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,
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 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-Pkdscid Il2rgtm1Wjl/Szh) (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’, TFT1 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’-AACT-GTG-GGC-TTC-GTA-ATC-A-3’ and reverse 5’-GCC-AGA-GTA-TGC-TCT-GTC-GTA-G-3’, TFF3 forward 5’-TCC-AGC-TCT-GCT-GAG-TAC-G-3’ and reverse 5’-ATC-GTG-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 (Milipore, #MAB1501), STAT5 (Santa Cruz, sc-835), LGR5 (Abcam, 7S850), 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 $log_2$ 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 (DEM) 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 vitro 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, 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.
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 O2 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
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) CD24high and CD44high 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. (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.
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-215 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-210, the most noted HRM, has been described to act as a negative feedback mechanism in neural progenitor cells, protecting against hypoxia. Here again, depending on the molecular context, HRMs can either amplify or work against the effects of hypoxia.

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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