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Original Articles

Tumor suppressor miR-215 counteracts hypoxia-induced colon cancer stem cell activity

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ABSTRACT

Cancer stem cells, also known as tumor-initiating cells (TICs), are a population of aggressive and self-renewing cells that are responsible for the initiation and progression of many cancers, including colorectal carcinoma. Intratumoral hypoxia, i.e. reduced oxygen supply following uncontrolled proliferation of cancer cells, is thought to support TIC activity by inducing specific hypoxia-responsive mechanisms that are not yet entirely understood. Using previously established and fully characterized patient-derived TIC cultures, we could observe increased sphere and colony formation under hypoxic conditions. Mechanistically, microRNA (miRNA)-profiling experiments allowed us to identify miR-215 as one of the main hypoxia-induced miRNAs in primary colon TICs. Through stable overexpression of miR-215, followed by a set of functional *in vitro* and *in vivo* investigations, miR-215 was pinpointed as a negative feedback regulator, working against the TIC-promoting effects of hypoxia. Furthermore, we could single out LGR5, a *bona fide* marker of non-neoplastic intestinal stem cells, as a downstream target of hypoxia/miR-215 signaling. The strong tumor- and TIC-suppressor potential of miR-215 and the regulatory role of the hypoxia/miR-215/LGR5 axis may thus represent interesting points of attack for the development of innovative anti-CSC therapy approaches.

1. Introduction

According to the cancer stem cell (CSC) model, tumorigenic properties are reserved to a rare subset of cancer cells, displaying extensive self-renewal behavior [1]. Such tumor-initiating cells (TICs) have first been described in the context of acute myeloid leukemia [2,3], but their existence has also been reported in different solid tumor types, including breast [4], brain [5], lung [6], and colon [7,8] cancer. Since the first discovery of colon TICs in 2007, many different isolation strategies (reviewed in Ref. [9]) and surface markers (reviewed in Refs. [10,11]) have been employed, with varying degrees of success. In particular leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), *a bona fide* Wnt target and accepted marker of non-neoplastic intestinal stem cells [12,13], has gained a lot of attention over the last years [14]. Barker and colleagues could show that APC deletion in LGR5$^+$ stem-like cells results in the massive formation of fast-developing adenomas, whereas the same genetic alteration showed much less effect in more differentiated cells [15]. Moreover, a cell lineage tracing study by Schepers and colleagues could identify LGR5$^+$ cells as the cellular origin of colonic neoplastic growth [16]. More recently, LGR5 expression has also been clearly associated with the initiation of both primary intestinal cancers and colorectal carcinoma (CRC)-derived liver metastasis [17–19]. Taken together, these studies deliver direct experimental evidence for the existence of stem cell-derived colon TICs. However, due to extensive plasticity and fast population dynamics, the functional regulation of colon TICs and the molecular control of LGR5 remain incompletely understood.

It has long been noted that cancer cells closely interact with surrounding non-tumor elements, commonly referred to as the tumor microenvironment (TME). Maintenance of phenotypic plasticity,

Abbreviations: CRC, colorectal carcinoma; CSC, cancer stem cell; DEG, differentially expressed gene; DEM, differentially expressed microRNA; FDR, false discovery rate; GEO, Gene Expression Omnibus; HRM, hypoxia-responsive microRNA; IBBL, Integrated Biobank of Luxembourg; LGR5, leucine-rich repeat-containing G-protein coupled receptor 5; miRNA, microRNA; RT, reverse transcription; SC, spheroid culture; TFF, trefoil factor; TIC, tumor-initiating cell; TME, tumor microenvironment

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protection from the immune system, and facilitation of metastatic spread are features that are strongly promoted by the TME [20]. Besides cellular elements of the TME, intratumoral hypoxia is recognized as one of the driving forces of cancer initiation and progression [21]. In this context, hypoxia-responsive microRNAs (miRNAs) are thought to play an important role in modulating cancer cell activity [22]. Besides miR-210, which is commonly accepted as the main hypoxia-responsive miRNA (HRM) [23] with known implications in colon TIC regulation [24], many additional HRMs are suspected to fine-tune the molecular interplay between cancer cells and their microenvironment [22].

In the context of glioma, miR-215 has recently been reported as a HRM that is post-transcriptionally regulated in a HIF-Drosha-dependent manner [25]. While displaying oncogenic activity in glioma patients, miR-215 is a suspected CRC suppressor that is frequently down-regulated in tumor samples, compared to normal colon biopsies [26–28]. Besides modulating chemoresistance [29] and inhibiting proliferation, migration, and invasion [30] of differentiated CRC cells, miR-215 was also shown to specifically target the (cancer) stemness regulator BMI1 [31]. On the other hand, the functional effects of miR-215 on TIC activity as well as a potential link to hypoxia signaling are less well studied in the context of colon CSCs. In the present study, we show that miR-215–5p is consistently induced by hypoxia in different primary colon TIC cultures. Furthermore, we identify LGR5 as a novel hypoxia/miR-215 downstream target of interest. Most importantly, we demonstrate that miR-215 has strong tumor- and TIC-suppressor potential, thus acting as a negative feedback regulator of hypoxia-induced TIC activity. Altogether, our results deliver new insights into the molecular response of colon TICs to hypoxic conditions and highlight miR-215 as an interesting point of attack for the development of future TIC-targeting therapy approaches.

2. Materials and methods

2.1. Patients

Following approval by the Comité National d'Éthique de Recherche du Luxembourg (Reference 201009/09) and the institutional Ethics Review Panel (ERP-16-032), written informed consent of all patients was obtained and tissue samples were collected by the Integrated Biobank of Luxembourg (IBBL), as previously reported [32].

2.2. Cell culture

Primary spheroid cultures (SCs) T6, T18, T20, and T51 were isolated from fresh CRC patient material, fully characterized, and maintained in SC conditions, as described previously [33]. TIC enrichment was achieved by passaging the SCs at least 5 times before further use in experiments.

2.3. Viral transductions

SCs with stable miR-215 overexpression were generated by using ready-to-use lentiviral particles (Biosettia) at a multiplicity of infection of 3–5. Puromycin was used to select transduced cells and transduction efficiency was evaluated via fluorescence microscopy and qPCR. The specificity of the LGR5 antibody was assessed by using primary TIC cultures with stable knockdown of LGR5 via application of a pool of 3 different target-specific short hairpin (sh) RNA constructs against LGR5 (Santa Cruz sc-62559-V) (at a multiplicity of infection of 3–5), followed by puromycin selection.

2.4. Sphere and colony formation assays

In vitro self-renewal and 2D colony formation capacity were tested as previously described [24,33]. Limiting dilution assays, with cell densities ranging from 1 to 1000, were performed in SC conditions and evaluated using the extreme limiting dilution analysis (ELDA) software [34]. 3D colony formation assays were performed using a serum-free mix of 60% SCM medium [33] and 40% methylcellulose medium, i.e. MethoCult® H4100 (StemCell Technologies), supplemented with bFGF (Miltenyi Biotec; 20 ng/mL) and EGF (Biomol; 20 ng/mL). After 14 days, resulting 3D colonies were counted under a microscope.

2.5. In vivo assays

Non-obese diabetic/severe-combined immunodeficient gamma (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) (NSG) mice were bred in-house and experiments were performed according to all applicable laws and regulations, after receiving approval by the institution's animal experimentation ethics committee and the veterinarian service of the Ministry of Agriculture (Permit Number: 14-MDM-02 and 16-MDM-01-EL). In vivo tumor formation assays and serial transplantations were performed, as previously described [33]. Extracted xenografts were dissociated and lyzed and gene, miRNA, and protein expression was assessed.

2.6. Flow cytometry

The expression of the surface markers CD24 and CD44 was checked via flow cytometry. The samples were prepared and analyzed, as previously described [33], using a FACS Canto II device. Dead cells were stained with 1 μM Sytox blue (Life Technologies) and CD24 FITC, FITC Mouse IgG2a, κ, CD44 PE-Cy7 and PE-Cy7 Mouse IgG2b, κ (BD Biosciences) were used as primary antibodies and respective isotype controls.

2.7. RNA extraction and real-time qPCR

After collection and dissociation of SCs, patient samples, and xenografts, RNA was extracted and reverse transcription (RT) as well as real-time qPCRs were performed, as previously described [24]. Briefly, RNA extraction and RT were done using the miRNeasy Mini Kit (Qiagen) and the miScript II RT Kit (Qiagen), respectively. Expression levels were assessed with specific primer pairs (sequences: *LGR5* forward 5′-AGC-CAT-GAC-CTT-GGC-CCT-GA-3′ and reverse 5′-GCT-GTG-GAG—CCC—ATC-AAA-GCA-3′, *TFF1* forward 5′—CCA-GTG-TGC-AAA-TAA-GGG-CTG-C-3′ and reverse 5′-AGG-CAG-ATC-CCT-GCA-GAA-GTG-T-3′, *TFF2* forward 5′-AAC-TGC-GGC-TTC-CCT-GGA-ATC-A-3′ and reverse 5′-GCC-ACA-GTT-TCT-TCG-GTC-TGA-G-3′, *TFF3* forward 5′-TCC-AGC-TCT-GCT-GAG-GAG-TAC-G-3′ and reverse 5′-ATC-CTG-GAG-TCA-AAG-CAG-CAG-C-3′) for genes and with miScript Primer Assays (Qiagen) for miRNAs. Samples with poor melting curves and/or threshold cycle (Ct) values > 35 were considered as not expressed and removed from the analysis. Quality control and normalization were performed with the qBase + software (Biogazelle) using the geometric mean of multiple reference genes (primer sequences available in Refs. [24,33]).

2.8. Cell lysis and Western blots

SCs and extracted xenografts were washed with PBS and lyzed in RIPA buffer (Thermo Fisher) with 1% SDS. After adding 1 x Laemmli buffer, lysates were processed, blotted and detected, as previously described [24]. Primary antibodies used in this study were β-Actin (Millipore, #MAB1501), STAT5 (Santa Cruz, sc-835), LGR5 (Abcam, 75850), and LGR5 (Miltenyi, 130-104-945).

2.9. mRNA and miRNA microarray analysis

Affymetrix GeneChip™ Human Gene 2.0 ST Array and GeneChip™ miRNA 4.0 Array were used to generate mRNA and miRNA expression profiles of different primary SCs. Filtering and normalization of

expression intensities were performed as previously described [33,35]. Hypoxia-responsive genes and miRNAs were identified by setting a false discovery rate (FDR) < 0.05 and a |log2 fold change| > 1 as the cutoff values. For identifying potential miR-215 target genes, less strict cutoff criteria (i.e. FDR < 0.1) were used. Differentially expressed genes (DEGs) and miRNAs (DEMs) were determined with an empirical Bayesian approach using the Bioconductor *limma* R package. Normalized expression values were deposited in the Gene Expression Omnibus (GEO) and ArrayExpress databases and are available under accession numbers GSE120422, GSE120424, and E-MTAB-7354.

2.10. Public datasets

GSE49246 [28], GSE39582 [36], GSE41258 [37], and the TCGA cohort (COAD project) were used to study the expression of miR-215 and *LGR5* in publicly available CRC datasets. Expression levels of matching cancer and healthy colon samples were analyzed with a paired Student's *t*-test, whereas unpaired Student's *t*-tests were used for datasets with non-matching CRC and normal tissue samples.

2.11. Data analysis

GraphPad Prism5 (GraphPad Software) and R 3.2 were used to analyze and graphically visualize our results. Student's *t*-tests were used to assess differences between two end-point conditions, Chi-square tests were used to analyze single cell assays, and 2-way ANOVA tests were used to evaluate *in vivo* tumor growth over time. Unless otherwise stated, three independent replicates were performed for each experiment and results are shown as mean ± SD.

3. Results

3.1. Hypoxia promotes TIC properties of primary CRC cultures

Increasing evidence supports the view that intratumoral hypoxia promotes cancer initiation and progression via different mechanisms, including metabolic reprogramming, immune evasion, and cancer stem cell maintenance [21]. In the context of CRC, it has been suggested that hypoxia promotes an immature gene expression profile [38] and that reduced oxygen levels press cell line-derived CSC-like cells into forming undifferentiated colonies [39]. However, despite the identification of individual hypoxia-responsive TIC regulators, such as DUSP2 [40] or miR-210 [24], the molecular connections between hypoxia and colon cancer stemness remain relatively poorly understood. Accordingly, we set out to study the effects of reduced oxygen levels on CRC patient-derived CSCs. Our methodology thereby comprised the isolation and in-depth characterization of primary colon TICs, followed by an investigation of the functional and molecular effects of hypoxic culture conditions on our different cultures (Fig. 1A). In addition to the previously described T6, T18, and T20 [28], we established another stable TIC culture, i.e. T51, derived from a stage IIIB CRC patient (age/sex: 47/m; MSI). The corresponding T51 spheroid culture (SC) displayed extensive tumorigenic properties (Fig. 1B) as well as high *in vitro* (Supplementary Fig. S1) and *in vivo* (Fig. 1C) self-renewal capacity, emphasizing its high TIC potential. Interestingly, T51 showed an increased sphere formation ability under hypoxic conditions (Fig. 1D), similar to T6, T18, and T20 [24]. Reduced oxygen levels also consistently enhanced the clonogenic capacity of our different TIC cultures, as demonstrated with a series of 3D colony formation assays (Fig. 1E). Taken together, our results thus clearly highlight the tumorigenic properties of CRC patient-derived SCs and demonstrate the TIC-promoting effect of hypoxic conditions.

3.2. miR-215 is upregulated under hypoxic culture conditions

With the aim to decipher the molecular mechanisms that cause the enhanced TIC potential under hypoxia, we performed gene and miRNA expression profiling experiments, comparing normoxic and hypoxic SCs for T6 and T18. As several miRNAs have been identified as important mediators of the hypoxic response in different cancer types [22], we primarily focused on the identification of hypoxia-responsive miRNAs (HRMs) in our two selected TIC cultures. Expression analysis via miRNA microarray experiments (data available under GEO accession number GSE120424) allowed us to identify multiple HRMs, both for T6 (Fig. 2A) and T18 (Fig. 2B). Interestingly, three candidate miRNAs, namely miR-210–3p, miR-3178, and miR-215–5p, were differentially expressed in both SCs (Fig. 2C). However, as miR-3178 showed a conflicting expression profile, being upregulated in hypoxic T6 (Fig. 2A) but downregulated in T18 (Fig. 2B), we removed this candidate HRM from our analysis. Moreover, as miR-210–3p had already been the main subject of a previous publication [24], we decided to focus our attention on miR-215–5p. Importantly, using qPCR analysis, we were not only able to validate its hypoxia-responsiveness in T6 and T18, but we could also show that miR-215–5p was upregulated in two additional primary SCs under hypoxia (Fig. 2D), emphasizing the need to better understand its regulation and role in colon TICs.

3.3. Tumor suppressor miR-215–5p decreases colon TIC activity

Previous studies have highlighted that miR-215 acts as a tumor suppressor that is frequently downregulated in CRC [26–28]. Similarly, using both our own patient cohort as well as publicly available expression data, we could confirm that miR-215–5p is significantly repressed in CRC samples, compared to matching normal counterparts (Fig. 3A). To further study the role of this miRNA in primary colon TICs, we generated several different SCs with stable overexpression of miR-215, using a lentiviral transduction system (expression control in Supplementary Fig. S2A). We first looked at the effect of miR-215 on the expression of accepted colon TIC surface markers. In this context, $CD44^+/CD24^+$ CRC cells are suspected to display increased *in vitro* colony formation and *in vivo* tumor formation potential [41,42]. Strikingly, miR-215 overexpression resulted in a significant depletion of the $CD44^+$ and $CD24^+/CD44^+$ populations in different SCs (Fig. 3B), hinting at the TIC-suppressive activity of this miRNA. Furthermore, we could also observe impaired TIC functionality after miR-215 overexpression, as demonstrated by reduced 2D (Fig. 3C) and 3D (Fig. 3D) colony formation as well as repressed self-renewal capacity (Fig. 3E). Moreover, as also *in vivo* tumor growth was significantly decreased after miR-215 overexpression (Fig. 3F; overexpression control in Supplementary Fig. S2B), our results allow us to conclude that this miRNA acts as a potent tumor- and TIC suppressor in the context of CRC.

3.4. Identification of hypoxia-responsive miR-215–5p target genes

With the aim of further dissecting the molecular regulation of colon TICs, we tried to identify common hypoxia- and miR-215-responsive genes. By leveraging our previously performed microarray experiments for T6 and T18 SCs, we could identify 502 genes that were significantly deregulated under hypoxic conditions in both TIC cultures (Fig. 4A, *left*). Furthermore, lentiviral transduction of miR-215 in T51 TICs led us to determine 66 genes that were differentially expressed after miR-215 overexpression (Fig. 4A, *right*). The intersection of both lists allowed us to identify 8 genes of interest that were both sensitive to hypoxia and miR-215-responsive. In this manner, we could observe that trefoil factor 2 (*TFF2*), a member of the TFF protein family, which is suspected to be involved in the initiation of different human cancer types [43], was significantly deregulated under hypoxic conditions, both in T6 and T18 TICs (Fig. 4A). The O_2-sensitivity of *TFF2* as well as of the other TFF members, i.e. *TFF1* and *TFF3*, was validated by qPCR (Fig. 4B–D). All three TFF family members thereby showed a significant upregulation under hypoxia, which was consistent in all the tested TIC cultures.

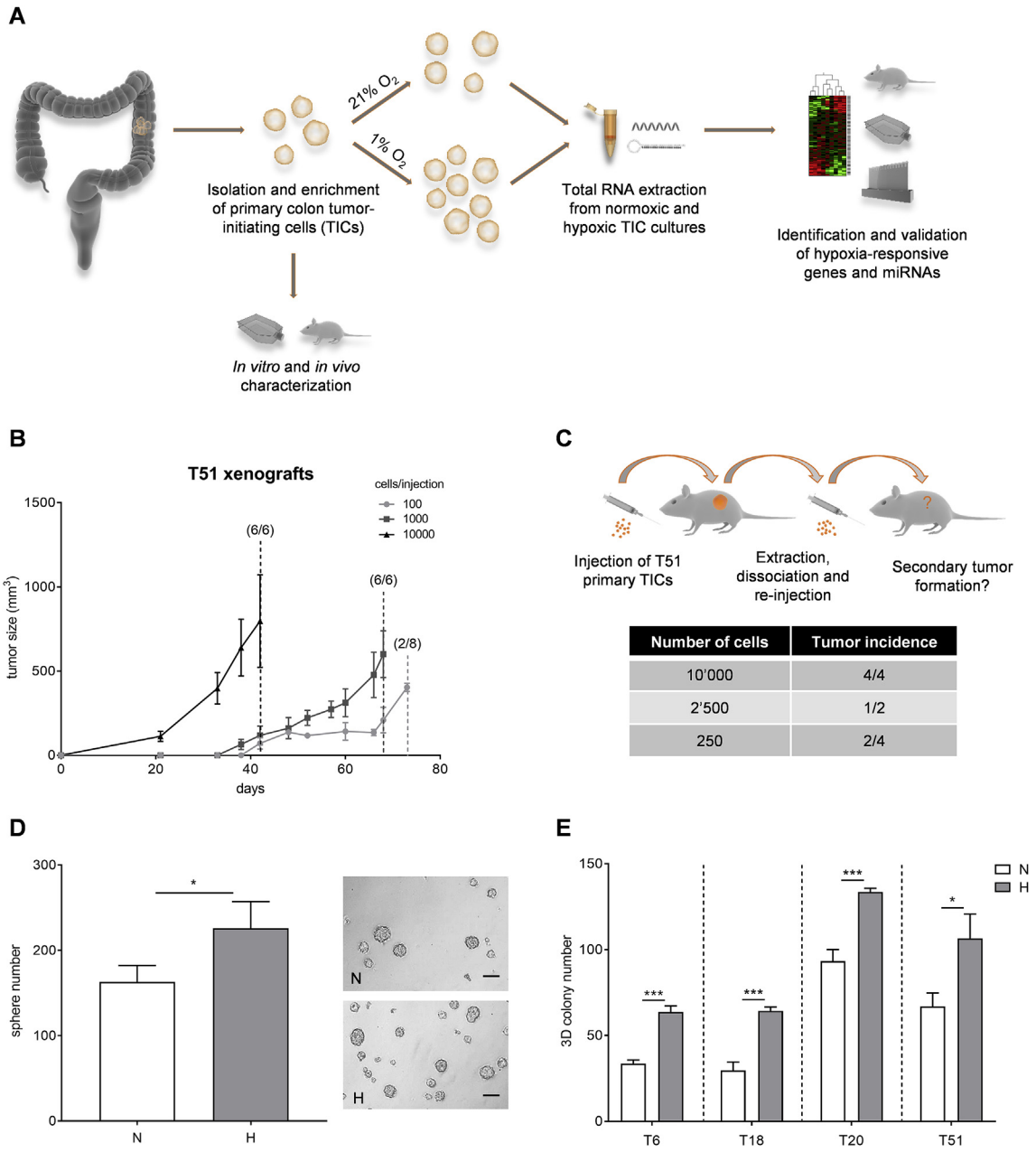

Fig. 1. Hypoxia promotes colon TIC expansion. (**A**) Study overview: Following *in vitro* and *in vivo* characterization, primary colon tumor-initiating cell (TIC) cultures are cultivated under normoxic and hypoxic (1% O_2) conditions. Functional effects as well as molecular alterations are analyzed, with a special focus on hypoxia-responsive miRNAs (HRMs). (**B**) *In vivo* tumor growth after injection of different T51 TIC doses. Tumor incidence for each dose is indicated in brackets, data shown as mean ± SEM. (**C**) *In vivo* self-renewal capacity, determined by serial transplantation of T51 TICs. Secondary tumor incidence was assessed, following injection of different doses of dissociated primary T51 xenografts. (**D**) Sphere formation assay with 1000 T51 TICs per well, following 7 days of normoxic (N) or hypoxic (H) conditions. Representative assay of 3 independent experiments, mean ± SD. Scale bar corresponds to 100 µm. (**E**) 3D colony formation assay with 250 cells per well for different primary TIC cultures, following 14 days of normoxia or hypoxia. Representative assay of at least 3 independent experiments, mean ± SD. Unpaired Student's *t*-tests were used to assess the statistical significance for (D) and (E); *p < 0.05 and ***p < 0.001.

Moreover, we were able to partially block the hypoxia-induced expression of the three TFFs by using HIF1A short hairpin RNA (Supplementary Figs. S3A–C; knockdown control in Supplementary Fig. S2C), further emphasizing the hypoxia-responsiveness of this protein family. Most interestingly, the well-accepted colon CSC marker *LGR5*, known to be essential for CRC initiation and metastatic spreading of the disease [17–19], was also among the identified candidates (Fig. 4A). Using a similar approach, we were able to confirm the regulatory link between O_2 and *LGR5*: although LGR5 did not respond to HIF1A repression (Supplementary Fig. S3D), we could observe a significant downregulation of both LGR5 gene (Fig. 4E) and protein (Fig. 4F, refer to Supplementary Fig. S4 for LGR5 antibody specificity) levels under hypoxic conditions. The fact that *LGR5*, in contrast to the different TFFs, did not respond to HIF1A modulation suggests that other hypoxia-responsive factors, such as HIF2A or miR-215, might be involved in the regulation of LGR5.

3.5. The colon TIC marker LGR5 is a hypoxia/miR-215 downstream target

The clinical relevance of LGR5 expression was examined using both our own CRC patient collection and large publicly available datasets. For each tested cohort, LGR5 was significantly increased in tumor

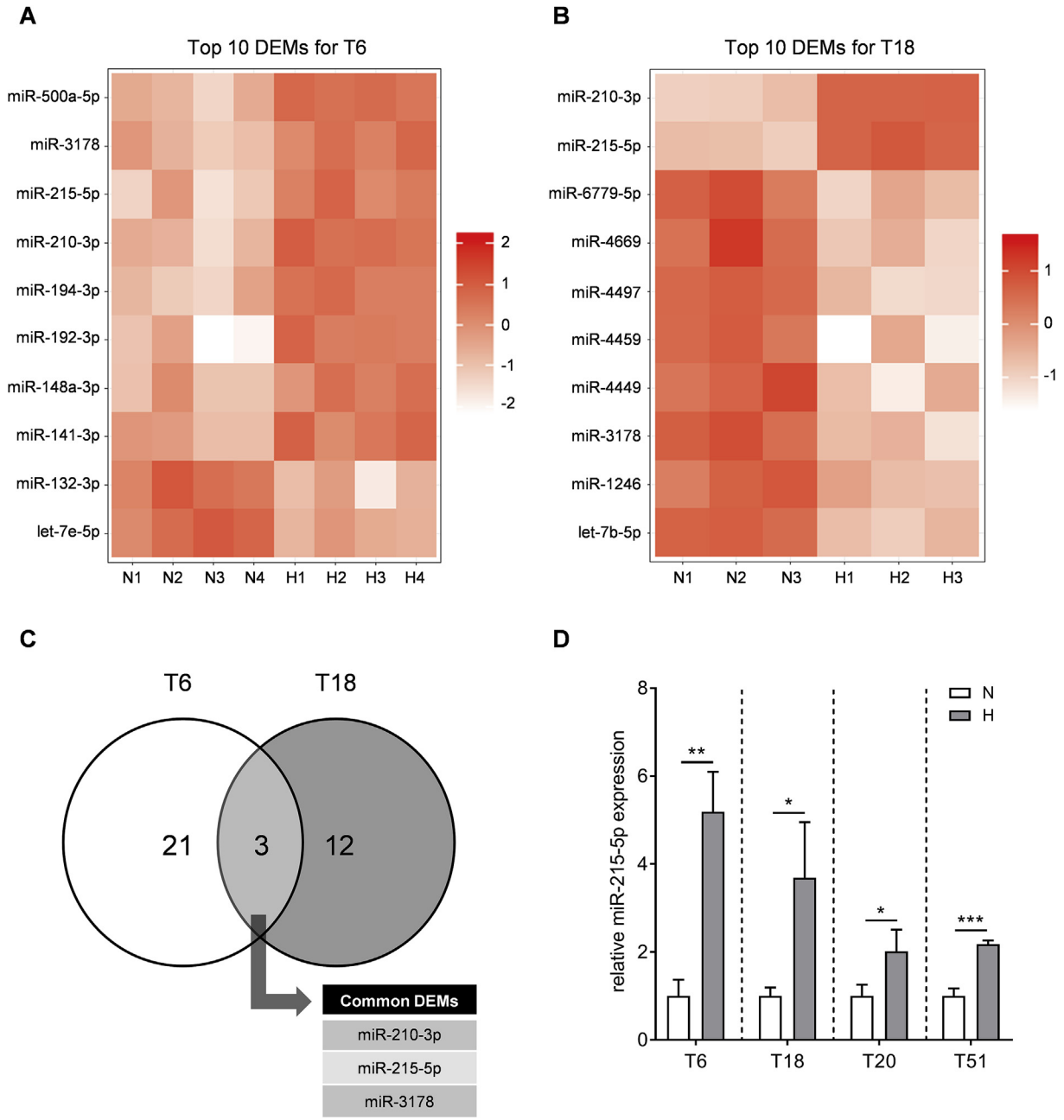

Fig. 2. miR-215-5p expression is induced under hypoxic conditions. Heatmap depicting the median-centered expression intensities of the 10 most significant differentially expressed miRNAs (DEMs), ranked according to their adjusted p-value, for (**A**) T6 and (**B**) T18 TIC cultures, following 72 h of normoxic (N) or hypoxic (H) conditions, respectively. Normalized $\log_2$ expression intensities are available under the GEO accession number GSE120424. (**C**) Determination of common hypoxia-responsive DEMs for T6 and T18 TICs. NB: While miR-210–3p and miR-215–5p were commonly upregulated under hypoxia in T6 and T18, miR-3178 showed an opposite expression behavior in both TIC cultures. (**D**) Relative miR-215–5p expression in different primary TIC cultures, following 72 h of normoxic (N) or hypoxic (H) conditions. Representative assay of at least 3 independent experiments, mean ± SD. Unpaired Student's *t*-test was used to assess the statistical significance for (D); *p < 0.05, **p < 0.01, and ***p < 0.001.

samples, compared to normal colon tissue biopsies (Fig. 5A and Supplementary Fig. S5A). The expression profile of LGR5 thus showed an opposite trend, compared to miR-215–5p (Fig. 3A), emphasizing its suitability as a potential miR-215–5p target gene. On the other hand, there was no observable difference in TFF gene expression between normal and cancerous samples (Supplementary Figs. S5B–D). Although other groups have linked individual TFFs to apoptosis resistance [44,45] as well as to CRC development and progression [46,47], we failed to observe such a correlation between TFF expression and CRC disease occurrence and thus decided to rather focus on LGR5 for the scope of the present study. Importantly, by using qPCR analysis to validate our microarray experiments, we could confirm the miR-215-responsiveness of LGR5 in two additional TIC cultures (Fig. 5B). Stable miR-215 overexpression under normoxia thereby diminished LGR5 expression, both on gene and on protein level, to a similar extent as hypoxia (Fig. 5C and D). The clinical relevance of our findings and the link of miR-215/LGR5 signaling to CRC tumor growth was further supported by the fact that LGR5 was significantly repressed in our previously extracted miR-215-overexpressing xenografts (Fig. 5E). We could thus consistently observe a strong hypoxia-responsiveness of miR-215 and LGR5 as well as a distinct regulatory connection between both factors, throughout all our *in vitro* and *in vivo* experiments. It is likely

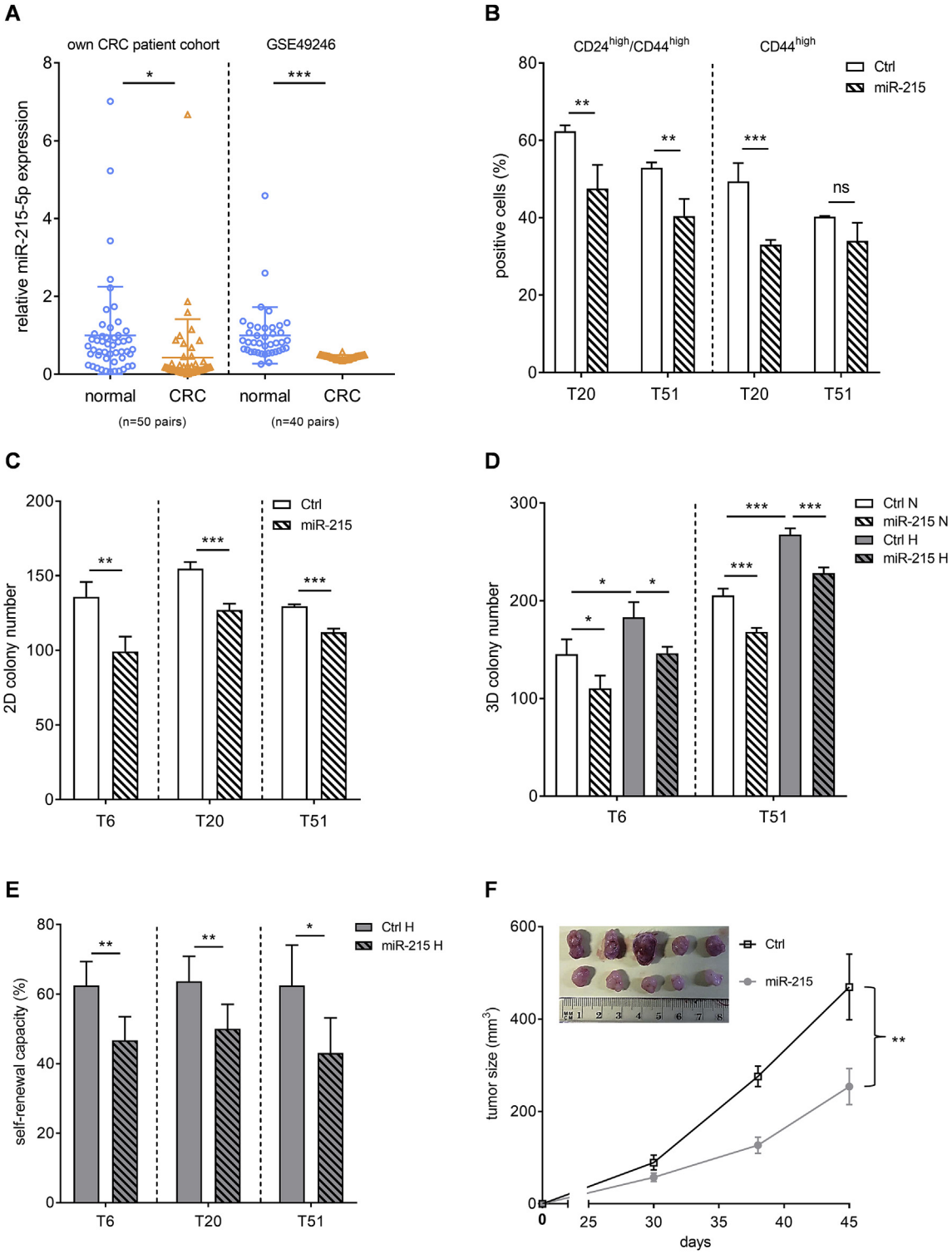

Fig. 3. Hypoxia-responsive miR-215 displays tumor- and TIC-repressive properties. (**A**) Relative miR-215–5p expression in matching colorectal cancer (CRC) samples and normal colon counterparts. Both own (*left*) and publicly available (*right*) patient cohorts were used for our analysis, n = 50 and n = 40 sample pairs, respectively; mean ± SD. (**B**) $CD24^{high}$ and $CD44^{high}$ populations were determined via FACS analysis in T20 and T51 TIC cultures, following lentiviral transduction of miR-215 or the respective control vector. Data shown as mean ± SD of 4 (for T20) or 3 (for T51) independent experiments. (**C**) 2D colony formation assay with 250 cells per well for different primary TIC cultures, following lentiviral transduction of miR-215 or the respective control vector. Colonies were counted after 10–14 days. Representative assay of at least 2 independent experiments, mean ± SD. (**D**) 3D colony formation assay with 400 cells per well for different primary TIC cultures, following lentiviral transduction of miR-215 or the respective control vector. Colonies were counted after 14 days, following normoxic (N) or hypoxic (H) culture conditions. Representative assay of at least 3 independent experiments, mean ± SD. (**E**) Single cell assay for different primary TIC cultures, following lentiviral transduction of miR-215 or the respective control vector. Spheres were counted after 10–14 days of hypoxia. Representative assay of at least 3 independent experiments, mean with 95% confidence interval. (**F**) *In vivo* tumor growth after injection of 100 T20 TICs, following lentiviral transduction of miR-215 or the respective control vector. Data shown as mean ± SEM. Statistical significance was assessed with a paired Student's t-test for (A), an unpaired Student's t-test for (B–C), a 2-way ANOVA test for (D) and (F), and a Chi-square test for (E); ns – not significant, *p < 0.05, **p < 0.01, and ***p < 0.001.

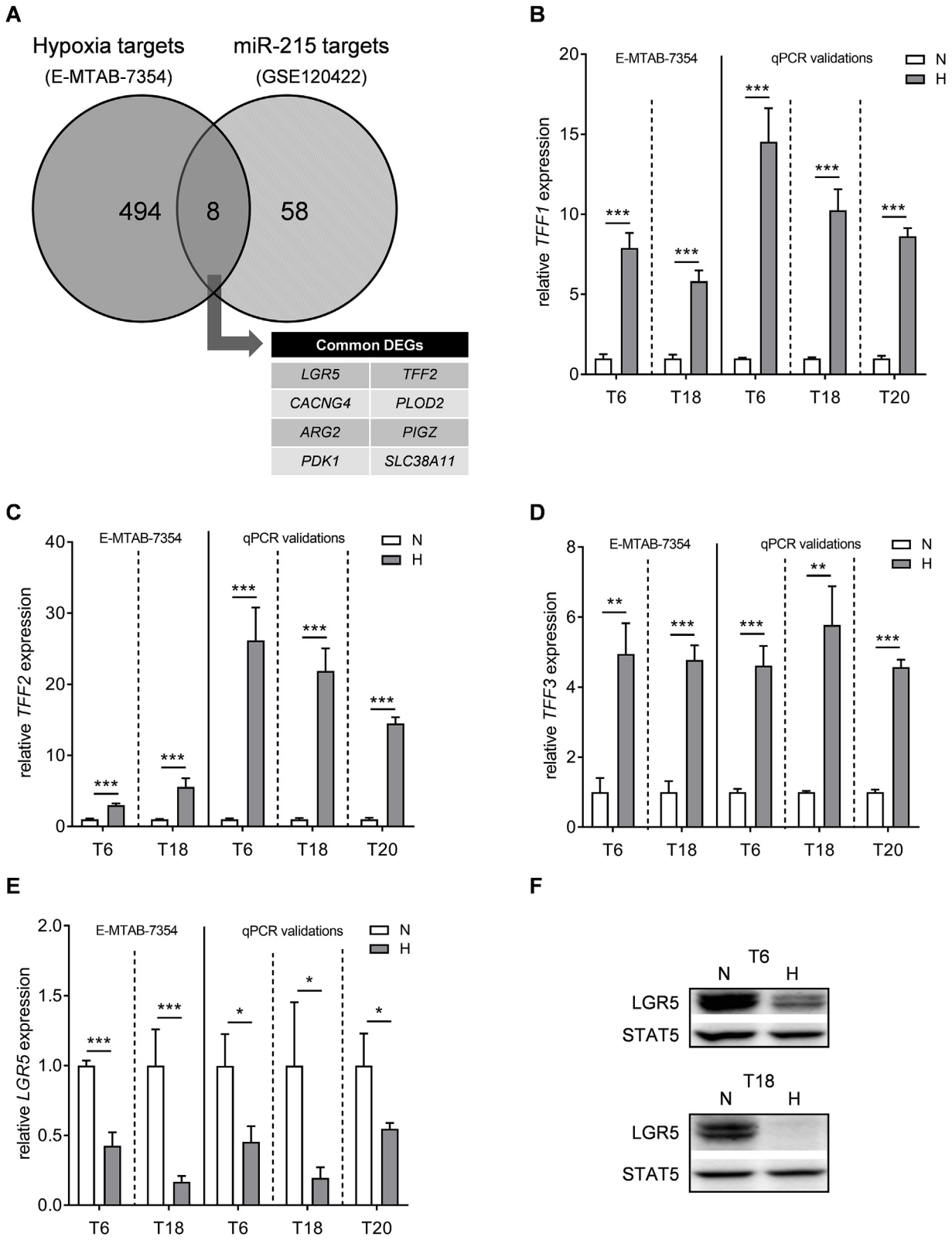

Fig. 4. Identification and validation of hypoxia-responsive genes of interest. (**A**) Determination of genes that are both hypoxia-responsive and potential miR-215 targets in colon TICs. Intersection of 502 hypoxia-responsive genes that are differentially expressed in T6 and T18 TICs under hypoxia (*left circle*), and 66 genes that are differentially expressed after lentiviral transduction of miR-215 in T51 TICs (*right circle*). Corresponding microarray data is available under accession numbers E-MTAB-7354 and GSE120422. Relative (**B**) *TFF1*, (**C**) *TFF2*, (**D**) *TFF3*, and (**E**) *LGR5* expression after 7 days of normoxic (N) or hypoxic (H) conditions. Microarray results for T6 and T18 (*left*) as well as one representative of at least 2 independent qPCR validation experiments for T6, T18, and T20 (*right*) are shown; mean ± SD. (**F**) LGR5 protein expression (Abcam antibody) in T6 and T18 TICs after 24 h under normoxic (N) or hypoxic (H) culture conditions, respectively. Representative picture of at least 2 independent Western blot experiments. Statistical significance for (B–E) was assessed with unpaired Student's *t*-tests; *p < 0.05, **p < 0.01, and ***p < 0.001.

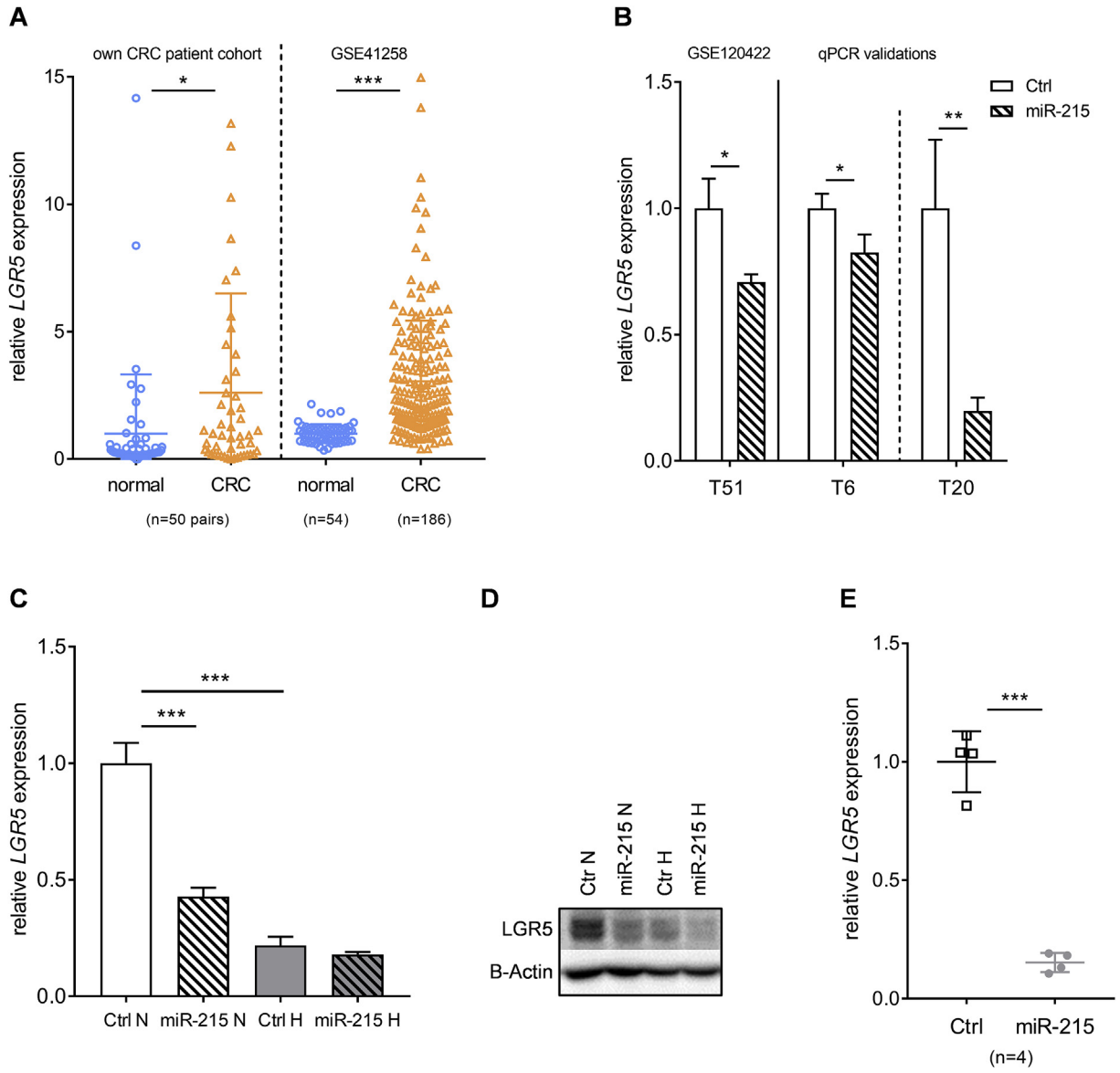

Fig. 5. The colon TIC marker LGR5 is a hypoxia/miR-215 downstream target. (**A**) Relative *LGR5* expression in colorectal cancer (CRC) and normal colon samples. Both our own paired (*left*) as well as non-matching publicly available (*right*) patient cohorts were used for the analysis; patient number (n) is indicated for the respective datasets; mean ± SD. (**B**) Relative *LGR5* expression following lentiviral transduction of miR-215 or the respective control vector. Microarray results for T51 (*left*) as well as one representative of at least 2 independent qPCR validation experiments for T6 and T20 (*right*) are shown; mean ± SD. (**C**) Relative *LGR5* gene and (**D**) LGR5 protein expression (Miltenyi antibody) in T20 TICs, after 72 h of hypoxia and/or following lentiviral transduction of miR-215 or the respective control vector. Representative assay of 3 independent experiments; qPCR data shown as mean ± SD. (**E**) Relative *LGR5* expression in extracted xenografts, 45 days after injection of 100 T20 TICs, previously transduced with miR-215 or the respective control vector; mean ± SD; n = 4. Statistical significance was assessed with a paired Student's *t*-test for (A, *left*) and (E), with unpaired Student's *t*-tests for (A, *right*) and for (B), and with a 2-way ANOVA test for (C); *p < 0.05, **p < 0.01, and ***p < 0.001.

that miR-215 represses a transcriptional coactivator, which could indirectly cause down-regulation of LGR5, as there are no reported binding sites for miR-215 at the three prime untranslated region (3′UTR) of LGR5 (www.targetscan.org). However, future studies are needed in order to unravel the missing link between miR-215 and LGR5. Altogether, our results highlight miR-215 as a potent TIC-suppressing HRM and identify the colon CSC marker LGR5 as a hypoxia/miR-215 downstream target. As all the molecular key players of this study have known clinical implications in CRC, modulating the hypoxia/miR-215/LGR5 axis might open up new avenues for therapeutic TIC-targeting interventions.

4. Discussion

Numerous research initiatives have focused on the relation between miRNAs and hypoxia signaling as well as on the regulatory role of HRMs in the context of cancer progression [22]. For instance, miR-424 was shown to inhibit the hydroxylation of HIF1A isoforms, thereby stabilizing HIF proteins in endothelial cells [48]. Similarly, miR-31 is known to increase the expression of HIF1A via downregulation of the HIF-repressor FIH-1 in head and neck carcinoma [49]. On the other hand, the oncogenic miR-17–92 cluster as well as p53-induced miR-107 were shown to decrease HIF protein levels via repression of the HIF α- or β-subunit, respectively [50,51]. These results illustrate that, depending on the context, miRNA-mediated upstream regulation of HIFs can either potentiate or repress the hypoxic response.

Other miRNAs act downstream of HIFs, which entails that their expression level can be directly or indirectly modified by tumor hypoxia. Here again, depending on the molecular context, HRMs can either amplify or work against the effects of hypoxia. For instance, miR-210, the most noted HRM, has been described to act as a negative feedback mechanism in neural progenitor cells, protecting against hypoxia-induced apoptosis [52]. On the other hand, miR-210 was shown to potentiate the TIC-promoting effects of hypoxia in the context of colon CSCs [24]. In a similar way, expression and functional impact of miR-215 also seem to depend on its molecular framework and interaction partners: although miR-215 is thought to display oncogenic activity in glioma TICs [25], the same miRNA is suspected to act as a tumor and CSC suppressor in the context of CRC [26–28]. Our own results thereby extend the findings of Hu et al. and Jones et al., consolidating miR-215 as both a HRM and a strong repressor of TIC activity. While hypoxia itself promoted the self-renewal capacity of our CSC cultures, hypoxia-induced miR-215 clearly worked against this effect, providing negative feedback and acting as a fine-tuning mechanism of the hypoxic response.

Most studies agree on the fact that LGR5 marks a population of cancer cells with increased tumor initiation and progression potential [15–19]. Reduced LGR5 levels after stable overexpression of miR-215, as observed here and elsewhere [53], could thus hint at a selective depletion of tumorigenic cells in the corresponding cultures. However, a study by Walker and colleagues suggests that inhibition of LGR5 leads to a rather enhanced proliferative and invasive capacity of CRC cells [54]. Hence it seems that the functional role of LGR5, while being a well-established CSC marker, still merits further investigation. Whether or not the CSC-repressing potential of miR-215 is mediated via inhibition of LGR5 remains to be determined and will be the subject matter of future studies. As it stands for now, our findings clearly demonstrate that LGR5 is a downstream target of hypoxia/miR-215 signaling and, most importantly, highlight miR-215 as a HRM with strong colon TIC-suppressing activity, paving the way for the development of novel anti-CSC treatment strategies.

Conflicts of interest

The authors declare no potential conflicts of interest.

Declarations of interest

None.

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Appendix A. Supplementary data

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References

[1] J.E. Visvader, Cells of origin in cancer, Nature 469 (2011) 314–322, https://doi.org/10.1038/nature09781.
[2] T. Lapidot, C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M.A. Caligiuri, J.E. Dick, A cell initiating human acute myeloid leukaemia after transplantation into SCID mice, Nature 367 (1994) 645–648, https://doi.org/10.1038/367645a0.
[3] D. Bonnet, J.E. Dick, Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell, Nat. Med. 3 (1997) 730–737, https://doi.org/10.1038/nm0797-730.
[4] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 3983–3988, https://doi.org/10.1073/pnas.0530291100.
[5] S.S.K. Singh, C. Hawkins, I.D. Clarke, J.A. Squire, J. Bayani, T. Hide, M. Henrelman, M.D. Cusimano, B.D. Peter, R.M. Henkelman, M.D. Cusimano, P.B. Dirks, Identification of human brain tumour initiating cells, Nature 432 (2004) 396–401, https://doi.org/10.1038/nature03128.
[6] C.F. Bender Kim, E.L. Jackson, A.E. Woolfenden, S. Lawrence, I. Babar, S. Vogel, D. Crowley, R.T. Bronson, T. Jacks, Identification of bronchioalveolar stem cells in normal lung and lung cancer, Cell 121 (2005) 823–835, https://doi.org/10.1016/j.cell.2005.03.032.
[7] C.A. O'Brien, A. Pollett, S. Gallinger, J.E. Dick, A human colon cancer cell capable of initiating tumour growth in immunodeficient mice, Nature 445 (2007) 106–110, https://doi.org/10.1038/nature05372.
[8] L. Ricci-Vitiani, D.G. Lombardi, E. Pilozzi, M. Biffoni, M. Todaro, C. Peschle, R. De Maria, Identification and expansion of human colon-cancer-initiating cells, Nature 445 (2007) 111–115, https://doi.org/10.1038/nature05384.
[9] K. Qureshi-Baig, P. Ullmann, S. Haan, E. Letellier, Tumor-Initiating Cells: a criTICal review of isolation approaches and new challenges in targeting strategies, Mol. Canc. 16 (2017) 40, https://doi.org/10.1186/s12943-017-0602-2.
[10] K. Kemper, C. Grandela, J.P. Medema, Molecular identification and targeting of colorectal cancer stem cells, Oncotarget 1 (2010) 387–395, https://doi.org/10.18632/oncotarget.101003.
[11] H. Clevers, The cancer stem cell: premises, promises and challenges, Nat. Med. 17 (2011) 313–319, https://doi.org/10.1038/nm.2304.
[12] N. Barker, J.H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P.J. Peters, H. Clevers, Identification of stem cells in small intestine and colon by marker gene Lgr5, Nature 449 (2007) 1003–1007, https://doi.org/10.1038/nature06196.
[13] J. Beumer, H. Clevers, Regulation and plasticity of intestinal stem cells during homeostasis and regenration, Development 143 (2016) 3639–3649, https://doi.org/10.1242/dev.133132.
[14] S.M. Dieter, H. Glimm, C.R. Ball, Colorectal cancer-initiating cells caught in the act, EMBO Mol. Med. 9 (2017) 856–859, https://doi.org/10.15252/emmm.201707858.
[15] N. Barker, R.A. Ridgway, J.H. van Es, M. van de Wetering, H. Begthel, M. van den Born, E. Danenberg, A.R. Clarke, O.J. Sansom, H. Clevers, Crypt stem cells as the cells-of-origin of intestinal cancer, Nature 457 (2009) 608–611, https://doi.org/10.1038/nature07602.
[16] A.G. Schepers, H.J. Snippert, D.E. Stange, M. van den Born, J.H. van Es, M. van de Wetering, H. Clevers, Lineage tracing reveals Lgr5$^+$ stem cell activity in mouse intestinal adenomas, Science 337 (2012) 730–735, https://doi.org/10.1126/science.1224676.
[17] C. Cortina, G. Turon, D. Stork, X. Hernando-Momblona, M. Sevillano, M. Aguilera, S. Tosi, A. Merlos-Suárez, C. Stephan-Otto Attolini, E. Sancho, E. Batlle, A genome editing approach to study cancer stem cells in human tumors, EMBO Mol. Med. 9 (2017) 869–879, https://doi.org/10.15252/emmm.201707550.
[18] M. Shimokawa, Y. Ohta, S. Nishikori, M. Matano, A. Takano, M. Fujii, S. Date, S. Sugimoto, T. Kanai, T. Sato, Visualization and targeting of LGR5+ human colon cancer stem cells, Nature 545 (2017) 187–192, https://doi.org/10.1038/nature22081.
[19] F. de Sousa e Melo, A.V. Kurtova, J.M. Harnoss, N. Kljavin, J.D. Hoeck, J. Hung, J.E. Anderson, E.E. Storm, Z. Modrusan, H. Koeppen, G.J. Dijkgraaf, R. Piskol, F.J. de Sauvage, A distinct role for Lgr5$^+$ stem cells in primary and metastatic colon cancer, Nature 543 (2017) 676–680, https://doi.org/10.1038/nature21713.
[20] V. Plaks, N. Kong, Z. Werb, The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? Cell Stem Cell 16 (2015) 225–238, https://doi.

org/10.1016/j.stem.2015. 02.015.
[21] G.L. Semenza, The hypoxic tumor microenvironment: a driving force for breast cancer progression, Biochim. Biophys. Acta Mol. Cell Res. 1863 (2016) 382–391, https://doi.org/10.1016/j.bbamcr. 2015.05.036.
[22] G. Shen, X. Li, Y. Jia, G a Piazza, Y. Xi, Hypoxia-regulated microRNAs in human cancer, Acta Pharmacol. Sin. 34 (2013) 336–341, https://doi.org/10.1038/aps. 2012.195.
[23] S.Y. Chan, J. Loscalzo, MicroRNA-210: a unique and pleiotropic hypoxamir, Cell Cycle 9 (2010) 1072–1083, https://doi.org/10.4161/cc.9.6.11006.
[24] P. Ullmann, K. Qureshi-Baig, F. Rodriguez, A. Ginolhac, Y. Nonnenmacher, D. Ternes, J. Weiler, K. Gäbler, C. Bahlawane, K. Hiller, S. Haan, E. Letellier, Hypoxia-responsive miR-210 promotes self-renewal capacity of colon tumor-initiating cells by repressing ISCU and by inducing lactate production, Oncotarget 7 (2016) 65454–65470, https://doi.org/10.18632/oncotarget. 11772.
[25] J. Hu, T. Sun, H. Wang, Z. Chen, S. Wang, L. Yuan, T. Liu, H.-R. Li, P. Wang, Y. Feng, Q. Wang, R.E. McLendon, A.H. Friedman, S.T. Keir, D.D. Bigner, J. Rathmell, X. Fu, Q.-J. Li, H. Wang, X.-F. Wang, MiR-215 is induced post-transcriptionally via HIF-drosha complex and mediates glioma-initiating cell adaptation to hypoxia by targeting KDM1B, Cancer Cell 29 (2016) 49–60, https://doi.org/10.1016/j.ccell.2015.12.005.
[26] C.J. Braun, X. Zhang, I. Savelyeva, S. Wolff, U.M. Moll, T. Schepeler, T.F. Orntoft, C.L. Andersen, M. Dobbelstein, p53-Responsive MicroRNAs 192 and 215 are capable of inducing cell cycle arrest, Cancer Res. 68 (2008) 10094–10104, https://doi.org/10.1158/0008-5472.CAN-08-1569.
[27] M. Karaayvaz, T. Pal, B. Song, C. Zhang, P. Georgakopoulos, S. Mehmood, S. Burke, K. Shroyer, J. Ju, Prognostic significance of miR-215 in colon cancer, Clin. Colorectal Cancer 10 (2011) 340–347, https://doi.org/10.1016/j.clcc.2011.06.002.
[28] J.X. Zhang, W. Song, Z.H. Chen, J.H. Wei, Y.J. Liao, J. Lei, M. Hu, G.Z. Chen, B. Liao, J. Lu, H.W. Zhao, W. Chen, Y.L. He, H.Y. Wang, D. Xie, J.H. Luo, Prognostic and predictive value of a microRNA signature in stage II colon cancer: a microRNA expression analysis, Lancet Oncol. 14 (2013) 1295–1306, https://doi.org/10.1016/S1470-2045(13)70491-1.
[29] B. Song, Y. Wang, M.A. Titmus, G. Botchkina, A. Formentini, M. Kornmann, J. Ju, Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells, Mol. Canc. 9 (2010) 96, https://doi.org/10.1186/1476-4598-9-96.
[30] Z. Chen, S. Han, W. Huang, J. Wu, Y. Liu, S. Cai, Y. He, S. Wu, W. Song, MicroRNA-215 suppresses cell proliferation, migration and invasion of colon cancer by repressing Yin-Yang 1, Biochem. Biophys. Res. Commun. 479 (2016) 482–488, https://doi.org/10.1016/j.bbrc.2016.09. 089.
[31] M.F. Jones, T. Hara, P. Francis, X.L. Li, S. Bilke, Y. Zhu, M. Pineda, M. Subramanian, W.F. Bodmer, A. Lal, The CDX1-microRNA-215 axis regulates colorectal cancer stem cell differentiation, Proc. Natl. Acad. Sci. U.S.A. 112 (2015) E1550–E1558, https://doi.org/10.1073/pnas. 1503370112.
[32] E. Letellier, M. Schmitz, K. Baig, N. Beaume, C. Schwartz, S. Frasquilho, L. Antunes, N. Marcon, P.V. Nazarov, L. Vallar, J. Even, S. Haan, Identification of SOCS2 and SOCS6 as biomarkers in human colorectal cancer, Br. J. Canc. 111 (2014) 726–735, https://doi.org/10.1038/bjc. 2014.377.
[33] K. Qureshi-Baig, P. Ullmann, F. Rodriguez, S. Frasquilho, P.V. Nazarov, S. Haan, E. Letellier, What do we learn from spheroid culture systems? Insights from tumorspheres derived from primary colon cancer tissue, PLoS One 11 (2016) e0146052, , https://doi.org/10.1371/journal. pone.0146052.
[34] Y. Hu, G.K. Smyth, ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays, J. Immunol. Methods 347 (2009) 70–78, https://doi.org/10.1016/j.jim.2009.06.008.
[35] P. Ullmann, F. Rodriguez, M. Schmitz, S.K. Meurer, K. Qureshi-Baig, P. Felten, A. Ginolhac, L. Antunes, S. Frasquilho, N. Zügel, R. Weiskirchen, S. Haan, E. Letellier, The miR-371 ~ 373 cluster represses colon cancer initiation and metastatic colonization by inhibiting the TGFBR2/ID1 signaling Axis, Cancer Res. 78 (2018) 3793–3808, https://doi.org/10.1158/0008-5472.
[36] L. Marisa, A. de Reyniès, A. Duval, J. Selves, M.P. Gaub, L. Vescovo, M.C. Etienne-Grimaldi, R. Schiappa, D. Guenot, M. Ayadi, S. Kirzin, M. Chazal, J.F. Fléjou, D. Benchimol, A. Berger, A. Lagarde, E. Pencreach, F. Piard, D. Elias, Y. Parc, S. Olschwang, G. Milano, P. Laurent-Puig, V. Boige, Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value, PLoS Med. 10 (2013) e1001453, , https://doi.org/10.1371/journal.pmed.1001453.
[37] M. Sheffer, M.D. Bacolod, O. Zuk, S.F. Giardina, H. Pincas, F. Barany, P.B. Paty, W.L. Gerald, D.A. Notterman, E. Domany, Association of survival and disease progression with chromosomal instability: a genomic exploration of colorectal cancer, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 7131–7136, https://doi.org/10.1073/pnas.0902232106.
[38] J. Mathieu, Z. Zhang, W. Zhou, A.J. Wang, J.M. Heddleston, C.M.A. Pinna, A. Hubaud, B. Stadler, M. Choi, M. Bar, M. Tewari, A. Liu, R. Vessella, R. Rostomily, D. Born, M. Horwitz, C. Ware, C.A. Blau, M.A. Cleary, J.N. Rich, H. Ruohola-Baker, HIF induces human embryonic stem cell markers in cancer cells, Cancer Res. 71 (2011) 4640–4652, https://doi.org/10.1158/0008-5472.CAN-10-3320.
[39] T.M. Yeung, S.C. Gandhi, W.F. Bodmer, Hypoxia and lineage specification of cell line-derived colorectal cancer stem cells, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 4382–4387, https://doi.org/10.1073/pnas.1014519107.
[40] P.C. Hou, Y.H. Li, S.C. Lin, S.C. Lin, J.C. Lee, B.W. Lin, J.P. Liou, J.Y. Chang, C.C. Kuo, Y.M. Liu, H.S. Sun, S.J. Tsai, Hypoxia-induced downregulation of DUSP-2 phosphatase drives colon cancer stemness, Cancer Res. 77 (2017) 4305–4316, https://doi.org/10.1158/0008-5472.CAN-16-2990.
[41] T.M. Yeung, S.C. Gandhi, J.L. Wilding, R. Muschel, W.F. Bodmer, Cancer stem cells from colorectal cancer-derived cell lines, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 3722–3727, https://doi.org/10.1073/pnas.0915135107.
[42] A. Jaggupilli, E. Elkord, Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity, Clin. Dev. Immunol. 2012 (2012) 708036, https://doi.org/10.1155/2012/708036.
[43] J.K. Perry, N. Kannan, P.M. Grandison, M.D. Mitchell, P.E. Lobie, Are trefoil factors oncogenic? Trends Endocrinol. Metabol. 19 (2008) 74–81, https://doi.org/10.1016/j.tem.2007.10.003.
[44] D.R. Taupin, K. Kinoshita, D.K. Podolsky, Intestinal trefoil factor confers colonic epithelial resistance to apoptosis, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 799–804, https://doi.org/10.1073/pnas.97.2.799.
[45] C. Hanisch, J. Sharbati, B. Kutz-Lohroff, O. Huber, R. Einspanier, S. Sharbati, Trefoil factor 3 mediates resistance to apoptosis in colon carcinoma cells by a regulatory RNA axis, Cell Death Dis. 8 (2017), https://doi.org/10.1038/cddis.2017.84 e2660.
[46] K. Morito, J. Nakamura, Y. Kitajima, K. Kai, T. Tanaka, H. Kubo, S. Miyake, H. Noshiro, The value of trefoil factor 3 expression in predicting the long-term outcome and early recurrence of colorectal cancer, Int. J. Oncol. 46 (2015) 563–568, https://doi.org/10.3892/ijo.2014.2755.
[47] G. Yu, P. Jiang, Y. Xiang, Y. Zhang, Z. Zhu, C. Zhang, S. Lee, W. Lee, Y. Zhang, Increased expression of protease-activated receptor 4 and Trefoil factor 2 in human colorectal cancer, PLoS One 10 (2015) e0122678, , https://doi.org/10.1371/journal.pone.0122678.
[48] G. Ghosh, I.V. Subramanian, N. Adhikari, X. Zhang, H.P. Joshi, D. Basi, Y.S. Chandrashekhar, J.L. Hall, S. Roy, Y. Zeng, S. Ramakrishnan, Hypoxia-induced microRNA-424 expression in human endothelial cells regulates HIF-alpha isoforms and promotes angiogenesis, J. Clin. Invest. 120 (2010) 4141–4154, https://doi.org/10.1172/JCI42980DS1.
[49] C.J. Liu, M.M. Tsai, P.S. Hung, S.Y. Kao, T.Y. Liu, K.J. Wu, S.H. Chiou, S.C. Lin, K.W. Chang, miR-31 ablates expression of the HIF regulatory factor FIH to activate the HIF pathway in head and neck carcinoma, Cancer Res. 70 (2010) 1635–1644, https://doi.org/10.1158/0008-5472.CAN-09-2291.
[50] A. Taguchi, K. Yanagisawa, M. Tanaka, K. Cao, Y. Matsuyama, H. Goto, T. Takahashi, Identification of hypoxia-inducible factor-1 as a novel target for miR-17-92 MicroRNA cluster, Cancer Res. 68 (2008) 5540–5545, https://doi.org/10.1158/0008-5472.CAN-07-6460.
[51] M. Yamakuchi, C.D. Lotterman, C. Bao, R.H. Hruban, B. Karim, J.T. Mendell, D. Huso, C.J. Lowenstein, P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 6334–6339, https://doi.org/10.1073/pnas.0911082107.
[52] F. Wang, L. Xiong, X. Huang, T. Zhao, L. Wu, Z. Liu, X. Ding, S. Liu, Y. Wu, Y. Zhao, K. Wu, L. Zhu, M. Fan, miR-210 suppresses BNIP3 to protect against the apoptosis of neural progenitor cells, Stem Cell Res. 11 (2013) 657–667, https://doi.org/10.1016/j.scr.2013.04.005.
[53] T. Nakaoka, Y. Saito, Y. Shimamoto, T. MUramatsu, M. Kimura, Y. Kanai, H. Saito, Cluster microRNAs miR-194 and miR-215 suppress the tumorigenicity of intestinal tumor organoids, Cancer Sci. 108 (2017) 678–684, https://doi.org/10.1111/cas.13165.
[54] F. Walker, H.H. Zhang, A. Odorizzi, A.W. Burgess, LGR5 is a negative regulator of tumourigenicity, antagonizes Wnt signalling and regulates cell adhesion in colorectal cancer cell lines, PLoS One 6 (2011) e22733, , https://doi.org/10.1371/journal.pone.0022733.