The highly expressed 5’isomiR of hsa-miR-140-3p contributes to the tumor-suppressive effects of miR-140 by reducing breast cancer proliferation and migration

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Abstract

Background: miRNAs are small noncoding RNA molecules that play an important role in post-transcriptional regulation of gene expression. Length and/or sequence variants of the same miRNA are termed isomiRs. While most isomiRs are functionally redundant compared to their canonical counterparts, the so-called 5’isomiRs exhibit a shifted 5’ end and therefore a shifted seed sequence resulting in a different target spectrum. However, not much is known about the functional relevance of these isoforms.

Results: Analysis of miRNA-seq data from breast cancer cell lines identified six pairs of highly expressed miRNAs and associated 5’isomiRs. Among them, hsa-miR-140-3p was of particular interest because its 5’isomiR showed higher expression compared to the canonical miRNA annotated in miRbase. This miRNA has previously been shown to control stemness of breast cancer cells. miRNAseq data of breast cancer patients (TCGA dataset) showed that both the canonical hsa-miR-140-3p and its 5’isomiR-140-3p were highly expressed in patients’ tumors compared to normal breast tissue. In the current work, we present the functional characterization of 5’isomiR-140-3p and the cellular phenotypes associated with its overexpression in MCF10A, MDA-MB-468 and MDA-MB-231 cell lines in comparison to the canonical hsa-miR-140-3p. Contrary to the effect of the canonical hsa-miR-140-3p, overexpression of the 5’isomiR-140-3p led to a decrease in cell viability. The latter observation was supported by cell cycle analysis, where the 5’isomiR-140-3p but not the hsa-miR-140-3p caused cell cycle arrest in G0/G1-phase. Additionally, 5’isomiR-140-3p overexpression was found to cause a decrease in cell migration in the three cell lines. We identified three novel direct target genes of the 5’isomiR-140-3p; COL4A1, ITGA6 and MARCKSL1. Finally, we have shown that knocking down these genes partially phenocopied the effects of the 5’isomiR-140-4p overexpression, where COL4A1 and ITGA6 knockdown led to reduced cell viability and cell cycle arrest, while MARCKSL1 knockdown resulted in a decrease in the migratory potential of cells.

Conclusions: In summary, this work presents evidence that there is functional synergy between the canonical hsa-miR-140-3p and the newly identified 5’isomiR-140-3p in suppressing growth and progression of breast cancer by simultaneously targeting genes related to differentiation, proliferation, and migration.

Keywords: Breast cancer, IsomiRs, MiRNA, Seed sequence, Proliferation, Migration, MiR-140
Background
Breast cancer is a complex heterogeneous form of cancer with lots of genetic alterations. According to the American Cancer Society, excluding the cancers of skin, breast cancer is the most common cancer among women, accounting for nearly one in three cancers diagnosed in US women [1]. In breast cancer, it is not the primary tumor, but its metastases at different distant sites that are the main cause of death [2]. Based on different phenotypes and gene expression profiling, breast cancer can be divided into five major so-called PAM50 subtypes: luminal A, luminal B, tumor enriched with human epidermal growth factor receptor 2 (Her2), basal-like and normal-like subtype [3–6]. Luminal subtypes are characterized by the expression of estrogen receptor (ER) and are treated with adjuvant endocrine therapy targeting the ER signaling such as tamoxifen and aromatase inhibitors [6]. The third subtype, Her2 positive, is classified by the absence of ER and progesterone receptor (PR) expression, high expression of Her2 and high proliferation rate [6]. Her2 positive tumors can be targeted using an antibody against Her2 such as trastuzumab [7, 8]. Basal-like breast cancer generally expresses none of the three markers (ER, PR and Her2), and largely corresponds to the group of triple negative breast cancer (TNBC). It is often highly aggressive and is associated with poor prognosis [9]. No targeted therapies are currently available for this breast cancer subtype and current regimes include the conventional chemotherapeutic approach. Nevertheless, promising strategies are being developed to treat TNBC, such as poly-ADP ribose polymerase-1 inhibitors [6, 10, 11].

Normal-like subtype is the rarest form of breast cancer. It is relatively poorly characterized, yet has been shown to express ER, Her2 and PR. Its clinical prognosis is believed to lie between TNBC and luminal A subtypes [6].

MicroRNAs (miRNAs) are single stranded RNA molecules of ~22 nucleotides that are associated with the Argonaute proteins (AGO). They serve a role in post-transcriptional gene regulation in both plants and animals. miRNAs are generated by the sequential action of two RNase III-type proteins (Drosha and Dicer) on short hairpin RNAs. Upon cleavage by Dicer, the small RNA duplex is incorporated into a complex known as RNA-induced silencing complex (RISC) or in case of miRNAs referred to as miRISC [12–14].

miRNAs control most of the protein-coding genes resulting in control of cancer-relevant processes such as cell cycle, proliferation, differentiation, apoptosis, and migration. miRNAs associated with cancers are termed oncomiRs. A single miRNA can target hundreds of mRNAs. Hence, even though the regulation of gene expression by miRNAs is generally rather mild, aberrant expression of a single miRNA may affect a multitude of transcripts involved in cancer-related signaling pathways. This fact makes the situation more complex, as the overall functions of miRNAs in oncogenesis can be context dependent. Accordingly, a particular miRNA may be found to be upregulated in some cancers while being downregulated in others [15]. These findings became more pronounced with the availability of whole genome mRNA and miRNA expression data from different cancers [16–20]. In addition, mouse models with miRNA overexpression or ablation have shown a direct causal link between miRNA expression profiles and cancer development. Clinically, miRNA are studied as clinical biomarkers and/or putative therapeutic targets [21, 22].

Deep sequencing technologies have enabled the more extensive study of miRNAs, leading to the identification of different variants of the same miRNA. Typically, miRNAs are annotated as a single defined sequence and despite that, for many miRNAs, length and/or sequence variants have been observed, these variants were believed to be experimental artefacts and were thus ignored or dismissed [23]. However, massive parallel sequencing and computational algorithms have confirmed the biological existence of such variants in different species [24, 25]. The term isomiR was coined to describe the different sequence and/or length variants of an individual miRNA. Additionally, studies demonstrated the active associations between isomiRs and RISC, suggesting that they can interact with miRNAs [26]. These observations pointed to the possible physiological role of isomiRs. Different reports pointed to gender and/or race dependence of the isomiRs expression [27, 28]. However, the regulatory mechanisms underlying the production of specific isomiRs are not fully understood. Moreover, to date, there have been only few reports on the functional role of lowly abundant 5’isomiRs and the capacity to modulate their targets relative to the canonical counterparts [28, 29]. However, the functional importance of highly expressed 5’isomiRs has not been addressed, yet.

Generally, isomiRs can broadly be classified into three main categories: 5’ isomiRs, 3’ isomiRs and polymorphic isomiRs. 3’ and 5’ isomiRs are those having variations at the 3’ and 5’ ends of the mature miRNA sequence respectively, whereas polymorphic isomiRs are harboring different internal nucleotide sequences [23]. Different mechanisms contribute to the generation of isomiRs. In the canonical miRNA biogenesis pathway 5’ and 3’ ends are specified by consecutive cleavage events of the primary transcript by the ribonucleases Drosha and Dicer. The alternative cleavage by Drosha and/or Dicer could result in length variation which has been shown to partially depend on individual features such as ethnic background [27, 28, 30]. Furthermore, the 3’ ends of the RISC-bound miRNAs are liable to trimming by
exoribonucleases [31, 32]. In the latter two cases the product is termed “templated”, since the miRNA sequence still matches the parent gene. Post-transcriptional addition of one or more bases might also take place with the help of nucleotidyltransferases. Most of the nucleotidyltransferases are 5’-3’ polymerases leading to the formation of 3’ isomiRs [33]. The addition of nucleotides that do not match the parent gene is termed as “non-templated” addition [34]. RNA editing, especially adenosine to inosine editing (A-to-I) is suggested to be a major driver in the generation of polymorphic isomiRs [35]. Most miRNAs, however, do not exhibit a high frequency of editing. The most frequently observed type of isomiR in animals and plants is the 3’ isomiR. 5’ and polymorphic isomiRs are generally rare. Nevertheless, they still represent a significant proportion of the population of some miRNAs [36, 37]. 3’ isomiRs or non-seed-based nucleotide substitutions are believed to be functionally redundant and mainly affect miRNA stability. In contrast, it has been shown that 5’ isomiRs can have different miRNA targets and thereby different phenotypes compared to their canonical counterparts [27, 28, 34].

MiR-140 is encoded within intron 16 of Wwp2, an E3 ubiquitin ligase. It was first identified as a key player in cartilage development and homeostasis in chondrocytes [38]. The regulation of miR-140 was reported to be tissue-dependent. Besides its role in chondrocytes, it was found to be expressed in numerous other tissues and cell types including brain, breast, lung, colon, ovary and testis [39–41]. Importantly, expression-profiling experiments revealed a potential tumor suppressor function for miR-140 in many cancers where its expression was found to be downregulated in cutaneous squamous cell carcinoma, basal cell carcinoma, osteosarcoma, ovarian cancer, colon cancer and lung squamous cell carcinoma. Inversely, Zou et al. revealed a correlation between miR-140-3p overexpression with chordoma invasion and recurrence suggesting poor prognosis [42].

In the majority of miRNA species, the 5’ miRNA is annotated as the guide strand, while the complimentary 3’ miRNA is degraded. Interestingly enough, stably expressed levels of miR-140-3p as well as miR-140-5p were found to be present. The expression was found to be tissue dependent and each strand had its own function owing to different seed sequences [43]. Wolfson et al. reported a tumor suppressor role for miR-140 mediated by Wnt, SOX2 and SOX9 stem cell regulator pathways [44]. Downregulation of miR-140 in breast cancer was attributed to methylation of Cpg islands in the promoter region of the miRNA gene. Inhibition of miR-140 allows for uncontrolled elevation of SOX2, which is known to be a stem cell self-renewal regulator causing an increase in stem cell populations and breast cancer progression, initiation and growth [44]. miR-140-3p is one of few miRNAs that showed high expression of 5’isomiR according to miRBase [45].

In the present work, we sought to characterize the effects of the overexpression of both the canonical hsa-miR-140-3p and the 5’isomiR-140-3p on breast cell lines through functional assays that assess cell viability, cell cycle, apoptosis and migration. Moreover, we aimed at identifying and validating target genes for 5’isomiR-140-3p that could account for the observed phenotypes. In summary, we could attribute tumor-suppressive phenotypes including reduced proliferation due to cell cycle arrest in G0/G1 phase of the cell cycle as well as reduced cell migration specifically to the 5’isomiR-140-3p. These phenotypes could be linked to direct targeting of MARCKSL1, ITGA6 and COL4A1. Analysis of two major breast cancer patient cohorts (METABRIC and TCGA) confirmed the relevance of both miRNA and the novel target genes, especially in TNBC.

Results
5’isomiR-140-3p is highly expressed in breast cancer cell lines and patients
With the advent of miRNA sequencing, the existence of isomiRs became increasingly clear. In addition, it is now possible to quantitatively assess expression of miRNAs and their isomiRs to allow for estimations of their biological relevance. Combining these two means of information, we analyzed miRNA sequencing datasets of several breast cancer cell lines that had been generated as part of the Illumina iDEA challenge to identify pairs of highly expressed miRNAs and 5’isomiRs. Here, we neglected variants at the 3’end of both species, treating all 3’isomiRs with a length of 18–24 nucleotides as miRNA or 5’isomiR, respectively, depending on their 5’ end. Using the notation suggested by Loher and colleagues [27], we refer for example to all miRNA species between hsa-miR-140-3p 0|-3 and 0|+3 (in Table 1 simplified as 0|x) as canonical hsa-miR-140-3p, whereas all species between hsa-miR-140-3p +1|-2 to +1|+3 (in Table 1 simplified as +1|x) are considered as 5’isomiR-140-3p. The sequencing data is provided as Additional file 1 and the raw data as well as more detailed information about the experimental setup can be found at ArrayExpress (E-MTAB-4539).

As criteria, we defined an expression of the canonical miRNA of at least 100 rpm on average and a ratio between 5’isomiR and miRNA of at least 1 to 5 on average. Thereby, we identified six potentially interesting miRNA/5’isomiR pairs (Table 1). Interestingly, we found that in case of one of these miRNAs, namely miR-140-3p, expression of the isomiR was even higher compared to the canonical form. Therefore, we decided to focus on this pair.
We analyzed the breast cancer patients’ sequencing data from TCGA for the expression of the canonical hsa-miR-140-3p and its 5’iso-miR-140-3p [46]. The clinical characteristics as well as the expression of canonical miRNA and 5’iso-miR are listed in Additional file 2. The abundance of the different 3’iso-miRs of the two 5’iso-miRs under investigation is detailed in Additional file 3 listing the average expression of each miRNA species in the analyzed patient samples.

Both isoforms were found to be strongly positively correlated ($p < 0.001, r = 0.85$; Fig. 1a). The patients from the same dataset were grouped according to ER expression and the levels of hsa-miR-140-3p and 5’iso-miR-140-3p were analyzed. It was found that both the hsa-miR-140-3p and 5’iso-miR-140-3p were higher in ER negative (ER-) patients (Fig. 1b). Furthermore, patients with distant metastases had lower expression of both isoforms compared to patients without metastases (Fig. 1c). Additionally, survival analysis in TNBC patients showed a trend of better survival among patients with higher expression levels of both hsa-miR-140-3p and 5’iso-miR-140-3p (Fig. 1d); a trend that was not observed when analyzing the overall population of breast cancer patients (data not shown).

5’iso-miR specifically inhibits proliferation and migration of breast cell lines

In order to evaluate the biological significance of both isoforms, we carried out a set of functional assays on MCF10A, MDA-MB-231 and MDA-MB-468 cells. All these cells are negative for ER, PR and HER2. MCF10A cells are non-transformed cells with higher genomic stability and generally higher susceptibility to perturbations of their normal growth and migratory behaviour when compared to the TNBC cell lines MDA-MB-231 and MDA-MB-468 that harbour more mutations. WST-1-based cell viability assay was used to assess the effect of overexpression of the canonical hsa-miR-140-3p and the 5’iso-miR-140-3p on the cells in both cell lines. hsa-miR-140-3p did not affect the cell viability as compared to the mimic-miRNA negative controls. In contrast, 5’iso-miR-140-3p caused a significant decrease in cell viability in all three cell lines (Fig. 2a).

The effects of the hsa-miR-140-3p and 5’iso-miR-140-3p overexpression on the cell cycle were also tested. MCF10A, MDA-MB-231 and MDA-MB-468 cells were transfected with miRNA mimics, (Fig. 2b). In all three cell lines, 5’iso-miR-140-3p overexpression resulted in a cell cycle arrest where more cells were found at the G$_0$/G$_1$ phase. Overexpression of the canonical hsa-miR-140-3p, however, showed no pronounced effect on the cell cycle. Analysis of baseline apoptosis in these cell lines showed no elevated activity of caspase-3/7 in 5’iso-miR overexpressing cells as determined by NucView-488 caspase-3/7 assay (Biotium, Hayward, CA, USA; data not shown).

In addition, we tested the impact of overexpression of both isoforms on cell migration in a transwell-based cell migration assay. Cell numbers were normalized to a seeding control and are shown as relative values compared to control transfected cells. A decrease in cell migration was observed upon the overexpression of 5’iso-miR-140-3p relative to hsa-miR-140-3p or the negative control in all three cell lines (Fig. 2c).

### Table 1 Expression (in rpm) of pairs of highly expressed miRNAs/5’iso-miRs in a panel of breast cancer cell lines

| Notation of isoform | Seed sequence | MCF7 | MDA-MB-231 | T47D | BT20 | BT474 | MDA-MB-468 | ZR-75 | MCF10A |
|---------------------|---------------|------|-------------|------|------|-------|-------------|-------|--------|
| mir-10a-5p 0’x      | canonical     | 81.9 | 4923.9      | 9254.5 | 7698.9 | 96.5  | 181.4       | 854.1 | 582.7  |
| mir-10a-5p +1’x     | isomiR        | 56.9 | 2158.4      | 3911.3 | 3660.0 | 64.2  | 65.0        | 477.0 | 273.0  |
| mir-1307-3p 0’x     | canonical     | 1159.2 | 737.2    | 2299.7 | 575.6  | 1894.2 | 554.8       | 2560.7 | 290.0  |
| mir-1307-3p +1’x    | isomiR        | 394.2 | 253.2      | 943.8  | 222.5  | 643.7  | 348.3       | 784.3 | 72.7   |
| mir-140-3p 0’x      | canonical     | 180.9 | 486.8      | 117.4  | 289.0  | 149.1  | 63.0        | 181.2 | 199.2  |
| mir-140-3p +1’x     | isomiR        | 407.7 | 851.6      | 284.8  | 568.7  | 2176.3 | 95.2        | 306.2 | 323.8  |
| mir-183-5p 0’x      | canonical     | 1187.2 | 91.7     | 1065.9 | 1132.5 | 2017.2 | 1028.1      | 849.9 | 518.7  |
| mir-183-5p +1’x     | isomiR        | 1102.0 | 44.0      | 831.3  | 822.5  | 1860.5 | 767.9       | 725.6 | 312.4  |
| mir-203-3p 0’x      | canonical     | 2327.2 | 3.1       | 2514.6 | 544.1  | 5319.0 | 427.8       | 2870.3 | 713.8  |
| mir-203-3p +1’x     | isomiR        | 2115.5 | 1.5       | 1137.8 | 329.8  | 2116.1 | 175.1       | 1104.8 | 23.1   |
| mir-30a-3p 0’x      | canonical     | 13.9  | 991.7      | 162.5  | 191.8  | 30.8   | 1033.2      | 1088.0 | 239.7  |
| mir-30a-3p +1’x     | isomiR        | 3.7   | 305.1      | 53.5   | 65.8   | 6.6    | 311.7       | 35.8  | 44.1   |

5’iso-miR-140-3p and its 5’iso-miR have overlapping but different target spectra

The 5’iso-miR is shifted by one nucleotide at the 5’ end resulting in a different seed sequence, and thus is expected to have different target mRNAs. In order to examine the different spectra of target genes of the canonical miRNA and the 5’iso-miR, a gene expression microarray was performed upon overexpression of both hsa-miR-140-3p and 5’iso-miR-140-3p in MCF10A as well as MDA-MB-231 cells and respective negative controls in two biological replicates. Genes were considered
to be downregulated by either miRNA, when their expression was reduced by at least 35% with a significant corrected p-value in both cell lines. Interestingly, 109 genes were downregulated in both cell lines specifically by 5’isomiR-140-3p, whereas 18 genes were downregulated specifically in both cell lines by hsa-miR-140-3p and 5 genes were downregulated by both (Fig. 3a and Additional file 4). Of note, genes downregulated by either miR-140-3p or 5’isomiR-140-3p were significantly enriched for genes containing predicted target sites for the respective miRNA species according to TargetScan (see Additional file 5) [47].

Based on the results of the microarray analysis, we aimed to identify genes targeted only by the 5’isomiR-140-3p that might explain the tumor-suppressive phenotypes observed upon overexpression of the 5’isomiR. The 109 genes identified from the microarray were subjected to literature research with the aim of defining genes that might potentially phenocopy the viability, cell cycle and migration phenotypes seen upon the overexpression of the 5’isomiR-140-3p. The 3’ UTRs of the candidate target genes were analyzed for seed sequence matches with the 5’isomiR-140-3p. Eight putative targets, namely SRF, RUVBL1, MARCKSL1, CHCHD4, COL4A1,
Fig. 2 (See legend on next page.)
ITGA6, CAP1 and CCT5 met the aforementioned criteria. The full length 3’UTRs of the target genes were cloned into the dual luciferase reporter plasmid psiCHECK-2, a vector that utilizes Renilla luciferase as the primary reporter gene (see Additional file 6 for primer sequences). The respective reporter vectors or empty psiCHECK2 vector (as a negative control) were co-transfected with has-miR-140-3p or 5’isomiR-140-3p or mimic miRNA negative controls in MCF7 cells. Seventy-two hours post transfection, relative luciferase activity (renilla luciferase activity normalized to firefly luciferase activity) was measured (Fig. 3b). RLU values of target genes were normalized to the RLU of the empty psiCHECK2 vector. We identified the 3’ UTRs of MARCKSL1, CHCHD4, COL4A1 and ITGA6 to be specifically affected by 5’isomiR-140-3p. Moreover, 3’ UTR of CCT5 showed a decrease in luciferase activity upon co-transfection with has-miR-140-3p or 5’isomiR-140-3p, indicating targeting by both forms. Therefore, it was excluded from further analyses. Additionally, SRF and CAP1 were excluded from further experiments since no reduction in luciferase activity was observed compared to the empty vector.

In order to further confirm direct targeting of the candidate genes, miRNA-binding sites within the respective 3’UTRs were mutated and luciferase activity was measured. Values were normalized to the empty psiCHECK2 vector (Fig. 3c). Luciferase activity was rescued in all of the target genes but CHCHD4 (data not shown). This means that the reduction observed in luciferase activity was potentially due to an indirect effect. Therefore, CHCHD4 was omitted from further studies.

To validate downregulation of the putative target genes on mRNA level, MCF10A and MDA-MB-231 cells were transfected with has-miR-140-3p, 5’isomiR-140-3p or miRNA mimic negative control. The mRNA expression levels of the candidate genes were then assessed by Taqman qRT-PCR (Fig. 3d). Consistent with the previous findings from microarray and luciferase assay, a reduction in the mRNA levels of the genes ITGA6, MARCKSL1 and COL4A1 was observed.

In summary, ITGA6, MARCKSL1 and COL4A1 were validated as direct targets of the 5’isomiR-140-3p and further investigated for their impact on the phenotypes observed upon overexpression of the 5’isomiR-140-3p. COL4A1 encodes for collagen, type IV, alpha1. MARCKSL1 encodes for a member of the myristoylated alanine-rich C-kinase substrate (MARCKS) family and ITGA6 encodes for the integrin subunit alpha 6 and is commonly found in heterodimers known as α6β4 integrin and α6β1 integrin. Figure 4 highlights the seed regions targeted by the 5’isomiR-140-3p in the 3’UTR of each of the target genes. Nucleotides that were mutated for the experiments shown in Fig. 3c are highlighted.

Target gene knockdown partially phenocopies overexpression of 5’isomiR-140-3p

Having identified and validated the targeting of several candidate 3’UTRs, we attempted to link the downregulated genes to the phenotypes observed upon the overexpression of the 5’isomiR-140-3p in MCF10A and/or MDA-MB-231 cells. To this end, siRNAs were used to knockdown candidate genes, and the effects on cell viability, cell cycle and cell migration were assessed. We used a set of 4 different siRNAs (numbered 1-4 according to the order numbers assigned by the Dharmacon) against each target gene. The knockdown efficiency of each individual siRNA was assessed (Additional file 7). In case of MARCKSL1, two siRNAs (MARCKSL1_3 and MARCKSL1_4) were found to reduce the mRNA level. In contrast, in case of COL4A1 and ITGA6, all four siRNAs were found to reduce its expression on the mRNA level by 70 % or more. We continued further analyses with COL4A1_2 and _4 and ITGA6_3 and _4.

Initially, the effect of candidate gene knockdown on cell viability was evaluated. COL4A1 and ITGA6 knockdown was observed to phenocopy the effect of 5’isomiR-140-3p overexpression on cell viability in both cell lines and for both siRNAs (Fig. 5a). The effect was not pronounced, yet was found to be
statistically significant relative to siRNA negative control siAllstar. In contrast, MARCKSL1 knockdown did not have any effect on cell viability.

Moving on to cell cycle analysis, effects of knockdown of candidate target genes were examined. Only COL4A1 knockdown showed cell cycle arrest with a greater fraction of cells in the G0/G1 phase and a reduction of the percentage of cells in S phase in both cell lines, similar to the 5’isomiR-140-3p (Fig 5b).

We proceeded next to examine the effect of candidate gene knockdown on cell migration using a transwell-based cell migration assay. We observed a significant decrease in cell migration upon the knockdown of MARCKSL1 for both cell lines with both siRNAs. In
addition, migration of MCF10A cells was reduced upon knockdown of ITGA6 (Fig. 5c). The synergistic effect of these two target genes might be a partly explanation for the enhanced phenotype of the 5′isomiR in transwell migration on MCF10A cells when compared to MDA-MB-231 (see Fig. 2c).

Discussion

IsomiR is a term coined to describe length and/or sequence variations of the same miRNA. Different mechanisms underlie the generation of isomiRs, stemming usually from the normal biogenesis pathway of miRNAs [34]. In the current work, we present the functional characterization of 5′isomiR-140-3p, where the phenotypes of different cell lines are examined upon the overexpression of the 5′isomiR-140-3p in comparison to the canonical hsa-miR-140-3p. TCGA breast cancer patients’ sequencing data revealed that the expression of both isoforms is strongly positively correlated indicating a stochastic post-transcriptional process leading to the formation of both forms [48]. Additionally, a trend of better survival among TNBC patients was observed with higher expression levels of both hsa-miR-140-3p and 5′isomiR-140-3p. This might point to a possible synergistic role of the canonical hsa-miR-140-3p and the 5′isomiR-140-3p in suppressing the growth or development of breast cancer.

The tumor suppressor role for miR-140 has been characterized and reported by Wolfson B et al., where they showed that miR-140 acts by negatively regulating Wnt, SOX2 and SOX9 [44]. The latter are known to be key stem cell self-renewal regulatory elements. Inhibition of miR-140 expression was found to be through an ER-mediated mechanism and/or through differential methylation of CpG islands in the miR-140 promoter region. This results in uncontrolled elevation of SOX2, causing an increase in stem cell populations and breast cancer initiation, progression and growth [44, 49]. Based on the hypothesis that canonical miRNAs and their functional isomiRs might synergistically target related phenotypes, we were interested in assessing the effects of overexpressing the hsa-miR-140-3p and its 5′isomiR on different cancer-associated cellular phenotypes including viability, cell cycle, and cellular migration.

We observed that, contrary to the effect of the canonical hsa-miR-140-3p, overexpression of the 5′isomiR-140-3p led to a decrease in cell viability. The latter observation was supported by cell cycle analysis, where the 5′isomiR-140-3p but not the hsa-miR-140-3p caused cell cycle arrest in G0/G1 phase evident by 7-AAD/BrdU cell cycle analysis. Co-transfection of the two forms did not result in synergistic effects on cell viability in any of these three cell lines (data not shown). Noteworthy, we found that the 5′isomiR downregulates a larger number of genes compared to the canonical miRNA annotated in miRBase. This corresponds to the observation, that the isomiR is considered to be the canonical form in several species including species closely related to humans such as gorilla.

We identified three novel direct target genes related to the observed phenotypes: COL4A1, ITGA6 and MARCKSL1. COL4A1 encodes for collagen, type IV, alpha1. Type IV collagen is a main component of basement membranes, where molecules attach to each other forming complex protein networks. These
networks help the basement membranes interact with nearby cells, playing a role in cell movement or migration, cell survival and proliferation, and cell differentiation [49]. We observed that knockdown of COL4A1 caused a slight reduction in cell viability, cell cycle arrest in the G0/G1 phase and decrease in cellular migration through transwells. Previous studies pointed to the effect of COL4A1 in tumor progression and metastasis. Chen et al. observed a change in morphology of the skin cancer cells upon knocking down the COL4A1 gene [50]. They also noted that knocking down COL4A1 conferred the cells with higher levels of elasticity and lower motility.

ITGA6 is the gene encoding for the α6 subunit of integrins and forms heterodimers preferentially with the β4 and β1 subunits. Integrins are a group of proteins that regulate the cell-cell adhesion as well as the cell-matrix adhesion. They also transmit chemical signals that modulate cell growth and can alter the activity of certain genes [51]. Similar to COL4A1, we observed that knockdown of ITGA6 caused a reduction in cell viability and in MCF10A cells a decrease in cellular migration through transwells. In the study of hepatocellular carcinoma (HCC), it was shown that Integrin α6 can mediate metastasis, and that it could be used as a therapeutic target for improved patients’ survival. Knockdown of
**ITGA6** using shRNA was found to inhibit the proliferation and metastasis of HCC cells though PI3K/AKT and MAPK/ERK, where p-ERK and p-AKT were reduced by shRNA targeting integrin α6 [52]. The effects of 5’isomiR-140-3p overexpression on the signaling molecules downstream of the collagen/integrin signaling pathway remain to be studied. Additionally, in esophageal squamous cell carcinoma (ESCC), **ITGA6** was found to be highly expressed. In agreement with the results reported in HCC, *in vitro* knockdown of **ITGA6** in ESCC cells resulted in inhibition of cell proliferation, invasion and colony formation [53]. Given the tight connection between integrins and collagens, their combinatorial downregulation by the 5’isomiR might be a reason for the reduced migratory potential of cells overexpressing the 5’isomiR.

**MARCKSL1** encodes for a member of the myristoylated alanine-rich C-kinase substrate (MARCKS) family. This family of proteins functions in cytoskeletal regulation, protein kinase C signaling and calmodulin signaling. **MARCKSL1** protein affects the formation of the intermediate junction (also called adherens junction or belt desmosome), which is a type of cell–cell junctions occurring in epithelial as well as endothelial cells [54, 55]. Our experiments revealed a significant reduction in cellular migration across the transwells upon the knockdown of **ITGA6**. Consistent with these results, Jonsdottir et al. studied the prognostic value of **MARCKSL1** in lymph node-negative breast cancer patients. Among different prognosticators studied (age, tumor diameter, grade, estrogen receptor, and proliferation), **MARCKSL1** protein expression was the strongest prognosticator. Patients with high **MARCKSL1** expression showed a 44 % survival versus 88 % in patients with low expression at 15-year follow-up. Moreover, distant metastasis free survival was found to be significantly higher in patients with low **MARCKSL1** protein expression (78 %), compared to 45 % survival for patients with high expression [56]. Additionally, in a separate study that involved breast cancer metastasis to the bone, **MARCKSL1** was reported to be upregulated. Björkblom et al. searched the *In Silico* Transcriptomics (IST) database for **MARCKSL1** expression data. They found that in normal tissues, **MARCKSL1** mRNA was highly expressed in the central nervous system, testis, ovary, and lymphatic organs. In addition to breast cancer, there was significant upregulation of **MARCKSL1** in lung cancer, rhabdomyosarcoma, leiomyosarcoma, prostate cancer and uterine cancer [55].

Counter-intuitively, knockdown of **MARCKSL1** in different tissue contexts, specifically the neural tissue, and prostate cancer cell line (PC-3) resulted in an abrupt increase in migration. This was explained by the fact that activation of **MARCKSL1** through phosphorylation leads to its interaction with actin, reducing actin turnover in cells and retarding cell migration [55]. Hence, it seems to depend on the cellular context and the phosphorylation status of **MARCKSL1** whether its downregulation results in increased or decreased migration of the cells.

**Conclusions**

In conclusion, in this work we present the characterization of 5’isomiR-140-3p. Its overexpression in breast cancer cells resulted in phenotypes counteracting the progression of cancer including reduction in cell viability, cell cycle arrest and inhibition of migration. Moreover, we were able to identify and validate novel targets for the 5’isomiR-140-3p: **COL4A1**, **ITGA6** and **MARCKSL1**. Finally, we have shown that knocking down these genes could partially phenocopy the effects of the 5’isomiR-140-4p overexpression. **COL4A1** and **ITGA6** knockdown led to less cell viability and cell cycle arrest, while **MARCKSL1** knockdown resulted in a decrease in the migratory potential of cells. Figure 6 represents a summary of our understanding to the regulation of the miR-140 expression and its targets.

This study shows – to our knowledge – the first example on the functional discrepancy of the two highly expressed isoforms a miRNA in the context of human cancer. Despite the difference in their regulatory roles, both the canonical miRNA and the 5’isomiR work in a tumor suppressor direction. It’s worth mentioning that the mechanism underlying the regulation of the canonical or the 5’isomiR expression is not yet fully understood. Interestingly, we found that the ratio of the canonical miRNA and the 5’isomiR was significantly shifted towards to canonical form when comparing normal breast tissue with breast cancer tissue data, both from the TCGA dataset. An open question to be asked here is whether and how the cell can decide for or control which variant of the miRNA to express at which circumstances.

Lastly, with **isomiRs** in picture, the study of miRNAome is way more complex than previously thought. A growing number of reports point to the biological significance of at least some isomiRs. One nucleotide shift changes the spectrum of targeted genes, changing thereby the functional regulatory role of the miRNA. Through further characterization of the canonical miR-140-3p and the 5’isomiR-140-3p and other miRNA/5’isomiR pairs, more insights will be gained into the carcinogenesis of breast cancers. This will potentially provide one more tool to be used by clinicians in diagnosis or treatment.

**Methods**

**Cell culture and reagents**

MCF10A, MDA-MB-231, MDA-MB-468 and MCF-7 cell lines were obtained from ATCC (Manassas, VA, USA) in 2009 and 2010. Cell lines were verified using the cell line authentication service at the DKFZ Core Facility by Multiplex human cell authentication in 2011 (23). Cells were
incubated in a humidified incubator at 37 °C and 5 % CO₂. MCF10A cells were cultured in DMEM F12 medium (5 % Horse serum, 20 ng/ml EGF (BD Biosciences, Bedford, MA, USA), 0.5 μg/ml Hydrocortisone (Sigma-Aldrich, Saint-Louis, MO, USA), 0.01 mg/ml bovine insulin (Sigma-Aldrich, Saint-Louis, MO, USA), 100 ng/ml Choleratoxin (Sigma-Aldrich, Saint-Louis, MO, USA), 50 units/ml penicillin, 50 μg/ml streptomycin sulfate). MDA-MB-231 and MDA-MB-468 cells were maintained in RPMI1640 medium (10 % FBS, 1 % L-glutamine, 1 % nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin sulfate). MCF-7 cells were cultured in MEM medium (10 % FBS, 1 % nonessential amino acids, 1 % L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin sulfate). Cell culture media and reagents were obtained from Invitrogen.

Transfections
All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. miRNA mimics were purchased from Ambion (Life technologies). The mature sequences for hsa-miR-140-3p and 5’isomiR-140-3p are 5’UACCACAGGGUAGAACCACGGG 3’ and 5’ ACCACAGGGUAGAACCACGGAC 3’ respectively. miRNA negative controls were obtained from Dharmacon (Lafayette, CO). siRNAs against COL4A1, ITGA6, and MARCKSL1 were purchased from Dharmacon as set of four individual siRNAs per gene. Two of them were used for phenotypic analyses. siAll-Star non-targeting control was obtained from Qiagen (Hilden, Germany). siRNAs and miRNA mimics were used at a final concentration of 30 nM.

Microarray-based analysis of deregulated genes
MCF10A and MDA-MB-231 cells were seeded in 6-well plates and transfected with miRNA mimics for hsa-miR-140-3p and 5’isomiR-140-3p in two biological replicates, each, as well as with one replicate of mimic-ctrl1 and mimic-ctrl2. 48 h after transfection, RNA was extracted.
using the RNaseq kit (Qiagen, Hilden, Germany) and submitted to the Microarray unit of the Genomics-Proteomics Core Facility of the German Cancer Research Center. Genes were considered to be downregulated by the canonical miRNA or the 5’isomiR, respectively, if they showed an expression of less than 65 % of the expression in control transfected cells and the corrected p-value was below 0.05. Results from the microarray were uploaded to GEO (GSE74539).

Luciferase reporter assays
To validate direct targeting of miRNAs, 100 ng/well psiCHECK2 vectors (Promega, Madison, WI, USA), containing the respective 3’UTRs, were co-transfected with mimic miRNAs in MCF-7 cells. 48 h after transfection Renilla and firefly luciferase activities were determined as previously described using a luminometer (Tecan, Linz, Austria) [57]. Mutations within each of the predicted target sites of the 3’-UTRs generated by site-directed mutagenesis using Quickchange Lightning kit (Aglient Technologies). Primers are listed in Additional file 6.

RNA isolation and real-time PCR
Total RNA was isolated from cells using the RNaseq mini kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription of mRNAs into cDNA was done using the Transcriptor Reverse Transcriptase kit (Roche). The qRT-PCR reactions for target genes were performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Weiterstadt, Germany), using probes from the Universal Probe Library (Roche Diagnostics). For normalization of the mRNA analysis, HPRT1 and GAPDH were used as housekeeping genes.

WST-1 based cell viability assay
5 × 10^4 MCF-10A, MDA-MB-468 or MDA-MB-231 cells/well were seeded into 96-well plates. After 24 h, cells were transfected with the respective miRNAs or siRNAs. Transfections were performed in 6 replicates. 72 h later, 10 μL/well WST-1 reagent were added and the absorbance of the plate was measured at 450 nm using the TECAN plate reader (Infinite® M200). As medium blank, 10 μL of WST-1 reagent was added to a well containing only medium without cells and the absorbance of this well was subtracted from all other values obtained.

Cell cycle analysis
Cells were seeded as a single cell suspension with 1.5 × 10^5 cells/well into 6-well plates and transfected as described above. 48 h after transfection, the cells were incubated with BrdU for 30 min before fixation with Cytofix/Cytoperm (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s recommendations. BrdU was stained with a FITC-conjugated anti-BrdU antibody (BD Biosciences, Heidelberg, Germany) and DNA was stained with 7-AAD. Samples were analyzed using a FACSCalibur (Becton-Dickenson) and their distributions in the distinct cell cycle phases G0/G1, S and G2/M phases was assessed based on their fluorescence intensity for both dyes.

Transwell-based cell migration assay
MCF10A cells were transfected with 30 nM siRNAs or miRNAs in 6-well plates as described above. 48 h after transfection, cells were detached and 2 × 10^5 cells were seeded in starvation medium into transwell inserts with 8.0 μm pore-sized membranes in the 24-well plates format (Corning). Full growth medium was used as chemo-attractant in the lower chamber. To control for the number of cells, cells from the same suspension were seeded into poly-L-lysine coated 0.72 μm glass bottom square well MatriPlates. After 6 h (to allow for cell attachment), cells were stained with Hoechst-33258 and counted using Olympus ScanR microscope. In the transwells, cells were allowed to migrate for 20 h and the number of migrating cells was determined by flow cytometry using a FACSCalibur (Becton-Dickenson). For that purpose, cells attached to the lower side of the membrane were trypsinized and transferred into FACS-tubes. Cells were centrifuged, washed once with PBS and resuspended in 150 μl PBS. Cells were counted for a total of 2 min at high flow rate. The number of cells migrating was normalized to the total cell number from the counting control and values were presented as fraction of cells migrating.

Analysis of small RNA sequencing of breast cancer cell lines
Small RNA sequencing data distributed during the Illu-mina iDEA challenge was submitted to ArrayExpress (E-MTAB-4539) with kind permission by Illumina; details on the experimental setup can be found in the respective metadata. Briefly, the data had been generated by Illumina on a Gene Analyzer instrument using total RNA from eight commonly used breast cancer cell lines. Adapters were trimmed using trimmomatic and bowtie was used for mapping of the sequences to the genome (GRCh38) without allowing for any mismatches. IsomiR notation was based on miRBase version 21 and reads were mapped to the specific isomiR using bedtools intersect.

We analyzed the data for the presence of highly abundant miRNAs with associated highly abundant 5’isomiRs using an average expression over all cell lines of 100 rpm and a ratio of isomiR to miR of at least 1:5 as threshold to define potentially interesting pairs.

To do so, we did not distinguish variants at the 3’end of both species, treating all 3’isomiRs with a length of
18-24 nucleotides as miRNA or 5’isomiR, respectively, depending on their 5’ end. Using the notation suggested by Loher and colleagues [27], we refer for example to all miRNA species between hsa-miR-140-3p 0|-3 and 0| + 3 (in Table 1 simplified as 0|x) as canonical hsa-miR-140-3p, whereas all species between hsa-miR-140-3p +1|-2 to +1| +3 (in Table 1 simplified as +1|x) are considered as 5’isomiR-140-3p.

Patient data analyses
Expression values of hsa-miR-140-3p and the 5’isomiR-140-3p were extracted from the TCGA breast cancer dataset (accessed August 2013) and correlated with clinical parameters such as ER- or metastasis status [46]. The reads for miR and isomiR as well as the clinical parameters are provided as Additional file 2. Survival analyses were performed using GraphPad Prism version 6.0; the upper and the lower quartiles of expression were considered as ‘high’ and ‘low’, respectively.

Statistical analyses
Data are presented as mean ± S.D. Samples were analyzed by two-tailed unpaired Student’s t test, unless otherwise mentioned, and p values < 0.05 were considered as being statistically significant. p values < 0.05, < 0.01, and < 0.001 are indicated with one, two, and three asterisks, respectively. Data obtained from breast cancer patient samples were analyzed using GraphPad Prism version 6.0.

Additional files

Additional file 1: miRNA seq dataset used to identify highly abundant miRNAs that have abundant 5’isomiRs. (XLSX 3320 kb)

Additional file 2: Clinical data and miR/isomiR expression of patients from the TCGA breast cancer cohort [48]. (XLS 7116 kb)

Additional file 3: Relative abundance of 31 isomiRs of miR-140-3p and 5’isomiR-140-3p in the TCGA breast cancer patient cohort. The table lists the genomic coordinates, the notation of the isomiR and the average expression of each individual form (in rpm). (XLS 9 kb)

Additional file 4: Genes deregulated by hsa-miR-140-3p, 5’isomiR-140-3p or both in both MCF10A and MDA-MB-231 cells. This table contains the genes which are downregulated in both cell lines by either hsa-miR-140-3p, 5’isomiR-140-3p or both by at least 35 % and with a significant corrected p-value. These genes are summarized in the Venn diagram in Fig. 2a. In addition, the genes that are upregulated in both cell lines by at least 65 % and with a significant corrected p-value by either hsa-miR-140-3p, 5’isomiR-140-3p or both are also listed in this table. Values are reported as log2 fold changes and Benjamini-Hochberg corrected p-values. (XLS 74 kb)

Additional file 5: Venn Diagrams of predicted and downregulated genes for both miRNA species. Predicted targets according to TargetScan irrespective of site conservation and downregulated genes as listed in Additional file 3 for hsa-miR-140-3p (A) and 5’isomiR-140-3p (B) are depicted. For both miRNA species, downregulated genes are significantly enriched for predicted targets of the respective miRNA (p < 0.001 as determined by Chi-square test). (JPG 62 kb)

Additional file 6: List of primers and probes. This file contains all primer sequences used for cloning and mutagenesis of the luciferase reporter plasmids, as well as the UPL primers and probes used for qRT-PCR. (DOCX 17 kb)

Additional file 7: Efficiency of target gene knockdown on the mRNA level using Taqman qRT-PCR. MCF-7A cells were transfected with different siRNAs or siRNA negative control (siAllstar). 72 h later, cells were lysed and total mRNA was isolated and purified using RNeasy kit (Qiagen). The mRNA expression levels of the candidate genes were then assessed by Taqman qRT-PCR. Gene expression was normalized to HPRT and GAPDH housekeeping genes. Normalized gene expression is depicted as relative expression to cells transfected with siAllstar. Values represent the mean of three technical replicates. (JPG 350 kb)

Abbreviations
COL4A1, collagen, type IV, alpha1; ER, estrogen receptor; FACS, Fluorescence-activated cell sorting; HER2, human epidermal growth factor receptor 2; ITG6, integrin alpha 6; MARCKSL1, myristoylated alanine-rich C-kinase substrate like 1; miRNA, microRNA; PBS, phosphate buffered saline; PR, progesterone receptor; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; TCGA, The Cancer Genome Atlas; UTR, untranslated region

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Authors’ contributions
OS did the cellular assays, all statistical analyses, and drafted the manuscript. NE, JJ, AW and HW did the cellular assays. EM cloned and mutated the luciferase reporter constructs. SW participated in the design of the study and helped to draft the manuscript. OS did the cellular assays, all statistical analyses, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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