when p<0.05.

Results

Hypoxia shifts transcriptional programs from TCF to Hif1α-dependent gene transcription

Hypoxia results in the stabilization of Hif1α and subsequent activation of Hif-regulated transcription by binding to HREs. Earlier studies have shown that under hypoxic conditions, β-catenin shifts its role as a transcriptional co-activator of TCF-mediated gene transcription, to Hif-mediated gene transcription (Kaidi, Williams & Paraskeva...
To confirm this, we measured TCF- and Hif-mediated gene transcription in a panel of malignant cells, consisting of HepG2 (hepatocellular carcinoma), Panc-1 (pancreatic carcinoma), A375 (melanoma), SK-N-AS (neuroblastoma), HCT116 (colorectal carcinoma), Res256 (medulloblastoma) and A549 (non-small cell lung carcinoma). TCF-mediated gene transcription was determined using the TOPFlash luciferase reporter gene construct with the FOPFlash construct, with mutated TCF binding sites, as a negative control. Hif-mediated gene transcription was determined using the HRE luciferase reporter gene construct. After transfection, cells were incubated in a normoxic chamber (5% CO₂, 20% O₂) or a hypoxic chamber (5% CO₂, 1% O₂) for 24 hours. As expected most cell lines tested, except the A375 melanoma cell line, showed an increase in HRE-luciferase, indicating enhanced Hif1α-mediated gene transcription (Fig. 1). In addition, a decrease in TOPFlash reporter gene expression was observed, indicating a decrease in TCF-mediated gene transcription. As the role of Wnt/β-catenin signaling is well established in colorectal carcinoma (Clevers, Nusse 2012) and we have demonstrated in an earlier study that SK-N-AS neuroblastoma cells critically require β-catenin for their survival (Chapter 3), we used for further experiments HCT116 colorectal carcinoma cells and SK-N-AS neuroblastoma cells.

In addition, we activated cellular Wnt/β-catenin with Wnt3a conditioned medium. In both cells lines, hypoxia decreased expression of the canonical Wnt/β-catenin target gene Axin2 (Fig. 2B). However, Wnt3a stimulation elicited a response only in the HCT116 cells and not in the SK-N-AS cells, possibly pointing to a low expression level of functional Wnt3a receptors in SK-N-AS cells. Importantly, hypoxia largely abrogated Wnt3a-induced Axin2 gene expression in HCT116 cells. Thus, hypoxia interferes with the canonical Wnt/β-catenin response and activates the Hif1α response in a distinct subset of tumor cells.

**Hypoxia regulates Epac2 and VEGFα expression**

In order to establish if hypoxia and canonical Wnt/β-catenin affect the expression of Epac proteins, we performed a qPCR for expression of the Epac subtypes Epac1 and Epac2. In HCT116 cells, hypoxia induced a marked increase in mRNA expression of Epac2 (Fig. 3A). In SK-N-AS cells, expression of Epac2 seems to be decreased under hypoxic conditions, however, the reduction in Epac2 expression did not reach statistical significance. The expression of the Epac1 subtype was not affected by hypoxia (data not shown). Stimulation with Wnt3a conditioned medium had no effects on expression of Epac2 (Fig. 3A) or Epac1 (data not shown).

To further determine the effect of Wnt3a on Hif1α-mediated gene transcription, we determined expression of the Hif1α target gene VEGFA. Expression of VEGFA was increased under hypoxic conditions in both cell lines (Fig. 3B). This process was unaffected by Wnt3a conditioned medium, indicating that canonical β-catenin activation leaves VEGFA expression largely unaffected.

**Inhibition of Epac2 attenuates canonical Wnt signaling**

As shown above SK-N-AS cells did not respond to Wnt3a conditioned medium (Fig. 2B) and show no alterations in Epac2 gene expression under hypoxia (Fig. 3A), we performed further experiments with the Wnt3a conditioned
Epac2 regulates hypoxia-induced gene transcription and β-catenin/TCF transcription

medium and pharmacological inhibition of Epac2 in HCT116 cells only. Since earlier studies (Kaidi, Williams & Paraskeva 2007) suggest that hypoxia shifts the balance from β-catenin/TCF to β-catenin/Hif1α-mediated transcription, we hypothesized that Epac2 plays a role in maintaining the balance between β-catenin/TCF and β-catenin/Hif1α and that loss of Epac2 potentially shifts the balance towards β-catenin/Hif1α. If this is the case, inhibition of Epac2 function should decrease β-catenin/TCF-mediated transcription.

To study the role of Epac2 under hypoxia in our cell model, we used the subtype selective Epac2 antagonist, ESI-05 (Tsalkova et al. 2012). We observed that Wnt3a conditioned medium induced TOPFlash reporter gene activation, a process being largely abolished by the Epac2 inhibitor ESI-05 (Fig. 4A). In addition, we observed that Axin2 gene expression was absent in ESI-05 treated cells, to a further extent compared to hypoxia (Fig. 4B). Strikingly, Wnt3a conditioned medium-induced Axin2 gene expression was also completely abolished, indicating that Epac2 is required for functional canonical Wnt/β-catenin gene transcription.

**Inhibition of Epac2 enhances VEGFA gene expression in and release from HCT116 colorectal cancer cells**

Further, we questioned whether pharmacological inhibition of Epac2 was also able to affect Hif1α-mediated transcription in HCT116 cells. In line with our findings on VEGFA gene expression (Fig. 3B), Wnt3a conditioned medium had no effect on HRE reporter gene activation in HCT116 cells (Fig. 5A), further confirming that canonical Wnt/β-catenin had no effect on the hypoxic response. However, pharmacological inhibition of Epac2 decreased HRE reporter gene activation, both under normoxia and hypoxia, suggesting that the hypoxia-induced increase in Epac2 gene expression plays a potential functional role in Hif1α-mediated transcription.

Therefore, we determined the effects of pharmacological inhibition of Epac2 on mRNA expression of pro-angiogenic factors. As Epac2 is upregulated during hypoxia in HCT116 cells (Fig. 3A), and pharmacological inhibition of Epac2 attenuated the hypoxia-driven HRE reporter gene activation (Fig. 5A), we expected that pharmacological Epac2 inhibition decreases the hypoxia-driven VEGFA expression. Interestingly however, we observed an increase in VEGFA gene expression in cells treated with the Epac2 inhibitor, both under normoxia and hypoxia (Fig. 5B). To confirm this, we performed an ELISA for VEGFA protein secretion by HCT116 cells. Similar to VEGFA gene expression, we observed a marked increase in VEGFA secretion in cell treated with the Epac2 inhibitor ESI-05, which was further increased by hypoxia (Fig. 5C). Thus, it appears that Epac2 activity is required to diminish acute hypoxic responses in HCT116 cells.
Chapter five

![Bar graphs showing fluorescence (Fluc) as percentage of normoxia for various cell lines under hypoxia (HRE and TOPFlash conditions).](image)

**HepG2**
- HRE: **+++**
- TOPFlash: *

**Panc-1**
- HRE: **+++**
- TOPFlash: *

**A375**
- HRE: **++**
- TOPFlash: *

**SK-N-AS**
- HRE: **+**
- TOPFlash: *

**HCT116**
- HRE: *
- TOPFlash: *

**Res256**
- HRE: *
- TOPFlash: **++**

**A549**
- HRE: *
- TOPFlash: *
Figure 1.

TOPFlash and HRE reporter gene assays for activation of canonical Wnt/β-catenin and Hif1α-mediated gene transcription, respectively. A panel of 7 different cell lines was used of hepatocellular carcinoma (HepG2), pancreatic carcinoma (Panc-1), melanoma (A375), neuroblastoma (SK-N-AS), colorectal carcinoma (HCT116), medulloblastoma (Res256), and non-small cell lung carcinoma (A549). Cells were incubated for 24 hours in either a normoxic (5% CO\textsubscript{2}, 20% O\textsubscript{2}) or a hypoxic (5% CO\textsubscript{2}, 1% O\textsubscript{2}) chamber. Data represent mean ± s.e. of the mean of 9 separate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to normoxic conditioned cells.

Figure 2.

A) Cell viability of HCT116 and SK-N-AS cells after 24 hours incubation in the hypoxic chamber. B) qPCR detection of AXIN2 expression in HCT116 and SK-N-AS cells after 24 hours incubation in the hypoxic chamber in the presence or absence of Wnt3a. Cells were treated with either 1:20 control conditioned medium (final FCS concentration 0.5%) or 1:20 Wnt3a conditioned medium. Data represent mean ± s.e. of the mean of 4 separate experiments. *** p < 0.001 compared to normoxic, control conditioned cells.
**Figure 3.**

A) qPCR detection of Epac2 expression in HCT116 and SK-N-AS cells after 24 hours incubation in the hypoxic chamber in the presence or absence of Wnt3a. B) qPCR detection of VEGFA expression in cells after 24 hours incubation in the hypoxic chamber in the presence or absence of Wnt3a. Data represent mean ± s.e. of the mean of 4 separate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to normoxic, control conditioned cells.

![Graph A](image1)

![Graph B](image2)

**Figure 4.**

A) TOPFlash reporter gene assay in HCT116 cells after 24 hours incubation in the hypoxic chamber in the presence or absence of Wnt3a and the Epac2 inhibitor ESI-05 (10 μM). B) qPCR detection of AXIN2 expression in HCT116 cells after 24 hours incubation in the hypoxic chamber in the presence or absence of ESI-05. Data represent mean ± s.e. of the mean of 4 (qPCR and ELISA) or 9 (HRE reporter gene) separate experiments. *** p < 0.001 compared to normoxic, control conditioned cells or between indicated groups.
A) HRE reporter gene assay in HCT116 cells after 24 hours incubation in the hypoxic chamber in the presence or absence of Wnt3a and the Epac2 inhibitor ESI-05 (10 μM). B) qPCR detection of VEGFA expression in HCT116 cells after 24 hours incubation in the hypoxic chamber in the presence or absence of ESI-05. C) ELISA assay for determination of VEGFA protein secretion in HCT116 cells after 24 hours incubation in the hypoxic chamber in the presence or absence of ESI-05. Data represent mean ± s.e. of the mean of 4 (qPCR and ELISA) or 9 (HRE reporter gene) separate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to normoxic, control conditioned cells or between indicated groups.
Discussion

Over the past decade it has become clear that Hif1α interacts with several transcriptional co-activators, including the canonical Wnt effector β-catenin. Here, we demonstrate by TOPFlash reporter gene expression and expression of the canonical Wnt/β-catenin target gene Axin2, that hypoxia inhibits β-catenin/TCF-mediated transcription in HCT116 colorectal carcinoma cells. Hypoxia did not only inhibit basal activity of β-catenin/TCF-mediated transcription, but also its induction by the canonical Wnt ligand Wnt3a, indicating that hypoxia effectively inhibits canonical Wnt/β-catenin activation. Kaidi and coworkers reported in colorectal carcinoma cells that hypoxia inhibits β-catenin/TCF complex formation and transcriptional activity resulting in a G1 arrest. Additionally, the authors have shown that nuclear Hif1α competes with TCF for direct binding to β-catenin and proposed that β-catenin enhances Hif1α-mediated transcription, promotes cell survival and adaptation to hypoxia (Kaidi, Williams & Paraskeva 2007). Our data indicate that activation of canonical Wnt/β-catenin, being characterized by increased stability and nuclear translocation of β-catenin, enhances the hypoxic response. However, we did not observe enhanced HRE-reporter gene expression, or enhanced expression of VEGFA, upon stimulation of colorectal carcinoma cells with Wnt3a. Thus, based on our findings, hypoxia seems not to shift β-catenin from TCF to Hif1α, but only seems to inhibit β-catenin/TCF-mediated transcription. Similarly, matrix metalloproteinase-13 expression known to be under the control of canonical Wnt/β-catenin, is exacerbated in chondrocytes from a conditional Hif1α knock-out mice model, indicating that Hif1α is able to block canonical Wnt/β-catenin (Bouaziz et al. 2016).

Several studies have aimed to decipher the mechanism by which Hif1α is able to affect β-catenin/TCF-mediated transcription. The Human arrest-defective-1 (hARD1) regulates cell cycle and proliferation, and physically associates with Hif1α (Arnesen et al. 2005). In addition, it was found that hARD1 binds and acetylates β-catenin, which in turn activates β-catenin/TCF and induces canonical Wnt/β-catenin-dependent proliferation of non-small cell lung cancer cells (Lim, Park & Chun 2006). In a follow-up study, the same group demonstrated that Hif-1α binding to hARD1, dissociated hARD1 from β-catenin, thereby preventing β-catenin acetylation (Lim, Chun & Park 2008).

In a recent study, it was found that hypoxia results in the phosphorylation of β-catenin at Y654. All β-catenin phosphorylated at this residue was found complexed with Hif1α and it was demonstrated that this β-catenin phosphorylation was required for Hif1α transcriptional activity (Xi et al. 2013). Hif1α and Hif2α are structurally very identical. However, growing evidence suggests that both Hif members regulate the expression of distinct set of genes (Lofstedt et al. 2007). In addition, Hif1α induces cell cycle arrest during hypoxia, while Hif2α promotes proliferation of renal carcinoma, lung carcinoma, and neuroblastoma cells (Gordan et al. 2007). A recent study proposed that the functional discrepancy between Hif1α and Hif2α might be due to a different association with β-catenin (Santoyo-Ramos et al. 2014). Both Hif isotypes are able to bind β-catenin, however, at distinct interaction sites. Hif1α bind to the C-terminus of β-catenin, while Hif2α with the N-terminus. Interestingly, Hif2α was found to assemble with β-catenin/TCF and facilitate gene transcription due to an enhanced recruitment...
of the transcriptional co-activator p300. The distinct binding properties of Hif2α oppose those of Hif1α on β-catenin, and this suggests that the Hif1α/Hif2α balance may determine cell cycle progression under coexistence of hypoxia and Wnt stimulation (Choi et al. 2010).

In chapter 4 we studied PGE2-induced nuclear accumulation of β-catenin. We found that Epac1 is required for nuclear translocation and activation of β-catenin/TCF-mediated transcription. Therefore, we were interested if Epac also plays a role in determining the outcome of hypoxia-driven inhibition of β-catenin/TCF-mediated transcription. Interestingly, we observed a marked increase in mRNA expression of Epac2, but not Epac1, in HCT116 colorectal carcinoma cells. Thus, we questioned whether pharmacological inhibition of Epac2 reduces β-catenin/TCF-mediated transcription. Indeed, the Epac2 inhibitor ESI-05 (Tsalkova et al. 2012), completely abolished Wnt3α-induced activation of the TOPFlash reporter gene and expression of the canonical Wnt/β-catenin target gene Axin2. Our data indicate that indeed Epac2 mediates canonical Wnt/β-catenin and β-catenin/TCF-mediated transcription. Importantly, the Epac2 inhibitor ESI-05 increased expression of the Hif1α target gene VEGFA and VEGFA secretion both under normoxia and hypoxia in HCT116 cells. Together, our data suggest that Epac2 mediates Wnt/β-catenin and β-catenin/TCF-mediated transcription, and blocks Hif1α-mediated transcription. Taken studies into consideration demonstrating that under hypoxia β-catenin shifts from TCF-mediated transcription to Hif1α-mediated transcription (Santoyo-Ramos et al. 2014, Kaidi, Williams & Paraskeva 2007, Bouaziz et al. 2016, Lim, Chun & Park 2008, Xi et al. 2013, Choi et al. 2010), our data indicate that Epac2 supports TCF/Hif1α balance maintenance in favor of β-catenin/TCF-mediated transcription. If our hypothesis turns out to be true, it implies that the hypoxia-induced Epac2 expression is not driving Hif1α-mediated gene transcription, but should be envisioned as a cellular adaptation to restore the balance TCF- and Hif1α-mediated transcriptional programs.

Once activated by cyclic AMP, Epac1 directly binds to the nuclear pore protein RanBP2 at the nuclear membrane (Liu et al. 2010, Gloerich et al. 2011, Qiao et al. 2002), through a sequence in the CDC25-HD domain of Epac1 (Parnell, Smith & Yarwood 2015). In chapter 4 we showed that a RanBP2-binding-deficient Epac1 decreased β-catenin/TCF-mediated transcription. Our data suggest that nuclear pore localization of Epac1 may enhance the nuclear import of β-catenin. Interestingly, even though Epac2 contains the same RanBP2 binding sequence, cyclic AMP failed to induce nuclear localization of Epac2 at least in HEK293 cells (Parnell, Smith & Yarwood 2015).

Interestingly, a recent study on the role of cyclic AMP-degrading phosphodiesterases (PDEs) in hypoxia in non-small cell lung carcinoma provided further evidence on the role of Epac in Hif1α-mediated transcription (Pullamsetti et al. 2013). The authors observed an increased protein expression of PDE4A and PDE4D in hypoxic cells. Using RNA interference for Hif1α, it was demonstrated that the expression of PDE4A and PDE4D is under direct control of hypoxia and Hif1α. Further, it is reported that increasing intracellular cyclic AMP by inhibition of PDE4A and PDE4D, resulted in a complete abolishment of Hif1α-mediated transcription. Moreover, treatment with cyclic AMP elevating agents significantly decreased Hif1α-mediated transcription. More specifically, treating cells with the Epac agonist 8-pCPT-2’-O-Me-cAMP also decreased Hif1α-mediated transcription, including VEGFA
expression, in non-small cell lung carcinoma cells. Thus, the authors showed that activation of cyclic AMP signaling, including Epac activation, resulted in an inhibition of hypoxia-induced Hif1α-mediated transcription. Here, we report that inhibition of the cyclic AMP effector Epac2, enhances Hif1α-mediated transcription.

In conclusion, we report here that hypoxia inhibits canonical Wnt/β-catenin and β-catenin/TCF-mediated transcription, but promotes Hif1α-mediated transcription. In HCT116 colorectal carcinoma cells, hypoxia is accompanied by increased expression of Epac2. Pharmacological inhibition of Epac2 results in a reduction of canonical Wnt/β-catenin, and further enhances Hif1α-mediated transcription. Our data suggest that Epac2 may thereby function to maintain a balance between β-catenin/TCF and β-catenin/Hif1α-mediated transcription.

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Conflict of Interest Statement

The authors declare that they have no competing interests.
Chapter five

A — W


