Identification of microRNAs miR-203 and miR-335 forming a network of regulation in breast cancer development

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Content

1 Summary

Human breast cancer, representing the most frequent tumor in women, can be divided into two major subclasses, the inherited and the sporadic form. Whereas the inherited subclass is predominantly characterized by mutations of the cancer susceptibility genes *BRCA1* and *BRCA2*, the underlying mechanisms leading to sporadic breast cancer remain undefined and are therefore subject of the present study.

Here, we focus on the decipherment of the complex network regulating *BRCA1*, involving the transcriptional activators ERα, IGF1R, AhR and SP1, as well as the dominant negative repressor ID4. Deregulation of individual or multiple components of this network promote breast cancer formation by repressing *BRCA1* as a key molecule for genomic stability and by activating mitogenic signal cascades.

In this study, we analyzed post-transcriptional control mechanisms mediated by microRNAs, which could previously be identified as regulator of crucial cellular functions. We investigated the role of two independent microRNAs, miR-203 and miR-335, for the formation of sporadic human breast cancer and their involvement in the regulatory network of the cancer susceptibility gene *BRCA1*.

MiR-335 was found at a decreased expression level in primary sporadic breast cancer specimens, positively correlating to the transcript level of *BRCA1*. Functionally, overexpression of miR-335 led to decreased cell viability, paralleled by an increase in apoptosis and downregulation of the *BRCA1* activators ERα, SP1, AhR and IGF1R and the repressor ID4, suggesting a tumor-suppressive function for miR-335 in breast cancer.

As microRNA miR-203 influences partly the same factors in the regulatory pathway of *BRCA1* and could also be connected to apoptosis and altered cell viability, both microRNAs form a network with superior regulating function for the homeostasis in breast tissue. Furthermore, the expression of miR-203 is regulated by SP1, binding to a methylation sensitive upstream motif. In addition, the expression of both microRNAs is controlled by estrogens, forming a tight network controlled by feedback mechanisms.

Taking the results together, both microRNAs affect the same targets in signaling pathways of breast cells with impact of apoptosis, proliferation and expression of the tumor susceptibility gene *BRCA1*. Misregulation during cancer development and progression may lead to an increased tumorigenic potential by suppression of tumor suppressing signals and activation of growth promoting cascades.

1.1 Zusammenfassung

Brustkrebs, der den größten Teil der weiblichen Krebserkrankungen ausmacht, wird in zwei Klassen unterteilt: die erbliche und die sporadische Form. Während sich die erbliche Form häufig durch Mutationen der Krebs-Suszeptibilitätsgene *BRCA1* und *BRCA2* auszeichnet, sind Mechanismen, die eine sporadische Erkrankung auslösen, weitestgehend unbekannt und daher Schwerpunkt dieser Studie. Dabei fokussieren wir auf die Entschlüsselung des komplexen Netzwerkes zur Regulation von *BRCA1*, welches aktivierende Faktoren wie ERa, IGF1R, AhR und SP1 beinhaltet, aber auch dominant negative Repressoren wie ID4. Fehlregulierungen einzelner oder mehrerer Komponenten dieses Netzwerkes unterstützen die Entwicklung von Brustkrebs durch die Verringerung der *BRCA1*-Expression, was zu einer genomischen Instabilität führt, aber auch durch die Aktivierung von wachstumsfördernden Signalkaskaden. In dieser Studie wurden post-transkriptionelle Kontrollmechanismen basierend auf MikroRNAs untersucht, die bereits als Regulatoren von wichtigen zellulären Prozessen identifiziert wurden. Hierbei wurde die Rolle zweier MikroRNAs, miR-203 und miR-335, bei der Entwicklung von Brustkrebs und der Regulierung des Brustkrebs-Suszeptibilitätsgens *BRCA1* untersucht.

MiR-335 wies eine verminderte Expression im primären Brustkrebsgewebe auf, wobei diese positiv mit der Expression von *BRCA1* korrelierte. Funktionell führte eine Überexpression von miR-335 zu geringerer Zellviabilität, gesteigerter Apoptose und verminderter Expression der *BRCA1*-Aktivatoren ERα, SP1, AhR und IGF1R sowie des Repressors ID4, was eine Tumorsuppressor-Funktion von miR-335 vermuten lässt.

Da miR-203 teilweise die selben Komponenten der *BRCA1*-Kaskade reguliert und ebenfalls mit verminderter Zellviabilität und gesteigerter Apoptose in Verbindung gebracht wird, bilden beide MikroRNAs ein Netzwerk mit übergeordneter Funktion bei der Erhaltung des Gleichgewichts im Brustgewebe. Dieses Netzwerk weist diverse Rückkopplungsschleifen auf, da die Expression von miR-203 von SP1 reguliert wird, dass an ein Motiv im Methylierungs-sensitiven Promoter der MikroRNA bindet, und beide MikroRNAs Östrogen-abhängig sind.

Zusammenfassend wirken beide MikroRNAs auf gleiche Zielmoleküle in Signalkaskaden von Brustzellen ein, mit Einfluss auf Apoptose, Wachstum und die Expression des Brustkrebs-Suszeptibilitätsgens *BRCA1*. Fehlregulierungen während der Krebsentstehung oder dessen Fortschreitens könnte zu erhöhtem tumorgenen Potential führen, indem Tumor-unterdrückende Signale vermindert und wachstumsfördernde aktiviert werden.

2 Introduction

Breast cancer is the most frequent tumor in human females, with only marginal appearance in men. The tumor derives in a multistep process from ductal epithelial cells by triggering mechanisms as yet poorly defined. Human breast cancers can be divided into two major subclasses, the inherited and the sporadic form, with the latter representing the greatest part. The inherited subclass is predominantly characterized by mutations of the cancer susceptibility genes *BRCA1* and *BRCA2*, favoring a high predisposition to develop breast cancer. Since *BRCA1* mutations are found in the sporadic form only rarely, mechanisms triggering the formation of these tumors are under intense investigation.

2.1 *BRCA1* and breast cancer formation

The inherited form of human breast cancer in 40-50% of cases can be associated with mutations of the cancer susceptibility genes *BRCA1* and *BRCA2* (1). Four years after the association of breast and ovarian cancer to one specific locus on chromosome 17q in 1990 (2), the coding sequence of *BRCA1* was identified by positional cloning (3). In addition, mutation studies revealed sequence aberrations for *BRCA1* in numerous inherited breast and ovarian cancers (4).

BRCA1 is involved in a number of cellular processes such as DNA repair (5), transcriptional regulation (6), ubiquitinylation (7), chromatin remodeling (8), X chromosome inactivation (9) and estrogen signaling (10). Specific domains within its protein structure mediate the nuclear localization and present interaction sites for a range of proteins with diverse cellular functions. Its N-terminal RING-finger domain dimerizes with BARD1 to form a complex with ubiquitinylation function upon the induction of cellular stress (11). Since the BRCA1/BARD1 complex polyubiquitinates its target with strong preference to an unconventional lysine, specific functions apart from protein degradation are hypothesized (12). Upon DNA damage, BRCA1 is phosphorylated by the signaling kinases ATR and ATM and co-localizes with the ssDNA binding protein RAD51 (5) and the helicase BACH1 (13) in the BRCA1-associated surveillance complexes (BASC) at the sites of double-strand breaks. Since no direct activity of BRCA1 could be

detected, a scaffolding function for repair proteins is speculated (14). In its unphosphorylated form, BRCA1 is incorporated in the BRCA1-associated transcriptional (BAT) complex (15). The C-terminal domain (CTD) supports the binding to the RNA polymerase II and the RNA helicase A and other proteins of the core transcription complex (16). Here, BRCA1 acts as an activator of transcription. Since evidence of a direct DNA binding of BRCA1 is lacking, it is thought to exhibit post-promoter activities in the transcriptional activation complex (17). Overexpression of BRCA1 leads to upregulation of stress-response genes and downregulation of estrogen-receptor-regulated genes resulting in cell cycle arrest and apoptosis (6, 10).

Its diverse functions in crucial cellular processes define BRCA1 as a key protein to maintain homeostasis and chromosomal integrity. Hence, downregulation or mutation promotes chromosomal instability (18) and tumorigenesis. In inherited breast cancers, mutations of *BRCA1* account for about 25% of all cases, with an estimated lifetime risk to develop breast cancer of as high as 80% (19). However, hereditary breast cancer is only responsible for 5-10% of the overall appearance. Despite the fact that mutations in *BRCA1* are the predominant cause in inherited breast cancer, only minimal numbers were determined in sporadic tumors (4, 20, 21). Nevertheless, due to its function as a tumor suppressor gene, *BRCA1* is thought to play a major role in the development of sporadic breast cancer as well.

Various attempts have been made to establish the role of *BRCA1* in the development of sporadic breast cancer. After Thompson and colleagues first described a decreased expression of *BRCA1* in tumor samples (22), a multitude of studies followed confirming downregulation of BRCA1 protein expression in a high percentage of sporadic breast tumor cases (23-25). The underlying mechanisms remain unclear, since almost no genomic mutations were detected. However, altered expressions of regulatory factors of *BRCA1* or epigenetic modifications have been suggested to be responsible. Here, Dobrovic and Simpfendorfer first described promoter methylation as a possible repression event in breast cancer (26). In subsequent studies, the number of cases with full or incomplete methylation status varies from 11% up to 30% in sporadic breast cancers (27-29).

A different attempt to explain aberrant expression of *BRCA1* is based on an altered transcriptional regulation. *BRCA1* expression is embedded in a tightly controlled network involving various activating and repressing factors. Here, the expression was determined to be hormone-dependent via direct activation through the estrogen (ERα), the aryl hydrocarbon (AhR), and the insulin-like growth factor 1 (IGF1R) receptor. They are

supported by hormone-independent factors such as the specific protein 1 (SP1) or inhibited via the inhibitor of DNA binding 4 (ID4). These factors were identified as potent regulators forming a tightly cross-linked network controlling the expression levels of *BRCA1* and are introduced in the following chapters.

2.2 The inhibitor of DNA binding (ID4)

ID4 is a negative regulator belonging to the family of helix-loop-helix proteins (30). Importantly, ID proteins harbor an HLH domain, but lack the DNA interaction site. ID proteins therefore act as the dominant-negative interaction partner of specific HLH factors by inhibiting their binding to promoter sequences. The ID family consists of four members, sharing the structural specialities identifying them as heterodimerization partners with an inhibitory function. Since the family members appear to be differentially expressed in tissue types and development stages, a distinct function of each member is assumed (31, 32). Functionally, ID4 was validated to abrogate the DNA binding ability of E47 and MyoE. Expression profiling determined ID4 to be involved in cell differentiation, especially in neurogenesis and osteogenesis (33, 34).

In diverse tumor types, an altered *ID4* expression could be detected. Here, some studies demonstrated downregulation of *ID4* expression, mostly due to promoter hypermethylation, in breast cancer (35-37), colon cancer (38), gastric cancer (39), and leukemia (40, 41). In contrast, others described *ID4* overexpression in bladder cancer (42), small cell lung cancer (43), and t(6;14)-associated leukemia (44). Accordingly, Shan and colleagues detected overexpression of ID4 in mammary cancers and its correlation with increased cell growth and higher tumorigenic potential in a rat model (45).

In a previous study, ID4 was identified to negatively regulate the expression of *BRCA1* (46). These data were further underlined by studies describing an inverse correlation of *BRCA1* and *ID4* in sporadic breast tumors (47, 48). Welcsh and colleagues expanded these findings to a feedback-loop after detecting an activation of *ID4* by BRCA1 (49).

2.3 The estrogen receptor α (ERα)

The family of estrogen receptors consists of the two members, ERα (gene: *ESR1*) and ERß (gene: *ESR2*), sharing a high homology in their DNA- and hormone-binding domains. Estrogen receptors interact directly with promoter regions harboring specific estrogen-responsive elements (ERE), thereby mediating mitogenic effects to the cells (50). Here, secreted growth factors, growth factor receptors, proteases, and cyclin/cdk factors have been identified as targeted genes (51). Interaction of ERα with other transcription factors, such as JUN (52), NFkappaB (53) or SP1 (54) transmits ERE-independent transcriptional activation. In addition, ERα transduces non-genomic effects by interacting with membrane-associated proteins involved in mitogenic signaling pathways (55). In normal breast tissue, ERα is necessary for mammary gland development. Its tumorigenic effect was primarily discovered after detecting an increased risk of breast cancer following excessive exposure to estrogens (56). In concordance with this, premalignant lesions showed an increase in the ERα level, suggesting a higher risk with elevating receptivity to estrogens. These hormones are thought to stimulate proliferation by inducing pathways of autocrine, paracrine or intracrine growth factors. In cell cultures, estrogens were able to directly stimulate proliferation by increasing the number of cells entering the S-phase (57).
Binding of an ERα antagonist results in an altered conformation of the protein, thereby inhibiting its DNA binding capacity. Estrogen-positive tumors show good response to anti-estrogen agents such as tamoxifen, leading to a more favorable prognosis. Furthermore, these tumors have a higher grade of differentiation, lower cell proliferation rates and a decreasing tendency towards metastasis (58). This was confirmed by *in vitro* experiments, revealing a lower invasiveness and motility of ERα-positive cancer cells (59, 60). Conversely, ERα-negative cancer cells implanted in mice showed a higher metastatic potential (61). Also, unliganded ERα has anti-invasive capacities (62), challenging the development of ERα antagonists for hormonal therapy. An effective treatment is obliged to block the mitogenic effect of the receptor without lowering the protein amount to avoid a progression to more unfavorable tumor types.
In contrast to the tumorigenic potential of estrogens and their receptors, the addition of estrogens to ERα-positive cancer cells induced the expression of the cancer susceptibility gene *BRCA1* (63). Despite lacking a direct interaction motif, *BRCA1* recruits the liganded receptor to its AP-1 binding site as part of an activating multi-protein complex including ERα, p300, JUN, and FOS (64). In a negative feedback loop, BRCA1 induces p53, which

itself interrupts the estrogen activation. This mechanism is thought to be a checkpoint to allow DNA repair or induction of apoptosis to ensure genomic stability in proliferating cells.

Summarizing, ERα stimulates cell growth in a ligand-dependent and -independent manner. Its activation and overexpression favors tumorigenesis at an early cancer stage. Later in tumor progression, ERα expression is associated with a good response to hormonal therapy and decreased aggressive tumor capacities.

2.4 The aryl hydrocarbon receptor (AhR)

AhR is a transcription factor belonging to the HLH family. Upon binding of its ligands, AhR translocates into the nucleus. Once activated, AhR associates with the nuclear factor ARNT through an HLH interaction. As an activated transcription factor, it binds to XRE/AhRE promoter elements, thereby activating or suppressing its target genes (65). CYP1B1 and other members of the P450 enzyme family have been identified as downstream targets, mediating the toxic effect by metabolizing the AhR ligands into mutagenic intermediates (66). CYP1B1 contains seven AhR binding motifs and was shown to be upregulated upon AhR activation (67). As AhR is highly conserved throughout its evolution, additional functions are assumed (68). This is supported by AhR activation even in the absence of specific ligands and by overexpression of the receptor in a range of cancer types (69). The oncogenic effect of AhR is probably mediated by its downstream targets, since the CYP1B1 protein level is also increased in these tumors. Expression of CYP1B1, which metabolizes endogenous estrogens to mutagenic 4-hydroxy-estradiol (70), directly correlates with the risk of breast cancer (71), supporting a specific role of AhR by induction of CYP1B1 during breast cancer pathogenesis. Functional studies further underlined its association with cancer development. Overexpression of AhR increased the proliferation rate of cancer cells, whereas knockdown of the expression converted the effect (72). In concordance with this, downregulated AhR led to a prolonged transition from G1- to S-phase by downregulating diverse cell cycle regulators (73). As additional effectors, epiregulin (74), overexpressed in breast cancer, and TGF-ß (75), which inhibits mammary tumor growth, were identified. Controversially, activation of AhR results in a reduced cell proliferation under some circumstances. Here, diverse effectors have been

identified mediating the inhibiting effect of AhR. For example, the growth-promoting ERα signaling cascade is abolished following interaction with ligand-bound AhR, thus leading to a degradation of the receptor (76). In contrast, other ligands activate both ERα- and AhR-induced transcription (77). Studying AhR-induced apoptotic mechanisms, a similar discrepancy appears. On the one hand, a pro-apoptotic function of AhR by inducing the BAX and the FAS cascade was identified (78). *Vice versa*, AhR was shown to repress apoptosis, for example by blocking pro-apoptotic E2F-1 signals (79).

Summarizing, these data support the idea that AhR might have oncogenic and tumor-suppressive functions depending on the cellular background and presence of ligands. In breast tumors, overexpression of AhR might lead to growth-promoting signals by activating the expression of CYP1B1. As it is described for the estrogen receptor α, AhR drives tumorigenesis, but simultaneously activates the tumor suppressor gene *BRCA1*. Hockings and colleagues demonstrated that estrogen-induced *BRCA1* expression is dependent on the availability of unliganded AhR, which binds to XRE elements in the promoter region (80).

2.5 The insulin-like growth factor 1 receptor (IGF1R)

The insulin-like growth factors IGF-I and IGF-II bind to receptors, which are important components in the growth signaling machinery, controlling development and cellular integration within the tissue structure. One major effector is the insulin-like growth factor 1 receptor (IGF1R) stimulated upon ligand binding. The receptor is tightly controlled by various binding proteins mediating an activating or suppressing effect on its function (81). IGF1R is associated with mitogenic and transforming activities following overexpression of the receptor (82), whereas inhibition leads to cell cycle arrest in the G0-G1 phase, induction of apoptosis, and a reduced tumor growth *in vivo* (83). IGF1R signaling is mediated by the Akt pathway leading to inhibition of pro-apoptotic mitochondrial enzymes, thereby promoting cell survival (84). Both overexpression of the receptor itself as well as increasing levels of the ligand IGF-II were associated with cancer formation (85, 86) including breast cancer (87). The expression of *IGF1R* is controlled by a network involving ERα, SP1 and BRCA1. Here, BRCA1 has a key regulatory function since interaction with ERα or SP1 can abrogate the activation of *IGF1R* gene expression (88-90).

Interestingly, the specific ligand IGF-I also downregulates the expression of its receptor IGF1R (91), probably by activation of *BRCA1*, indicating a negative feedback loop (92). The participation of IGF1R in diverse growth and survival-promoting pathways and its association with cancer formation defines it as an interesting therapeutic target. Targeted inhibition of the receptor (83), its binding proteins (93) or the ligands is a potent approach to abolish mitogenic signals leading to cancer formation or progression. Some of these therapeutic strategies are currently the subject of first clinical trials (94).

2.6 The specificity protein 1 (SP1)

The transcription factor SP1 is one of four members of the SP family, sharing three zinc finger DNA binding domains. SP1 and Sp3 are ubiquitously expressed in mammalian cells. However, despite being united through conserved protein domains, they fulfill distinct cellular functions. SP proteins regulate gene expression by binding to specific GC-rich motifs in the promoter regions of target genes. The regulatory effect of SP1 was shown to be activating or repressing, with the specificity of its functions depending on the cellular background and its interacting partners (95). The regulatory effects of the SP family are in part hormone-dependent through an interaction with the estrogen receptor. Specific knockdown of SP proteins abrogated the estrogen-mediated gene regulation of two thirds of genes regulated by the hormone (96). Furthermore, SP1 can be activated by an interaction with other regulatory factors such as RB or E2F, for example in the regulation of IGF-II (97).

An increased DNA-binding activity of SP1 could previously be associated with the formation of breast carcinomas (98). In other cancer types, an overexpression of SP1 was detected and was accompanied by an unfavorable prognosis (99). Conversely, interference of the SP1 function results in decreased cancer cell growth (100).

Activation of *BRCA1* expression depends on the ligand binding of the estrogen receptor and its interaction with SP1. This is thought to depend on the activation of the MAPK pathway, leading to an activating phosphorylation of both transcription factors (101). In addition, the recruitment of SP1 to the proximal promoter of *BRCA1* was determined to be activated by IGF-I (92). In return, BRCA1 inhibits SP1 function following physical interaction (88).

2.7 The tightly cross-linked network of *BRCA1*

The expression of the cancer susceptibility gene *BRCA1* is incorporated in a tightly controlled network of transcriptional activators and repressors. Importantly, these transcription factors are also involved in diverse cellular processes regulating cell proliferation and apoptosis, defining them as crucial elements for the formation of cancer. In the following, the network regulating *BRCA1* expression is summarized in detail (Fig. 1).

Activation of *BRCA1* depends on the assembly of a multi-protein complex including ERα, SP1, AhR and other cofactors. The formation of the transcriptional activator complex was shown to be regulated by ligand binding to the receptors and the expression level of the single components. While estrogen favors the binding of its specific receptor, ligand binding to AhR disrupts the initiation complex. IGF-I is responsible for SP1 recruitment to the promoter, probably by activating its receptor IGF1R. In contrast to the activating role on the tumor suppressor *BRCA1*, the factors are associated with increased proliferation rates and reduced apoptosis. In concordance with this, their overexpression was detected in a range of tumors including breast tumors. Among the predominantly growth-promoting functions of the factors, BRCA1 might act as a checkpoint ensuring an error-free replication cycle of proliferating cells. BRCA1 coordinates induction of apoptosis in damaged cells or the release into the next cycle phase. Multiple negative feedback loops initiated by BRCA1 form a tightly regulated downstream cascade ensuring genomic stability during cell growth. Here, BRCA1 disrupts its own activating complex by directly interacting with the components of the initiation complex (ERα, SP1) and by activation of p53, which inhibits ERα binding. BRCA1 blocks the IGF1-induced activation by interacting with SP1, a potent activator of *IGF1R*. This interaction further interrupts the negative regulation of *ID4* through SP1. ID4 might act as a dominant-negative inhibitor by binding to HLH proteins in the initiation complex of *BRCA1*.

BRCA1 regulation is embedded in a tight network with self-regulating feedback mechanisms. Here, *BRCA1* and its activators are involved in crucial mechanisms ensuring cellular and genomic integrity. Hence, alteration of this network might support tumor formation and progression. However, mechanisms leading to an altered expression of the single components are yet poorly defined. As microRNAs are described as post-transcriptional regulators of a high number of protein-coding genes, they might participate

in the control of *BRCA1* and its regulators. Therefore, microRNAs are of great interest in this study and are introduced in detail in the following chapters.

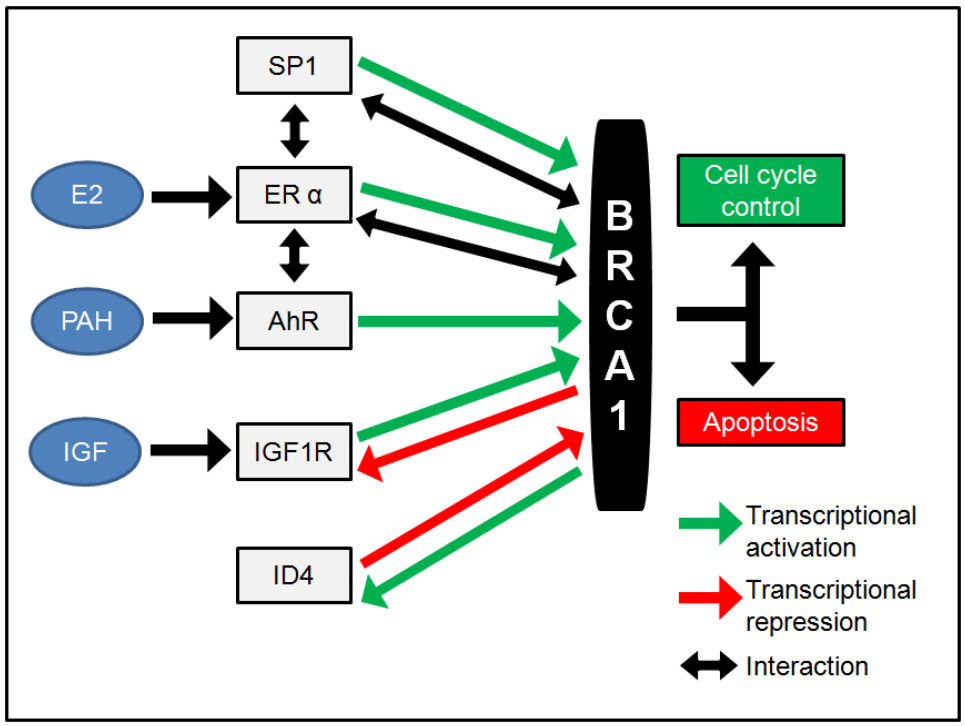


Fig. 1: Schematic overview of the complex *BRCA1* regulation mechanism by the transcriptional activators SP1, AhR, ERα, IGF1R and the repressor ID4.

2.8 MicroRNAs and their function

The discovery of microRNAs in *C. elegans* (102-104) and later in humans and most other eukaryotic species (105, 106) defined a new era in the understanding of post-transcriptional regulation of protein-coding genes. Previously, protein binding to the 3´ UTR, differential adenylation and splicing as well as RNA editing mediated the fine tuning of message delivery to the polysomes. With microRNAs binding to the 3´ UTR of mRNAs, another variable but specific post-transcriptional system was discovered. Regarding the high number of different microRNAs with multiple predicted targets, the functional dimension of these small RNAs can only be estimated.

MicroRNAs are non-coding RNAs transcribed by the RNA polymerase II as large pri-microRNAs (Fig. 2). Alternatively, these primary molecules excise from intronic regions of protein coding genes, thereby circumventing the transcription process. Pri-microRNAs fold in an imperfect hairpin structure and are further processed by the RNase III type endoribonuclease Drosha to approximately 70 nucleotide pre-microRNA hairpins. Rarely, intronic microRNAs (mirtrons) fold directly into pre-microRNAs following splicing (107, 108). These canonical hairpins are transported from the nucleus to the cytoplasm by exportin 5 (109), where they are further processed by the endonuclease Dicer. In collaboration with the TAR RNA-binding protein (TRBP), the precursors are sliced in double-stranded, approximately 21 bp long oligonucleotides with protruding 2-nucleotide 3´ ends. In general, the strand with the 5´ terminus, inhabiting the thermodynamically less stable end, is incorporated in the microRNA-induced silencing complex (miRISC), whereas the complementary strand is degraded as a passenger strand (110-112). However, sequencing efforts of small cellular RNAs indicated that the passenger strand is also present in the cells (113, 114) and, furthermore, the so-called microRNAs* were proven to be active in the post-transcriptional regulation process (115). MicroRNAs exhibit a functionality as part of the multi-protein complex miRISC. The major and best characterized members of the microRNA ribonucleoproteins (miRNPs) are members of the Argonaute family (AGO1-4) (116). AGO2 has previously been associated with the RNA interference machinery using siRNAs as mediators of gene silencing. It is the only family member harboring an RNaseH-like P-element induced wimpy testis (PIWI) domain, enabling the protein to slice double-stranded RNAs. For RNA cleavage, a perfect complementarity of the small RNA and the target molecule is required, which is obligatorily involved in siRNA-mediated silencing, but rarely found in microRNA regulation. Except for plants (117), where a perfect complementarity between the microRNA and its target is frequently found, microRNAs generally pair imperfectly to their targets (118). Consequently, the result of the microRNA:RNA interaction is not a degradation of the target, but rather a block of the translation process. The exact mechanism inhibiting the ribosomes from mRNA processing is not entirely understood. One model is based on the interference with initiation components such as the eukaryotic translation initiation factor 4E (eIF4E). This major member of a multi-protein complex, initiating ribosomal binding, binds to the cap structure of mRNAs. Interaction with the poly(A)- binding protein 1 (PABP1) facilitates the formation of a loop structure to attract the 60S ribosomal subunit to the complex. AGO proteins contain limited sequence

homology to the cap binding region of eIF4E, suggesting a competition of the initiation complex and miRISC. The involvement of the poly(A) tail in the repression activity of the miRNP complex is discussed controversially. While some studies described the requirement of both cap and tail structures (119), others revealed a tail-independent repression of translation (120). Another model of translational inhibition is based on an interrupted elongation process. In this so-called "drop-off" model, miRISC serves as a terminator for ribosomal elongation (121). Alternatively, mRNA degradation following destabilization through deadenylation or decapping is one proposed model for microRNA function (122). A key factor during the target decay is GW182 located in P-bodies previously connected to mRNA degradation. Regarding the fact that all models are experimentally validated, different effects of microRNAs on the translational repression are likely.

These aspects together with an imperfect base pairing sufficient to inhibit translation make it extremely challenging to predict functional microRNA targets. Therefore, specific features that are required for microRNA binding and may enhance the prediction accuracy have been defined: First, the "seed" region on positions 2-8 in the 5´ end of mature microRNAs has to pair perfectly to the target molecule (123). Second, mismatches (bulges) in the center of the RNA duplex prevent the argonaute-mediated cleavage. Third, the interaction is stabilized by base pairings in the 3´ end of the microRNA, where mismatches are tolerated. These criteria form the background for algorithms, predicting a functional microRNA:target interaction. Databases such as TargetScan (124) and miRBase (125) give an idea of how crucial microRNAs are in virtually all cellular mechanisms by targeting approximately 30% of all protein-coding genes. However, characterizing microRNAs as potent repressors in the 3´ UTR of mRNAs represents a too simplified view of its function. In addition to the mechanisms described above, microRNAs can act as repressors or activators (126, 127) of gene expression by binding to the 3´ and 5´ UTR (128, 129). Furthermore, microRNA can be transported into the nucleus (130) or even secreted (131) from the cell with as yet unknown functions.

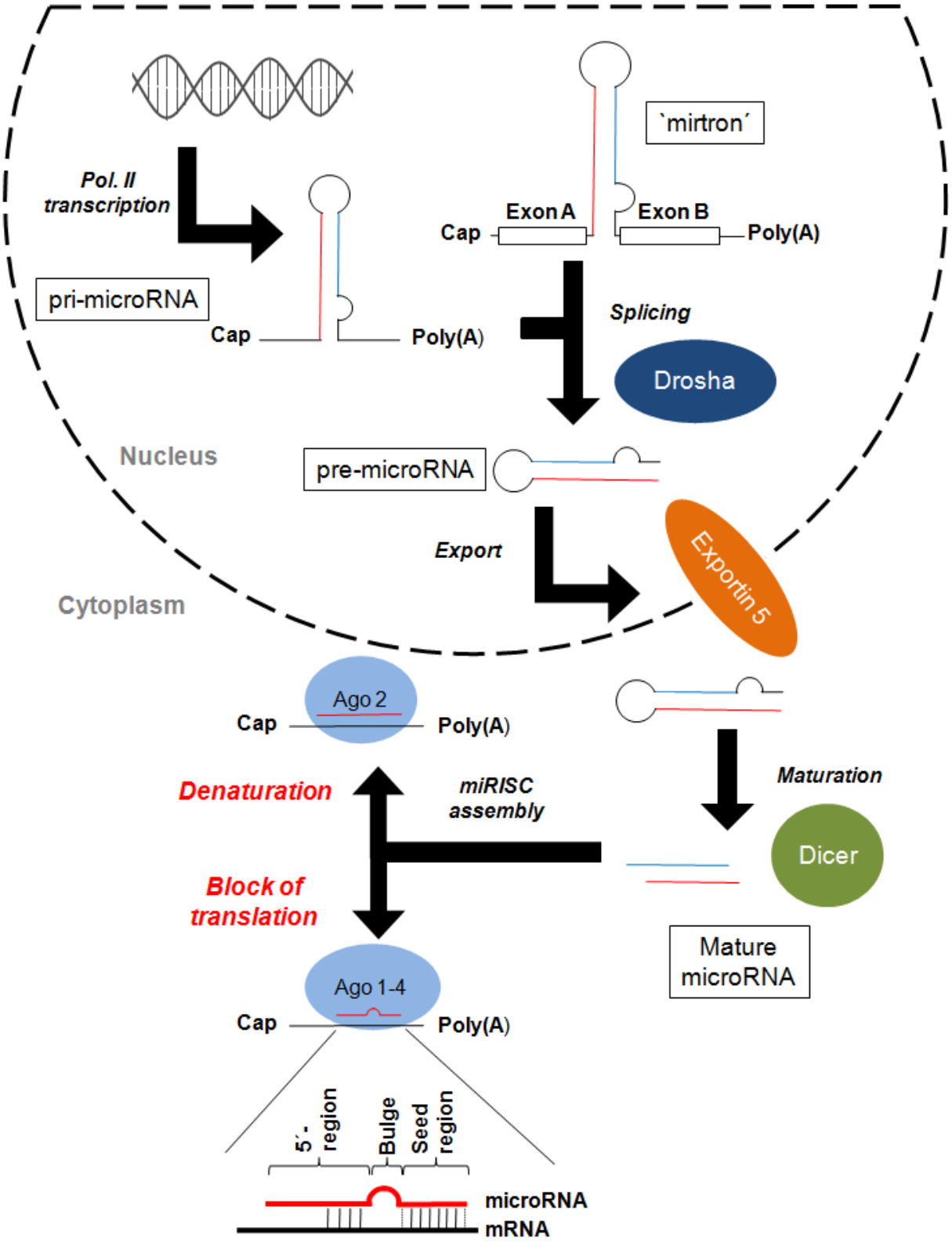


Figure 2: MicroRNA biogenesis in eukaryotic cells. MicroRNAs are transcribed as pri-microRNAs or are spliced from intronic gene regions. They are processed to mature oligonucleotides with a length of approximately 21 nt functioning as a translational repressor or leading to target degradation.

2.9 Control of microRNA expression and biogenesis

Among the 546 identified human microRNAs, some are broadly expressed in diverse tissue types or developing stages, suggesting a substantial role for the maintenance of the various cellular functions (132). In contrast, numerous microRNAs are expressed in a cell type- or timepoint-dependent manner. Their expression is associated with specific cell types, differentiation states or development stages, suggesting a distinct function in the cellular context. Furthermore, expression of single microRNAs could be directly linked to specific diseases and cancer types.

MicroRNA expression can be regulated in different steps, from transcription of the pri-microRNA up to biogenesis of the mature molecule. Global functional studies could link a number of processes to cancer formation. Here, the machinery responsible for microRNA processing could be connected to cancer progression. Cancer cells with impaired microRNA processing enzymes DGCR8, Drosha, Dicer, thus decreasing the overall level of mature molecules, revealed an increased tumorigenic potential in a mouse model system (133). Aberrant expression of Dicer was determined in a range of lung cancers (134) and is in concordance with a reduced global microRNA level in the cancer tissues compared to normal controls (135). As microRNAs are an important part of the post-transcriptional regulation process, it was surprising to find out that they themselves are post-transcriptionally controlled. Comparing the level of precursor molecules with that of mature microRNAs occasionally produced confusing results, since no linearity could be observed (136, 137). In one example, detailed analysis could link the stem cell protein Lin-28 to specifically regulate the microRNA Let-7 (138). Here, one model describes the degradation of the precursor molecule following uridylation of the 3´ terminus (139), another favors the impaired processing by binding of lin-28 to the loop-structure (140). A further example involves the mir-17-92 gene cluster, where the RNA-binding protein hnRNP A1 is required for processing of mir-18a but not the other microRNAs of the cluster. Considering the existence of 28 human hnRNPs, specific protein binding might be an important mechanism in microRNA biogenesis (141). Further post-transcriptional regulation is achieved by RNA editing of microRNAs. Here, an adenosine is converted to an inosine leading to blocked downstream processing (142) or differential target selection (143).

As is common for protein-coding genes, microRNAs are pre-transcriptionally regulated by activator and repressor molecules. MicroRNAs that are located in intronic regions of

protein-coding genes, representing approximately 25% of all microRNAs, are in general excluded from this mechanism (144). These microRNAs are predominantly expressed in correlation with their host genes without the requirement of further regulation. However, it was shown that DNA polymerase II also interacts with the proximal regions of intronic microRNAs, suggesting a host gene independent expression (145). The remaining number of microRNAs is located in intergenic regions, often clustering together in a multigene region transcribed simultaneously. Several studies determined their regulation to be closely associated with transcription factors binding to the 5´ region of the transcription start. For example, E2F was validated as an activator of the gene cluster *mir-17-92* representing an example for a feedback mechanism since -*vice versa*- the encoded microRNAs inhibit the transcription factor (146, 147). Furthermore, MYC was identified as a potent regulator of the gene cluster. In contrast, MYC also functions as a transcriptional repressor downregulating numerous targets by binding directly to their promoter regions (148). The repressed microRNAs were proven to exhibit tumor-suppressing features, suggesting MYC as a superior key molecule during cancer formation. Further molecules involved in apoptosis, proliferation and genomic stability have been described as transcriptional microRNA regulators. Here, p53, the key factor for genomic integrity, was validated to activate the expression of three miR-34 family members. ChIP experiments verified its binding to highly conserved sites in the proximal promoter region (149-151). Further examples of a specific activation of microRNAs are the regulation of miR-15b by the androgen receptor (152), the activation of miR-155 by NFkappaB (153), and the binding of Twist1 to the promoter of miR-10b (154). In summary, the regulation of microRNAs is defined as a multistep process involving numerous supporting factors. Interestingly, not only the proteins regulated by microRNAs but also microRNAs themselves could be linked to cancer formation and are discussed in the following chapter.

2.10 MicroRNAs involved in cancer

MicroRNAs are one of the largest class of gene regulators. With 546 different products they represent 1-4% of all expressed human genes (155). As regulators of approximately 30% of all protein-coding RNAs, microRNAs have a major influence on probably all important processes within the cell. Here, microRNAs regulate important cellular

pathways, e.g. proliferation, apoptosis, differentiation, and development. Therefore, they are crucial for a variety of different diseases including cancer (156). Single microRNAs are predicted to bind to different targets with a wide range of functions, suggesting that the small regulators have a key position for cellular homeostasis in the tissue environment (157).

The importance of microRNAs for the development of cancer is not only theoretical but is experimentally established. Expression analysis from various tumor types revealed an aberrant expression profile compared to normal control samples (158). Narrowing down the function to specific targets mediating the tumorigenic effect of microRNAs, oncogenes and tumor suppressors were validated as direct or indirect targets. Here, entire pathways important for the cellular fate are affected by single microRNAs (159). Genomic analysis further supported their tumorigenic potential by linking their genomic locations to fragile sites of the human genome that have been previously associated with cancer (160).

The direct or indirect association of microRNAs with key factors or pathways involved in cancer development and progression led to the nomenclature as “oncomirs” (161). In this context, the *mir-17-92* gene cluster is probably the most popular one combining all attributes described above. The gene cluster encodes for a polycistronic pri-microRNA, harboring sequences for the six mature microRNAs miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR92-1. The cluster is located in the third intron of the primary transcript of the gene *C13orf25* (162). Since this transcript is predominantly conserved in the microRNA-coding sequences, an exclusive function for microRNA transcription is suggested. Amplification of the genomic locus 13q31.4 is a common event in several types of lymphomas and diverse solid tumors and is accompanied by an increased *C13orf25* gene product and its associated microRNAs (163). Upregulation of the pri-microRNA miR-17-92 is observed in 65% of B-cell lymphomas, suggesting a direct contribution to cancer formation (164). Surprisingly, loss-of-heterozygosity of the locus has been observed for breast and ovarian cancers and melanomas. Detailed analysis revealed a suppression of the *AIB1* gene, accompanied by a decreased proliferation rate following overexpression of the cluster in breast cancer cells (165). These and previously described conflicting results demonstrate a dual role of the *mir-17-92* gene cluster in the formation of cancer. Depending on the cellular background and tissue type, it functions as an oncogene or tumor suppressor gene. The *mir-17-92* cluster is a good example of how microRNAs are differentially expressed in cancer, thereby influencing downstream targets involved in

crucial processes that lead to tumor formation. Their misregulation can be due to genomic alterations or varying expression of transcription factors.

Up to now, numerous examples of microRNAs associated with cancer development by deregulating key molecules have been described (166-168). Some microRNAs represent key molecules themselves by regulating multiple factors of specific pathways involved in cancer. Here, microRNA let-7, strongly associated with lung cancer with poor prognosis (169), not only directly regulates the oncogene *RAS* but also crucial members of the associated pathway (170). Another example indicates miR-21, upregulated in most human cancers (163), as a key factor involved in p53, TGF-ß, and apoptotic signaling by targeting numerous molecules of the pathway (159).

2.11 MicroRNAs as diagnostic tools and therapeutic targets

The aim of basic microRNA research is not only an improved understanding of the post-transcriptional regulation, but also the transfer of knowledge to develop new clinical approaches. Diagnosis, prognosis and therapy are crucial fields where microRNAs deliver new tools to advance treatment. Along with the characterization of novel cancer-specific microRNAs, a multitude of new targets for therapeutic approaches appear. Since microRNAs function as "oncomirs", specific downregulated or restored expression might achieve therapeutic effects in cancer cells. Here, problems arise that were already present in classical gene therapy and RNAi therapeutics. An effective therapeutic agent has to be specific and efficient to successfully regulate its target without causing severe side effects. Delivery of the construct remains another critical task of the treatment. These challenges are addressed by a number of fast-forward studies transferring modulation of microRNA expression from the cell culture to *in vivo* applications.

The development of potent microRNA inhibitors presents the basis for future studies (171). The so-called "antagomirs" are designed as complementary constructs to the mature microRNA sequence. Their delivery in significant amounts into the cytoplasm blocks the endogenous microRNA function by specifically and stably binding to the targeted molecules. Upregulation of mature microRNAs can be achieved by delivering sense oligonucleotides into the cells, which mimic the endogenous function. These modifying

approaches can be performed transiently with oligonucleotides or stably by using specific expression vectors.

One of the first *in vivo* applications used chemically modified "antagomirs" to downregulate single microRNAs in mice (172). Intravenous administration of the constructs resulted in a downregulation of the targeted microRNA in various tissues. The biological relevance was validated by analyzing downstream effects of the treatment. In detail, inhibition of the endogenous miR-122 resulted in downregulation of specific mRNAs enriched with recognition sites for miR-122. In concordance with the association of miR-122 to cholesterol biosynthesis, the plasma level of cholesterol was affected by the treatment. In a continuative study, chemically modified "antagomirs" for miR-122 were injected into primates (173). Here, the constructs blocked endogenous microRNA miR-122 and reduced the cholesterol level in hepatocytes. A similar technique was performed in an *in vivo* mouse model using reintroduction of Let-7 as treatment of KRAS-dependent lung cancers. The delivery of Let-7, a direct regulator of KRAS expression, resulted in reduced lung tumor growth in treated mice (174). These and other studies (175, 176) suggest microRNA-mediated therapy as a promising technique for the future treatment of cancer. Identification of potential targets is a crucial prerequisite for targeted therapy and is therefore addressed in this work.

However, besides the development of a specific therapy, cancer diagnostics has benefited from the emerging field of microRNA research. Expression analysis of diverse tumor types revealed a unique profile, clustering in distinct subgroups representing tumor origin, prognosis, and response to specific treatments. Determination of the expression level of approximately 200 microRNAs is sufficient to accurately classify human cancers (158). In contrast, this could not be achieved by a parallel analysis of 16,000 protein-coding genes. Applying this question to various cancer types and normal tissues, microRNAs may offer a high potential in tumor diagnosis (177-180). Highly relevant for the patient and for defining new therapeutic strategies is the evaluation of cancer-free survival. Several studies, screening high numbers of cancer specimens, present single or groups of microRNAs as a reliable factor for cancer prognosis (181). For example, the reduced expression of Let-7 serves as an indicator for shortened post-operative survival in lung cancer (182). High expression of miR-21 is associated with advanced clinical stage and poor prognosis (183). Another critical point in cancer therapy is the response to specific treatments. MicroRNAs were proven as reliable prediction factor of certain therapies. Here, in some cases, the microRNA expression status could be directly correlated to the

therapeutic response. Overexpression of Let-7 for example abolishes radiation-induced cell death (184). Radiation therapy for lung cancer patients might therefore be a promising treatment solely for tumors with a reduced Let-7 expression level. Another study identified miR-221 and miR-222 as potential indicators for cancer cell resistance against tamoxifen (185, 186). Summarizing, microRNAs might be a potent target for specific cancer therapies and an important indicator for cancer diagnosis, prognosis and therapeutic stratagies.

3 Aim of the study

Mutations in the breast cancer susceptibility gene *BRCA1* are predominantly associated with inherited breast cancers with only marginal appearance in the sporadic form. Although, a decreased expression of *BRCA1* was detected in the sporadic form, the underlying mechanisms remain undefined and are therefore the subject of this study.

Here, we focus on the decipherment of the complex network regulating *BRCA1*, representing a promising candidate to be deregulated in sporadic breast cancer. The transcriptional activators ERα, IGF1R, AhR, and SP1, as well as the dominant negative repressor ID4, were previously described as powerful regulators of *BRCA1*, with additional involvement in mitogenic signaling pathways in breast tissue. Deregulation of individual or multiple components of this network might therefore promote cancer formation by repressing *BRCA1* as a key molecule for the genomic stability and by activating growth-promoting signal cascades.

In this study, the traditional view of signaling cascades is extended to post-transcriptional control mechanisms mediated by small regulatory RNAs. The so-called microRNAs could previously be identified as regulators of crucial cellular functions by controlling key molecules, such as oncogenes or tumor suppressor genes. Furthermore, their deregulation was determined for a variety of cancers including breast cancer.

The aim of this study is to verify the importance of microRNA function for *BRCA1* regulation and its validation as key component within the network with a superior role for breast tumor formation. To identify microRNAs with crucial regulatory function, this study focuses on the identification of microRNAs with multiple targets in the regulatory cascade of *BRCA1*. As the transcriptional regulators ERα, IGF1R, AhR, SP1, and ID4 harbor multiple predicted binding sites for diverse microRNAs, a post-transcriptional control can be speculated and is therefore subject of this study.

A detailed characterization of microRNA function and targets might lead to an improved understanding of breast cancer development and might be important for future diagnostics and therapy.

4 Material and Methods

4.1 Cell culture and modification

The cell lines used in this study were maintained in their individual growth media in 10 cm cell culture dishes (Thermo Fisher Scientific, Rochester, NY, USA) in a CO_2 incubator (Binder, Tuttlingen, Germany). Medium was changed every 48 h and cells were split after reaching approximately 90% confluence using trypsin EDTA (0.5%) (Biochrom, Berlin, Germany).

4.1.1 Cell culture

MCF7 cells were obtained from ATCC (LGC Prochem, Wesel, Germany) and maintained in MEM (PAA Laboratories, Pasching, Austria), supplemented with 10% FCS (Gibco, Carlsbad, CA, USA), 1x sodium pyruvate (Sigma, St. Louis, MO, USA), 1x nonessential amino acids (Gibco), and 1% penicillin/streptomycin (PAA). HCC1937 cells were obtained from DSMZ (Braunschweig, Germany) and maintained in RPMI (Biochrom), supplemented with 10% FCS and 1% penicillin/streptomycin. HeLa and PA-1 cells (both LGC Prochem) were maintained in DMEM (PAA), supplemented with 10% FCS, 1x nonessential amino acids, 1x L-glutamine (PAA), and 1x penicillin/streptomycin. MiaPaCa2, Panc1, ASPC1 and Capan1 cells (all LGC Prochem) were maintained in DMEM supplemented with 10% FCS, 1x L-glutamine, and 1x penicillin/streptomycin. SK-BR-3 and T47D cells (both LGC Prochem) were cultured in McCoy`s 5A modified medium (Biochrom) supplemented with 10% FCS, 1x sodium pyruvate, and 1x penicillin/streptomycin. HBL100 cells (LGC Prochem) were maintained in McCoy`s 5A modified medium supplemented with 10% FCS and 1x penicillin/streptomycin. HEK293 cells (LGC Prochem) were maintained in DMEM, supplemented with 10% FCS, 1x L-glutamine, 1x sodium pyruvate, and 1x penicillin/streptomycin. Cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere.

4.1.2 5-Aza treatment

For the promoter methylation studies, 1 x 10^5 cancer cells were subcultured onto one well of a 6-well plate (Sarstedt, Nümbrecht, Germany) for 24 h and subsequently treated with 2 µM 5-Aza-2´-deoxycytidine (Sigma) for 72 h (media change every 24 h), followed by incubation in normal growth media for 24 h.

4.1.3 Estradiol stimulation

For hormonal stimulation, 4 x 10^4 MCF7 cells were subcultured onto one well of a 24-well plate (Sarstedt) for 24 h, followed by a cell starvation step in phenol-red and serum free DMEM for 48h. Subsequently, cells were treated with 10 nM ß-estradiol (Sigma) or an equal volume of ethanol (control) for 24 h.

4.1.4 Oligonucleotides and plasmids

In this study, different experimental setups required varying types of expression-modifying constructs. Overexpression of specific genes or *in vitro* transcriptions were performed using eukaryotic expression plasmids carrying the full-length mRNA or solely the coding sequence of the gene of interest. The expression of reporter genes was performed using an expression vector for the green fluorescent protein (GFP) or the firefly luciferase protein. For knockdown experiments mediated by transcript degradation, siRNAs were used. The overexpression of microRNAs was achieved by transfecting modified oligonucleotides with sequence identity to their endogenous counterparts. Antisense molecules, here termed inhibitors, blocking the endogenous microRNAs were transfected for microRNA-silencing experiments.

4.1.4.1 microRNAs

Overexpression and knockdown of the microRNAs miR-203 and miR-335 were performed using miScript microRNA Mimics (Qiagen, Hilden, Germany) or miScript microRNA Inhibitors (Qiagen), respectively. The negative control oligonucleotides for both constructs were also purchased from Qiagen.

For protein and cell cycle analysis, 1 x 10^5 MCF7 cells were seeded onto one well of a 24-well plate by simultaneous reverse transfection with 50 nM miScript microRNA Mimics or control oligonucleotides using 3 µl HiPerfect (Qiagen) according to the manufacturer`s protocol in a total volume of 500 µl normal growth medium. 26 h or 48 h later, the cells were collected by trypsinization.

To assess the proliferation rate and apoptotic activity, 7.5 x 10^3 MCF7 cells or 1 x10^4 HCC1937 cells were seeded onto one well of a 96-well plate (Sarstedt). Simultaneous reverse transfection with 50 nM miScript microRNA Mimics or control oligonucleotides was carried out using 0.75 µl HiPerfect following the manufacturer`s instructions in a total volume of 50 µl growth medium. 24 h after transfection, 50 µl culture medium were added to MCF7 cells or a medium change was performed after 6 h for HCC1937 cells.

4.1.4.2 siRNAs

Knockdown experiments for *SP1* were performed using siRNA oligonucleotides purchased from Qiagen. For the control setup, the AllStars Negative Control siRNA (Qiagen) was transfected. For specific silencing of *SP1*, the siRNA Hs_SP1_5 HP (5´-CAGCAAGTTCTGACAGGACTA-3´) was used. 1 x 10^5 MCF7 cells were subcultured onto one well of a 24-well plate for 24 h. Transfections were performed with 5 nM siRNA using 3 µl HiPerfect in a total volume of 500 µl. 48 h after transfection, cells were collected by trypsinization,

The silencing oligonucleotides specific for *ID4* (#1: 5´-GGAAAGGAAAAAACAUCGGtt-3´, #3: 5´-GGUGCAGUUAAACUUUUAAtt-3`) were purchased from Ambion (Austin, TX, USA) and were co-transfected with an *LNGFR* expression plasmid (Miltenyi Biotec, Bergisch Gladbach, Germany) to enrich for transfectants. The experiments were analyzed relative to cells transfected with Silencer Select Negative Control siRNA (Ambion).

4.1.4.3 Plasmids

For the construction of pcDNA3-NRAS-Full or pcDNA3-NRAS-ORF, the full-length mRNA or the ORF of *NRAS* was extracted from the clone IRAUp969B1241D (Imagenes, Berlin, Germany) with *Sfi*I (NEB, Ipswich, MA, USA) or *Sfi*I/*Dra*I (NEB), respectively.

Following blunt ending using the T4 DNA polymerase (NEB), the fragments were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA). The plasmid pcDNA3-ID4-ORF was created by subcloning *ID4* from pLPC-ID4 (46) in pcDNA3 using *Eco*RI (NEB) and *Not*I (NEB). The 3´ UTR of *ID4* was amplified by PCR: FF 5´-GTGAACAAGCCGCGGGACAGCATTCTGTG-3´; RV 5´-TTTGAATTCCAAGACAGAGAAATCTACTTTAATATTCAC-3´ (Operon, Huntsville, AL, USA), digested with *Not*I and *Xba*I (NEB), and subcloned into the *Not*I-*Xba*I-digested pcDNA-ID4-ORF to create pcDNA-ID4-Full.

For the cloning of multiple MREs in the luciferase reporter vector pGL3 (Promega, Madison, WI, USA) a polylinker was introduced at the *Xba*I site to create pGL3-MCS (Operon, Tab. 1). The MREs of *ESR1*, *AhR*, *IGF1R*, *SP1* and *ID4* were amplified with specific primers (Operon, Tab. 1) using the AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) and subcloned into pGL3-MCS using *Xba*I or *Sac*II (NEB) and *Eco*RI (NEB).

The expression plasmid for the surface marker LNGFR was obtained from Miltenyi Biotec.

Tab. 1: Oligonucleotide sequences for the cloning of the polylinker (pGL3-MCS) or the amplification of the diverse MREs in the 3´ UTRs of ESR1, AhR, IGF1R, SP1 and ID4. The restriction sites used for cloning into pGL3 (*Eco*RI: GAATTC; *Sac*II: CCGCGG; *Xba*I: TCTAGA) are indicated in bold letters.

	MRE	No. MRE	Primer forward	Primer reverse
pGL3-MCS	-	-	GACC**TCTAGA**CGAT**CCGCGG**TGAC**GAATTC**CAGT**TCTAGA**CGAG	CTCG**TCTAGA**ACTG**GAATTC**GTCA**CCGCGG**ATCG**TCTAGA**GGTC
ESR1	203	1	GAAGCAC**TCTAGA**TTGTTTAAGAAGCACC	GGC**TCTAGA**TGGTATTACATCGTCTAGTCC
	335	1	TAA**CCGCGG**CCATGTTCCAAACCCATCGTC	GTT**GAATTC**TTTATTTGAACATCAAATAGGTTGAG
AhR	203	1	CCGT**CCGCGG**CCAGACCTTTTCCTGATTTG	GGG**GAATTC**ATAAGAATCCAGTAGCTCAAAAC
	203	2	CC**TCTAGA**TCCTGGGTTCAAGTGATTC	GCCTATTTTCC**TCTAGA**CAATTTATCCAG
	203	3	GAC**TCTAGA**TGTTGCTATGTGCCTTATG	GTAT**CTAGA**GCCCCAACAAATTAAGTGC
IGF1R	203	1+2	GTC**CCGCGG**CTTTCTCTAGTCTCTATCCC	CCAG**GAATTC**TCTGGTGCCCTCGTTGGCC
	203	3	CTATC**CGCGG**TTCTAGCCTAACTTCATGC	CATC**GAATTC**AGTGTGATCTTGCCACAGAG
	203	4	GCTC**CGCGG**CAAGATCACACTGAGATCG	CACT**GAATTC**TCCAAATGACGCAACCAGTC
	203	5	CTC**CGCGG**TATCTCTATCATGGGAAACACC	CAT**GAATTC**CTTTACTAGAAAAAAGCAGAG
	335	1	GTAC**CGCGG**TCTCCCTGGTCCTGCTGCTCAC	GGA**GAATTC**CACAACTCAGCTTAAGATTATTTGG
SP1	203	1	AC**TCTAGA**CAATAGTTCACTCCATTTGGTCC	GC**TCTAGA**CTGCTGGGAGACAAAAGAAGC
	335	1	GCCCTAAGT**TCTAGA**TAAGTATCAGGGGAA	GGT**CTAGA**CTGGTTATTGCCAGTGTTAAGAGAG
ID4	203/335	1	CAT**CTAGA**ACTGCTGTGAATTGTACATTTC	GCAAGACC**TCTAGA**TATAACTGTTACTTCCG

4.1.4.4 Co-transfections

For protein analysis of ID4, 2 x 10^5 MCF7 cells were seeded onto one well of a 24-well plate by simultaneous reverse transfection with 20 nM miScript microRNA Inhibitors or control oligonucleotides and 0.75 µg plasmid DNA using 3 µl HiPerfect in a total volume of 500 µl growth medium. After 12 h, the cells were collected by trypsinization.

For the rescue experiment, 0.3 µg of plasmid DNA was co-transfected with 50 nM miScript microRNA Mimics using 0.75 µl HiPerfect by simultaneously seeding 7.5 x 10^3 MCF7 cells onto one well of a 96-well plate in a total volume of 50 µl growth medium. As a control experiment, an empty expression vector and control oligonucleotides were transfected. 24 h after transfection, 50 µl culture medium was added.

For luciferase assays 8 x 10^3 HEK293 cells were seeded onto one well of a 96-well plates and grown for 24 h. Subsequently, 25 ng of the pGl3 constructs were co-transfected with 3 ng of a renilla luciferase plasmid (pGl-4.7), 50 nM miScript microRNA Mimics or control oligonucleotides and 222 ng MIGR1 plasmid using 0.5 µl Lipofectamine 2000 (Invitrogen) according to the manufacturer`s protocol. Cells were incubated for 24 h before determination of the luciferase activity.

For the enrichment of cells transfected with siRNA specific for ID4, an expression plasmid for the truncated surface receptor LNGFR was co-transfected simultaneously with the oligonucleotides. Therefore, 5 x 10^5 MCF7 cells were plated onto one well of a 6-well plate 24 h prior to the transfection. Transfections were performed using the CalPhos Mammalian Transfection Kit (BD, Franklin Lakes, NJ, USA) following the manufacturer's instructions. Cells were transfected with a negative control siRNA or two independent *ID4*-specific siRNAs at a concentration of 150 nM. To enrich transfectants, cells were cotransfected with 3 µg *LNGFR* expression plasmid DNA. Transfections were carried out in OptiMEM (Gibco) supplemented with 5% FCS for 14 h and stopped by adding MEM with 10% FCS after washing once with 1x PBS. Subsequently, cells were selected using the MACSelect LNGFR Transfected Cell Selection Kit (Miltenyi Biotec) following the manufacturer's instructions. Separation efficiency was assessed by FACS analysis with MACSelect control antibodies (Miltenyi Biotec) in a FACSCalibur Flow Cytometer (BD).

4.2 Patient material

Sporadic breast tumors were randomly selected from the tumor bank of the Institute of Pathology at Hannover Medical School. For quantitative analysis, fresh frozen specimens from forty-one post-menopausal patients with a median age of 66 years were used. These samples were all invasive ductal carcinomas with six tumors having a G2 and thirty-five a G3 grade of differentiation. For microRNA expression analysis only G3 tumors were used. Eight breast tissues from healthy women undergoing breast reduction surgery were taken as control samples. The study was authorized by the Ethics Committee of Hannover Medical School.

4.2.1 Clinical features

Following surgery, sporadic breast tumors are analyzed routinely for their expression status of Her-2, the estrogen and the progesterone receptor by immunohistochemistry. Furthermore, proximal lymph nodes are analyzed for the occurrence of metastatic cells. These data and the age of the patient were assessed by a detailed search in an internal database of Hannover Medical School and are authorized by the Institute of Pathology.

4.3 MicroRNA co-precipitation

The interactions between microRNAs and mRNAs on a cellular background were detected by microRNA co-precipitation. The gene of interest was transcribed in the presence of biotin-labeled UTPs, introduced into HeLa cells and specifically rescued using avidin-agarose beads.

4.3.1 *In vitro* transcription

For *in vitro* transcription, plasmids pcDNA3-NRAS-Full and -ORF were linearized overnight with *Xho*I. The plasmids pcDNA3-ID4-Full and pcDNA3-ID4-ORF were

linearized with *Xba*I or *Not*I, respectively. The control plasmid pcDNA3-GFP was digested with *Xho*I prior to transcription. DNAs were ethanol-precipitated (2 vol. ethanol, 1/20 vol. 0.5M EDTA, 1/10 vol. 3M NaAc) and washed once with 70% ethanol. Dried pellets were resuspended in RNase-free water. The transcription was performed using the mMESSAGE mMACHINE Transcription kit (Ambion) and 1 mM Bio-16-UTP (Ambion) following the manufacturer´s instructions. Transcripts were further processed using the Poly(A)-Tailing kit (Ambion). After transcription, the RNAs were extracted using the RNeasy Mini kit (Qiagen) and quantified photometrically. The quality was determined using a Bioanalyzer (Agilent, Santa Clara, CA, USA).

4.3.2 mRNA transfection

4 x 10^5 HeLa cells were seeded onto one well of a 6-well plate and cultured overnight. For transfection, 6 µl of HiPerfect were preincubated with 100 µl OptiMEM for 5 min, followed by the addition of 3 µg mRNA and a further 10 min of incubation at room temperature. The pre-seeded cells were washed once with 1x PBS (PAA) and cultured in 900 µl OptiMEM prior to transfection. The transfection-RNA mix was added dropwise and the cells were cultured for 24 h. To determine the translation efficiency and the stability of the labeled mRNA, transfected cells were cultured for 48 h, trypsinized, and analyzed in FACSCalibur Flow Cytometer.

4.3.3 Co-precipitation

For co-precipitation, cells were lysed as described in 4.3.5. The RIPA buffer was supplemented with 100 U/ml RNaseOUT (Invitrogen). Samples were sonified five times for 15 sec each on ice, followed by centrifugation for 15 min at 13,200 x g and 4°C. The supernatant was precleared with proteinA-agarose beads (Roche, Mannheim, Germany) for 1 h on ice. Avidin-agarose beads (Pierce, Rockford, IL, USA) were washed three times in cold RIPA buffer and blocked for 1 h on ice with 1% BSA (Serva, Heidelberg, Germany) and 0.2 mg/ml carrier RNA (Qiagen). Subsequently, the beads were washed once with RIPA buffer. The precleared lysate was supplemented to final concentrations of 500 mM NaCl, 0.1 mg/ml carrier RNA and 0.1% BSA, followed by the addition of 50 µl avidin beads. After an incubation of 2 h rocking on ice, the beads were pelleted and washed five

times with RIPA buffer (including 500 mM NaCl, proteinase inhibitors and 10 U/ml RNaseOUT) for 5 min each rocking on ice. After changing the reaction cup, beads were washed three times in 0.1 x SSC for 5 min rocking at room temperature. Finally, the total RNA was extracted using the miRNeasy Mini kit (Qiagen) following the manufacturer´s instructions.

4.4 Expression analysis

Expression analysis was performed for specific genes on both the transcript and protein level. The data for mRNA expression were normalized to the housekeeping gene *GAPDH*, whereas the protein amount was normalized to the ubiquitously expressed structural protein ß-actin. As microRNAs are small RNAs with a number of processing steps, the expression level of mature microRNAs was detected and normalized to the small nuclear RNA U6 (sn-U6).

4.4.1 Microdissection

For the analysis of mRNA expression in cancer samples, all tissues were microdissected prior to RNA extraction. To determine the expression of microRNAs, all samples with a tumor fraction of less than 80% were microdissected prior to RNA extraction. In both studies, epithelial breast cells from control tissues were enriched using microdissection. Therefore, frozen tissues were cut in 6 µm cryosections, mounted on pre-cooled membrane slides and immediately frozen on dry ice. After dehydration in 70% ethanol, sections were stained on ice using 1% cresyl violet (Aldrich, Munich, Germany). After additional dehydration steps, sections were air-dried. Samples for *ID4* and *BRCA1* analysis were isolated using a PALM MicroBeam (P.A.L.M. Microlaser Technologies GmbH, Bernried, Germany), whereas the Cellcut Smart (Olympus, Hamburg, Germany) was used for the microRNA experiments.

4.4.2 RNA isolation

For the expression analysis of microRNAs from methylation experiments, RNA was isolated using 500 µl Trizol (Invitrogen) according to the manufacturer`s instructions. MicroRNAs from primary tissue, cancer cell line experiments, and co-precipitations were extracted using the miRNeasy Mini Kit (Qiagen). For microdissected samples, carrier RNA (Qiagen) was added during the isolation steps. MessengerRNAs from microdissected material were isolated using the RNeasy Micro Kit (Qiagen) as described in the manufacturer's instructions. RNA was quantified on a spectrophotometer (Implen, Munich, Germany).

4.4.3 Reverse transcription

To identify the expression levels of mRNAs, the reverse transcription reactions were performed as described by the manufacturer using SuperScriptII Reverse Transcriptase (Invitrogen) and random hexamer primers (MWG, Ebersberg, Germany). For the amplification of 3´ UTR fragments the RNA of various cell lines was pooled and reverse transcribed using oligo-d(T) primers (Invitrogen). Prior to the reaction, samples were DNaseI digested using the RNase-free DNase Set (Qiagen). For reverse transcription of microRNAs, the specific primers (Applied Biosystems) for the analyzed microRNAs were pooled in one reaction. Reactions were performed using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer´s instructions. For microdissected and co-precipitated material, the maximum volume of RNA solution was applied during the process, replacing the water in the reaction.

4.4.4 Quantitative real-time PCR (qRT-PCR)

For quantitative analysis of microRNAs, the Taqman microRNA assays (Applied Biosystems) were used following the manufacturer´s instructions with some modifications for microdissected and precipitated samples. To maximize sensitivity, the maximum amount of sample volume was used in the reaction.

For the quantitative analysis of protein-coding genes, specific primers (Tibmolbiol, Berlin, Germany) and FAM/TAMRA-labeled Taqman probes (Tibmolbiol) were used. For

BRCA1 detection, the primers (FF:5`-CTGCTCAGGGCTATCCTCTCA-3`; REV:5`-TGCTGGAGCTTTATCAGGTTATGT-3`) and the probe (5`-TGACATTTTAACCACTCAGCAGAGGGATACCA-3`) were used. For *ID4* detection, the primers (FF:5`-CGCTGTCCAGGTGTGCG-3`; REV:5`-GGCTTTTTTTCTCTAACTTCTGCTCTT-3`) and the probe (5`-CTGAGCCCGAGCCAGGAGCA-3`) were used. For *SP1* detection, the primers (FF:5`-GCCTCCAGACCATTAACCTCA- 3`; REV:5`-AGCCCCTTCCTTCACTGTCTT- 3`) and the probe (5`-CAAATGCCCCAGGTGATCATGGA-3`) were used.

The qRT- PCR was performed using the qRT-PCR Core Kit (Eurogentec, Seraing, Belgium) with a reaction volume of 20 µl for microRNA and 25 µl for mRNA detection, running on an I-Cycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). After an initial denaturation step at 95°C for 10 min, reactions were run for 50 cycles (95°C for 15 sec; 60°C for 1 min). Expression data were assessed in two independent reactions performed as doublets and are given as mean values +/- SD. The co-precipitation experiments were analyzed in triplets and are displayed as mean values +/- SD from three independent experiments.

To detect the reference gene *GAPDH*, the Human *GAPDH* Endogenous Control Assay (Applied Biosystems) was used according to the manufacturer´s instructions.

4.4.5 Protein isolation

For the isolation of total proteins from cell culture experiments, the cells were trypsinized, washed once with cold 1x PBS, and lysed in 100 µl 1x RIPA buffer (10 mM Tris, pH: 7.5; 150 mM NaCl; 1% NP40, 0.5% sodium deoxycholate; 0.1% SDS; 1mM EDTA) supplemented with proteinase inhibitors (1 mM PMSF, 2.8 µg/ml aprotinin, 1.46 µM pepstatin A, 16.8 µM leupetin (all: Sigma)). Lysates were incubated for 1 h on ice and cleared by centrifugation at 13,200 x g at 4°C for 20 min. Proteins from primary tissue were extracted using the Cell Grinding kit (Amersham, Little Chalfont, UK) following the manufacturer`s instructions.

4.4.6 Western blotting

Proteins from cell culture experiments were separated on a 10% SDS-PAGE (Invitrogen) and transferred onto a nitrocellulose membrane (Invitrogen) using the iBlot Gel Transfer Device (Invitrogen). Total cell lysates from primary tissues were separated on a 10% SDS-PAGE (Nunc, Wiesbaden, Germany). Proteins were blotted onto a nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany) using a semi-dry blotting system (Biometra, Göttingen, Germany). SP1 (Upstate, Billerica, MA, USA), ERα (SP1; Neomarkers, Fremont, CA, USA) were diluted and incubated 1:5000 in 5% NFDM. c-MYC (N-262; Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) was diluted and incubated 1:500 in 5% NFDM. AhR (H-211, Santa Cruz), IGF1R (C20; Santa Cruz) and ID4 (H70; Santa Cruz) were diluted 1:250 and incubated in 3% Slim Fast (Unilever, Hamburg, Germany). Samples were normalized to ß-actin (MP Biomedicals, Solon, OH), diluted 1:5000 in 5% NFDM. Detection was performed using Lumigen TMA-6 (Amersham). Signal intensities were scanned and quantified with ImageJ software (NIH, Bethesda, MA, USA).

4.4.7 Isolation of genomic DNA and MSP

For the promoter methylation studies, cancer cells were treated with 5-Aza as described in 4.1.2, trypsinized, and washed once in 1 x PBS. Fresh frozen primary tissues were sliced into small fragments and processed immediately. To isolate the genomic DNA, cell pellets or tissue materials were incubated in proteinase K buffer (50 mM Tris, pH:7.6; 25 mM EDTA; 0.5% Igepal; 0.5% SDS) supplemented with 0.5 mg/ml proteinase K (Merck, Darmstadt, Germany) for 4 h at 55°C. To ensure complete lyses, the solution was vortexed frequently. After the addition of 1.7 M NaCl, the solution was cleared by centrifugation. Samples were RNaseA (20 µg/ml) (Qiagen) digested and DNA was precipitated by adding 0.7 vol. isopropanol. DNA was pelleted, washed with 70% ethanol, airdried and dissolved in water.

Genomic DNA was converted using the Epitec Bisulfite kit (Qiagen) following the manufacturer`s instructions. Methylation-specific PCR was performed with specific primers (FF: 5´-GAGTATTTTCGGTTTAGACGAGAC-3´; RV: 5´-GAAAATAACCCTAACTCAACGAC-3´, Operon), at 56°C using the AmpliTaq Gold polymerase. Unmethylation-specific PCR was performed under the same conditions with

the specific primers (FF: 5´- GAGTATTTTTGGTTTAGATGAGATGG-3´ and RV: 5´-CAAAAATAACCCTAACTCAACAAC-3´, Operon).

4.5 Functional analysis

The effect of the overexpression or knockdown of certain genes or microRNAs was assessed by functional assays determining the viability, the caspase activity or the cell cycle status of the cells. The functionality of a microRNA:mRNA interaction was validated by luciferase reporter assays. Therefore, the cells were treated as described in 4.1.4.

4.5.1 Viability assay

To determine the amount of vital cells on days one to three after transfection, 1/10 vol. of WST-1 reagent (Roche) was added to the wells and incubated at room temperature. WST-1 is processed in the mitochondria of vital cells into a product with altered absorbance properties. After 1 h, the absorbance is measurement at 460 nm for the specific product and 600 nm to determine the background in a microplate autoreader (BioTek, Winooski, VT, USA). All assays were performed in triplicate and repeated at least twice. Data are given as mean ± SD.

4.5.2 Apoptotic assay

The rate of apoptosis induced by different treatments was assessed by using the caspase activity as an indicator of programmed cell death on days one to three after transfection. Therefore, the activities of caspase 3 and 7 were determined by the addition of Caspase 3/7 Glo substrate (Promega) to the wells according to the manufacturer`s instructions. Pro-caspases are cleaved to their active form, if apoptosis is induced. The substrate is processed into a luciferase substrate by active caspases, which is enzymatically converted to measurable bioluminescence. 1 h after addition of the substrate, the lysates were transferred to white-walled 96-well plates (Greiner Bio-one, Frickenhausen, Germany) and the relative light units were measured in a luminometer (Lumat; Berthold, Bad Wildbad,

Germany). All assays were performed in triplicate and repeated twice. Data are given as mean ± SD.

4.5.3 Cell cycle analysis

For cell cycle analysis, cells were collected 48 h after transfection by trypsinization, washed once with cold 1x PBS, and resuspended in 200 µl 1x PBS. After the addition of 800 µl ice-cold absolute ethanol, cells were stored at -20°C. For analysis, cells were collected by centrifugation, resuspended in 1 ml 1x PBS supplemented with 0.1% Triton X-100 (Sigma) and 1 µg/ml RNaseA (Qiagen), and incubated for 30 min at 4°C. After the addition of 1 ml of a 50 µg/ml propidium iodide solution and incubation for 30 min at 4°C, the cellular DNA content was measured by FACS cytometry in a FACS Calibur and was evaluated using CellQuest Pro software (BD). Experiments were repeated three times and data are given as mean ± SD.

4.5.4 Luciferase assay

To assess the luciferase activity, the Dual-Glo luciferase assay (Promega) was performed 24 h after transfection, following the manufacturer´s instructions, with the following time intervals. The firefly luciferase activity was measured 10 min after addition of its specific substrates. After the renilla luciferase substrate was added, the reaction was incubated for another 10 min, followed by the determination of the renilla activity. Measurements were performed in a Synergy 2 (BioTek). Luciferase experiments were repeated at least in three independent experiments and samples were measured as triplets. Data are given as mean ± SD.

4.6 Statistics

Statistical analysis was carried out using the Mann-Whitney U test, Student´s t test or Pearson´s correlation using GraphPad software (San Diego, CA, USA). Values of $p < 0.05$

were considered as significant. The expression data of primary cancer samples are displayed as a box plot with lower quartile (25%), median (gray bar) and upper quartile (75%) values. Whiskers extend from each end of the box to the adjacent values in the data; by default, the most extreme values within 1.5 times the interquartile range from the ends of the box.

5 Results

5.1 MicroRNAs control the regulatory cascade of *BRCA1*

Bioinformatic analysis (TargetScan 4.2 software (124)) was used to identify microRNAs with predicted response elements (MRE) that occured in the 3´ UTR of genes involved in the regulatory cascade of *BRCA1*. Most frequently, miR-203 was predicted to post-transcriptionally regulate the cascade by controlling *AhR*, *ESR1* (protein: ERα), *IGF1R*, *ID4*, and *SP1*. With several binding sites in the 3´ UTR of *IGF1R* and *AhR*, miR-203 had a high probability to regulate the expression of the predicted targets (Fig. 3). As a binding site was also predicted for *ESR1*, *ID4* and *SP1*, a superior function of miR-203 in an upstream pathway of *BRCA1* could be speculated. A second microRNA with predicted binding sites occurring numerous times in the 3´ UTR of the analyzed genes was miR-335. Therefore, the significance of this microRNA was also evaluated in this study. With the exception of *AhR*, microRNA miR-335 revealed binding sites in all analyzed regulators of *BRCA1*.

The binding probability was not only associated with the number of binding sites within a 3´ UTR, but was also influenced by different criteria improving the success of the prediction. Context scores, respecting the effect of various aspects of microRNA binding, were included in TargetScan 4.2 software. These criteria considered the seed region complementarity, the nucleotide composition near the site, the proximity to sites for coexpressed miRNAs leading to a cooperative action, the proximity to residues pairing to miRNA nucleotides 13-16, and the positioning within the 3´ UTR (124). They resulted in the context score, presented as increasing probability with decreasing values. In the cascade of *BRCA1* regulation, miR-203 revealed high context scores for *AhR* and *ID4*, intermediate values for *IGF1R* and *ESR1* and low values for *SP1* (Tab. 2). MicroRNA miR-335 showed intermediate score values for *SP1*, *ESR1*, and *ID4* (Tab. 2). The binding affinity of miR-335 to *IGF1R* revealed only low context scores, whereas this microRNA is not predicted in the 3´ UTR of *AhR*.

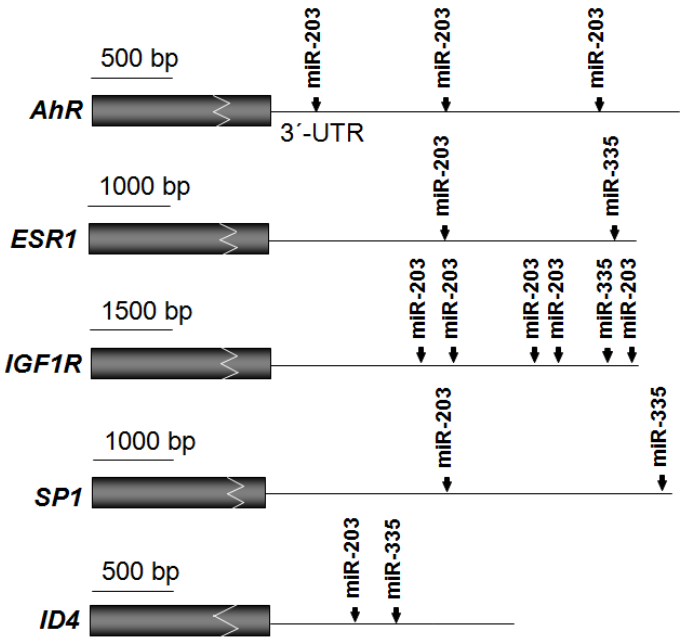


Figure 3: MicroRNAs miR-203 and miR-335 were predicted to regulate the expression of *AhR, ERa, IGF1R, SP1,* and *ID4*. The schematic overview displays the predicted microRNA response elements of miR-203 and miR-335 in the 3´ UTRs of *AhR*, *ESR1*, *IGF1R*, *SP1* and *ID4*. The gray boxes represent the coding sequence of the respective gene, whereas the arrows map the specific position of the microRNA binding site. The 3´ UTRs are displayed in the indicated magnifications.

Tab. 2: MicroRNA binding prediction was improved by considering different criteria. The context score values representing the probability of a microRNA:mRNA interaction were assessed using TargetScan 4.2 software. The values display the sum of different prediction criteria, with a score of -1 representing the highest likelihood of a functional target match.

	Context score	
Gene name	**miR-203**	**miR-335**
SP1	-0.04	-0.29
AhR	-0.81	-
IGF1R	-0.14	-0.08
ESR1	-0.18	-0.28
ID4	-0.57	-0.35

Since the 546 identified microRNAs differ in their specificity to distinct tissue types, the expression status of the analyzed microRNAs miR-203 and miR-335 was determined for breast (MCF7, SK-BR-3, HCC1937, HBL100, T47D), pancreas (Capan1, ASPC1, MiaPaCa2, Panc1), cervix (HeLa) and ovarian (PA-1) cancer cell lines (Fig. 4). Except for the breast cancer cell line T47D, miR-335 was ubiquitously expressed in all tested cell lines. Notably, two of the pancreatic cell lines (MiaPaCa2 and Capan1) revealed a low expression level, whereas the breast cancer cell line SK-BR-3 showed the highest miR-335 expression. For miR-203, there was only a very weak or no expression detectable in all pancreatic cell lines and PA-1. HeLa and HBL100 cells revealed moderate expression levels. Interestingly, four out of five breast cancer cell lines showed high levels of miR-203 expression, suggesting a specific function of the microRNA in this tissue. For subsequent analysis, the cell lines were assigned to three subgroups representing varying levels of expression. The three groups were termed high, moderate and low, indicating expression levels more than 10-fold higher, similar, or more than 10-fold smaller than the reference cell line HeLa, respectively.

A **B**

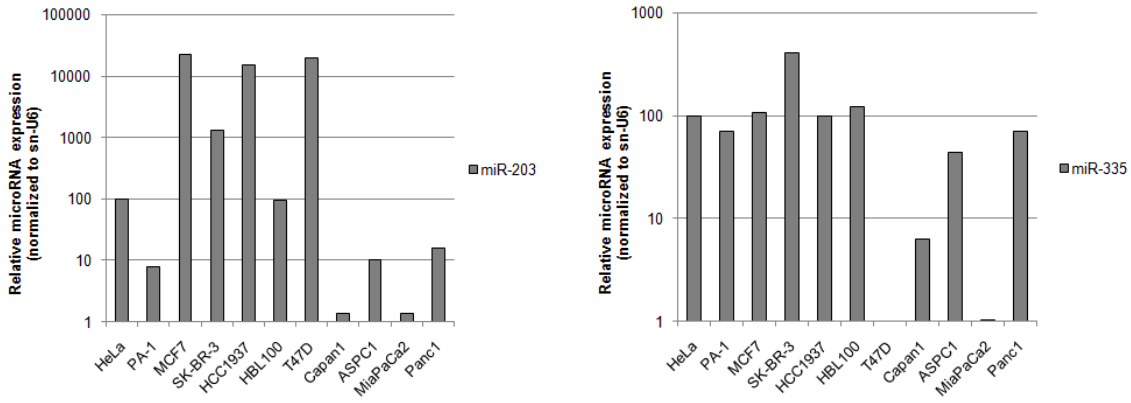


Fig. 4: MicroRNAs miR-203 and miR-335 revealed varying expression levels in human cancer cell lines. The expression level of miR-203 (A) and miR-335 (B) was determined for various human cancer cell lines from different tissue types. The expression was normalized to small nuclear RNA U6 (sn-U6) and is displayed relative to HeLa cells.

To determine the functional effect of miR-203 and miR-335 on the *BRCA1* regulating factors, their protein expression was analyzed by Western blotting following overexpression of the microRNAs. 48 h after transfection of mature microRNA miR-203,

MCF7 cells revealed a decreased amount of protein for the targets ERα (-93%), AhR (-73%) and IGF1R (-62%) compared to control-miR treated cells (Fig. 5). The protein expression of SP1 was slightly upregulated following treatment (+44%). To exclude a secondary effect caused by an altered cell cycle, apoptosis or proliferation status, c-MYC as a key regulator for the cellular fate was also detected. Revealing no change in the expression of c-MYC, the regulatory effect of miR-203 was specific to the genes analyzed.

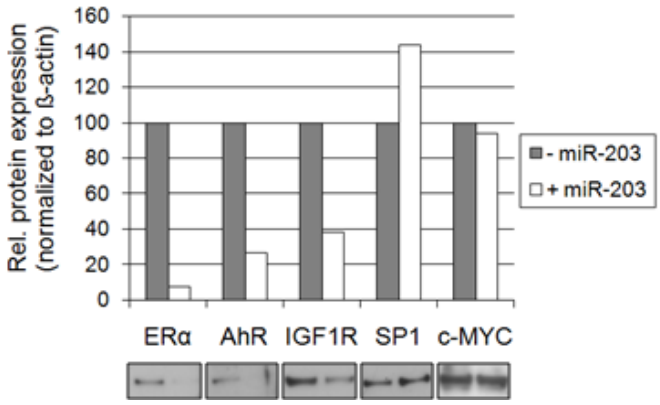


Fig. 5: MicroRNA miR-203 regulated the expression of ER*a*, AhR and IGF1R. The expression levels of the *BRCA1* regulating factors were assessed by Western blotting following overexpression of the microRNA miR-203 for 48 h in MCF7 cells. The band intensity was quantified and normalized to ß-actin. The results are displayed as one representative Western blot and as protein expression values relative to control-miR transfected cells.

To determine the effect of miR-335 overexpression, an earlier timepoint was used to avoid unspecific effects caused by induction of apoptosis. Therefore, protein analysis was performed 26 h following transfection of the mature microRNAs. The protein amounts of ERα (-55%), AhR (-32%), IGF1R (-91%), and SP1 (-74%) were reduced as a consequence of overexpression of miR-335 compared to the control-miR transfected cells (Fig. 6). At that timepoint, microRNA overexpression had no effect on the expression of c-MYC.

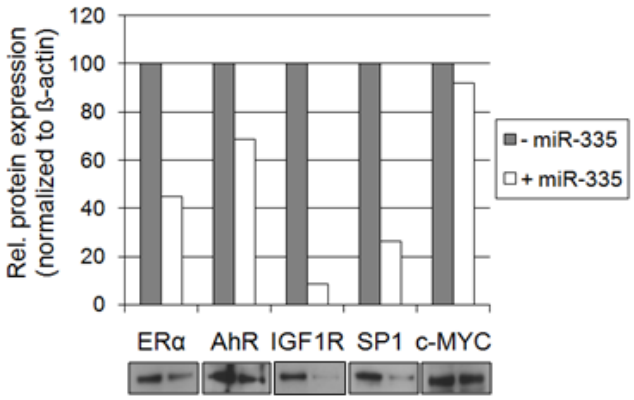


Fig. 6: Overexpression of miR-335 reduced the expression of ER*a*, AhR, IGF1R and SP1. Protein amounts of the indicated factors were determined by Western blotting 26 h after transfection of mature microRNA miR-335. The band intensities were quantified and normalized to ß-actin. The results are displayed as one representative Western blot and as protein expression values relative to control-miR transfected cells.

5.2 MicroRNA co-precipitation enabled the detection of predicted microRNA:mRNA interactions

Interactions between microRNAs and their specific targets can currently be detected by standard protein analysis or reporter gene assays. However, protein quantification using Western blotting or immunohistochemistry (IHC) are limited to the availability of specific antibodies. Furthermore, these methods cannot distinguish between primary and secondary targets. Reporter assays on the other hand specifically determine the activity of certain microRNAs for one gene, but do not consider naturally occurring modifications, e.g. mRNA folding. Therefore, the microRNA co-precipitation technique was developed as a tool to directly and specifically validate microRNA:mRNA interactions on a cellular background (Fig. 7). For the specific precipitation, the gene of interest was transcribed *in vitro* in the presence of biotin-labeled UTPs. The resulting tagged mRNAs were introduced into cells where endogenous microRNAs specifically bound to the bait molecule. These complexes were isolated using avidin-linked agarose beads incubated with the cell lysate. The specific isolation of labeled mRNAs was followed by a subsequent quantitative analysis.

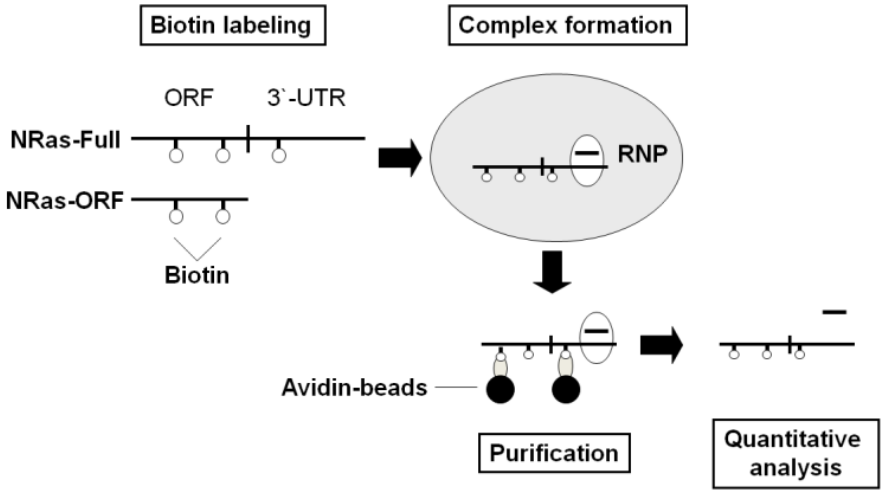


Fig. 7: MicroRNA:mRNA interactions could be detected by specific precipitation. The schematic overview of the microRNA co-precipitation technique presents a method with four subsequent steps. The *in vitro* transcription of the gene of interest with simultaneous biotin-labeling was followed by an introduction into a cellular system, leading to a complex formation with microRNAs. The endogenous microRNAs guided by RNPs were co-purified with the tagged mRNA using avidin-agarose beads. The isolated RNAs were quantified by qRT-PCR. Results were normalized to control constructs lacking the 3´ UTR.

Prior to microRNA co-precipitation, the effect of biotin-labeled UTPs on the *in vitro* transcription reaction and the translation efficiency in a cellular system was determined. Therefore, the reporter gene *GFP* was transcribed in the presence or absence of labeled UTP. The integrity of the resulting mRNA was analyzed on a Bioanalyzer revealing transcripts of the expected length and no detection of incomplete transcription products (Fig. 8). The efficiency of intracellular translation was assessed after transfection of the transcripts into HeLa cells. Both labeled and unlabeled mRNAs showed similar signal intensities for GFP 48 h after transfection, suggesting that the biotin-UTP had no influence on translation efficiency or stability of the mRNA (Fig. 9).

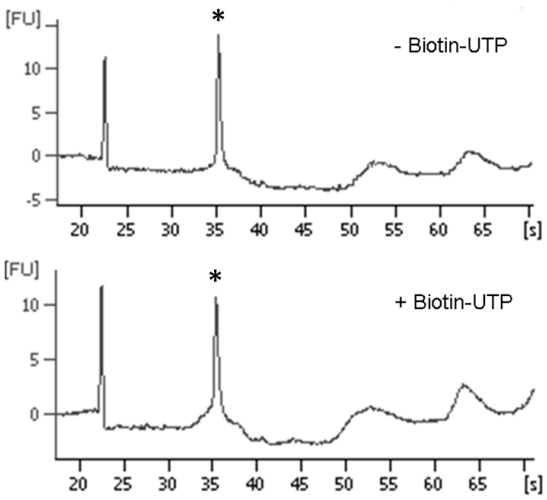


Fig. 8: The quality of the *in vitro* transcribed mRNA was not influenced by labeled UTPs. The coding sequence of *GFP* was cloned in a T7 RNA polymerase transcription vector and *in vitro* transcribed in the presence and absence of biotin-labeled dUTPs. The integrity of the mRNA was assessed using a Bioanalyzer and is displayed as a chromatogram. Completely processed transcripts are indicated (*).

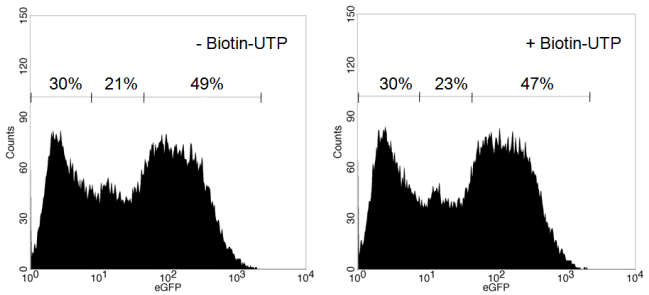


Fig. 9: The integration of biotin-labeled UTPs had no influence on mRNA stability or translation efficiency. Labeled or unlabeled mRNA of *GFP* was transfected into HeLa cells. The signal intensity was assessed by FACS analysis 48 h following transfection. The data are displayed as histograms with the indicated cell numbers representing different magnitudes of signal intensity.

5.2.1 MicroRNA Let-7 co-immunoprecipitated with *NRAS*

To verify a co-precipitation of associated microRNAs with a labeled mRNA, the published interaction between the oncogene *NRAS* and the microRNA Let-7 (166, 187) was used as a

positive control in a proof of principle. Two variants of *NRAS* were cloned and transcribed, one of which contained the coding sequence and the 3´ UTR including three predicted binding sites for Let-7 (Fig. 10), whereas the other contained only the coding sequence without the 3´ UTR and therefore served as a negative control.

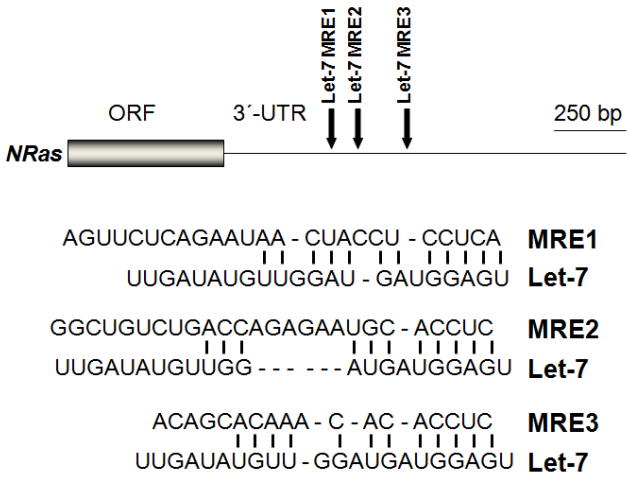


Fig. 10: MicroRNA Let-7 was predicted to bind to the 3´ UTR of the oncogene *NRAS*. According to Johnson et al. (166), the mRNA of *NRAS* harbors three active binding sites for the microRNA Let-7, located in the 3´ UTR, downstream of the coding sequence of the oncogene. The predicted Watson-Crick base bindings of Let-7 and *NRAS* are displayed.

Both transcripts were transfected into HeLa cells, predetermined to express microRNA Let-7, and rescued after 24 h using avidin-linked agarose beads. Both constructs were successfully extracted from the cell lysates at similar amounts as demonstrated by quantitative real-time PCR (Fig. 11), whereas avidin beads incubated with a lysate from untreated cells failed to isolate *NRAS*. Quantitative real-time PCR of microRNA Let-7 following microRNA co-precipitation resulted in a significantly higher signal in the full-length *NRAS* mRNA rescue compared to the ORF control.

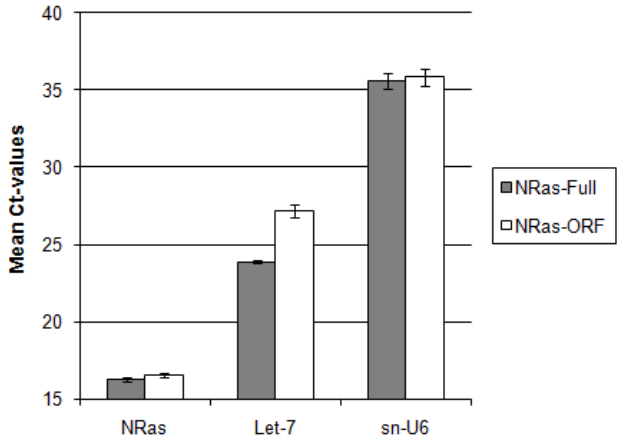


Fig. 11: MicroRNA Let-7 could be specifically co-precipitated with full-length *NRAS* mRNA. Following transfection of the labeled full-length *NRAS* mRNA (NRAS-Full) or the control lacking the 3´ UTR (NRAS-ORF), constructs were purified after 24 h. The amount of isolated *NRAS* mRNA, its associated microRNA Let-7 or unspecifically bound sn-U6 was determined by qRT-PCR. The results are displayed as Ct-values following amplification.

Quantification of the isolated microRNA, precipitated by the *NRAS* full-length mRNA, resulted in a 7.6-fold increased amount of Let-7 after normalization to the purified amount of *NRAS* ($p < 0.05$; Fig. 12A). To exclude the effect of altered unspecific binding capacity based on longer bait molecules or breakage products, unspecific bound small nuclear RNA U6 (sn-U6) was used for normalization in parallel. At low-level, unspecific precipitation of sn-U6 was found for both constructs (Fig. 11). However, no specific enrichment of sn-U6 could be detected in one of the variants. Normalizing the enriched microRNA Let-7 to sn-U6 or *NRAS* revealed comparable results (Fig. 12B). In this proof-of-principle experiment, the novel precipitation technique was able to specifically enrich microRNA Let-7 binding to *NRAS* at a significant amount above the background.

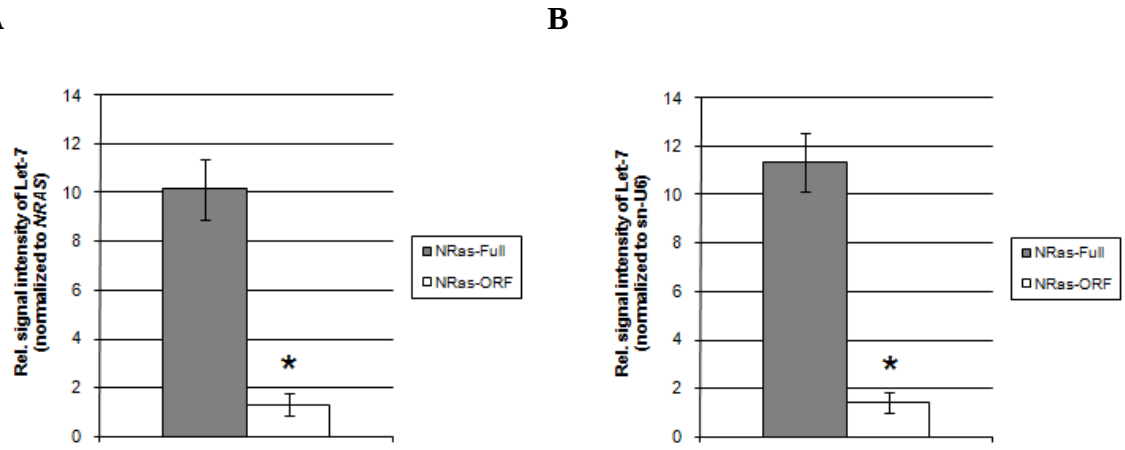


Figure 12: Normalizing the level of specific enriched microRNA Let-7 revealed a signal intensity significantly above the background. (A) Quantification of co-precipitated Let-7 normalized to the amount of isolated *NRAS*. (B) Quantification of isolated Let-7 normalized to the sn-U6. P-values < 0.05 are indicated (*).

5.2.2 MicroRNA miR-335 bound to *ID4* mRNA

The influence of a specific microRNA on the expression of ID4 could not be detected by standard protein analysis, since there was no antibody available that showed adequate sensitivity to quantify endogenous ID4 protein. Therefore, the microRNA co-precipitation technique was used to verify its part in the regulatory network involving miR-203 and miR-335. Both microRNAs are predicted to bind to the 3´ UTR of *ID4* (Fig. 3) and were expressed in HeLa cells (Fig. 4).

The full-length mRNA of *ID4* was transcribed in the presence of biotin-labeled UTPs. In parallel, a truncated *ID4* mRNA lacking the complete 3´ UTR sequence served as a negative control. The transcripts were transfected into HeLa cells and extracted from cell lysates using avidin-linked agarose-beads. After co-precipitation of labeled *ID4*, the amount of co-purified microRNAs was quantified. The sn-U6 and microRNA Let-7 were used as controls for unspecific binding of small RNAs. After normalizing the data of each microRNA to the negative control precipitation (ID4-ORF), the signal intensity was compared to both control RNAs (Fig. 13). Here, miR-335 was particularly enriched by co-precipitation with the full-length *ID4* transcript, whereas miR-203 had signal intensities comparable to the two control RNAs (Let-7 and sn-U6). The enrichment of miR-335 reached statistical significance ($p < 0.05$) in comparison to sn-U6 (2.3-fold) and microRNA

Let-7 (2.6-fold), clearly suggesting an interaction with the ID4 3´ UTR in the cellular system.

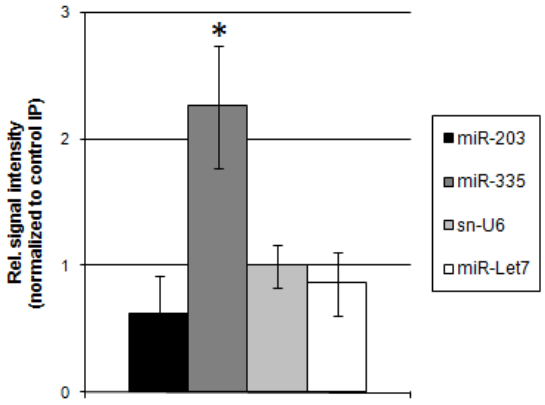


Fig. 13: MicroRNA miR-335 specifically bound to the 3´ UTR of *ID4*. Quantification of selected microRNAs and controls after precipitation of labeled *ID4* mRNA. The signal was normalized to a truncated negative control transcript lacking the 3´ UTR (ID4-ORF) and is displayed relative to sn-U6. P-values < 0.05 are indicated (*).

For validation, the full-length mRNA of *ID4* was overexpressed in MCF7 cells in the presence of antisense oligonucleotides directed against miR-335. ID4 protein expression was detected by Western blot analysis followed by quantification of the band density (Fig. 14). According to the previous experiments, the expression of ID4 could be increased when endogenous microRNA miR-335 was blocked. The specificity was approved by co-transfection experiments using an ID4-ORF control plasmid. As expected, no increased expression was detected for the negative control, suggesting that the post-translational regulation is specifically mediated through elements in the 3´ UTR of *ID4*.

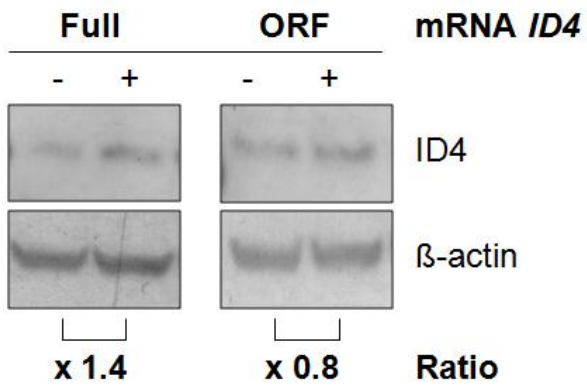


Fig. 14: Inhibition of miR-335 increased levels of exogenously expressed ID4. Western blot analysis after co-transfection of full-length (ID4-Full) or control (ID4-ORF) *ID4* expressing plasmids with antisense oligonucleotides inhibiting endogenous miR-335 (+) or control molecules (-). A representative immunoreaction is displayed for ID4 and ß-actin. The ratios represent the level of the differential expression of ID4 after transfection with anti-miR-335 or control molecules. Ratios are mean values normalized to ß-actin.

5.3 Reporter assays demonstrated direct microRNA:target interaction

To further validate the impact of the microRNAs miR-203 and miR-335 on the expression of the single components in the regulatory cascade of *BRCA1*, reporter assays were performed. The luciferase reporter system enabled the identification of direct microRNA:mRNA interactions and allowed the identification of active MREs in the genes of interest. Therefore 500 bp-fragments flanking the microRNA binding sites in the 3´ UTRs of *ESR1*, *AhR*, *IGF1R*, *SP1*, and *ID4* were amplified and cloned downstream of the luciferase reporter gene. Here, overexpression of miR-203 led to a significant decreased luciferase expression of *AhR* (-12%), *IGF1R* (-10%), and *SP1* (-15%) reporter constructs in HEK293 cells (Fig. 15A). For 3´ UTRs harboring more than one MRE, the most active site is displayed. The reporter activity of *ESR1* and *ID4* reporter plasmids were unaffected by the microRNA miR-203. Following overexpression of miR-335, the *ESR1* (-20%), *IGF1R* (-18%), and *SP1* (-18%) reporter constructs exhibited reduced luciferase

expression (Fig. 15B). In contrast to previous results the *ID4* reporter plasmid activity remained unchanged after co-expression of the miR-335.

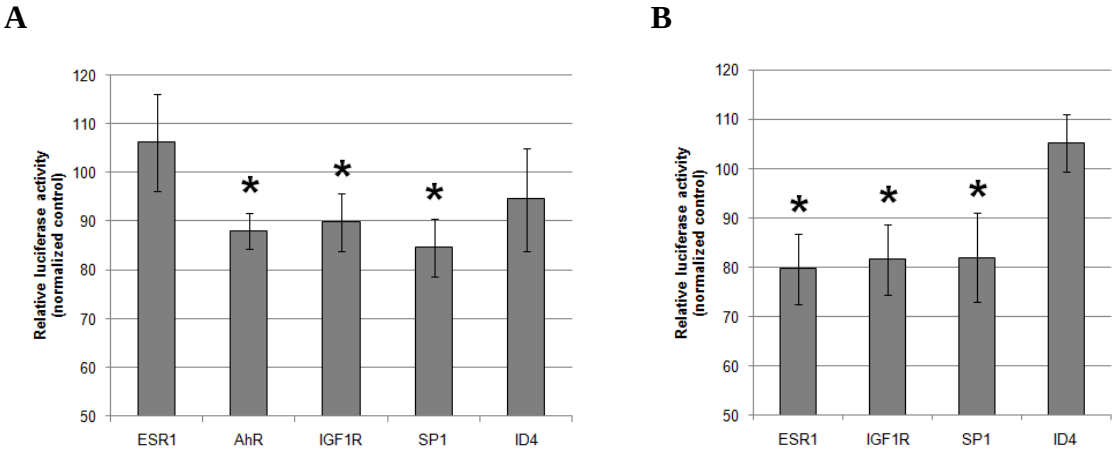


Fig. 15: Overexpression of miR-203 and miR-335 led a decreased reporter gene activity. Luciferase assays following co-transfection of mature microRNA miR-203 (A) or miR-335 (B) and reporter constructs harboring the respective microRNA target sites of *ESR1*, *AhR*, *IGF1R*, *SP1*, and *ID4*. The results represent mean values normalized to control-miR transfected cells and are displayed relative to a control plasmid lacking MREs. P-values < 0.05 (compared with control transfections) are indicated (*).

5.4 The microRNAs miR-203 and miR-335 influenced the expression of *BRCA1*

AhR, ERα, IGF1R and SP1 were all known regulators of the breast cancer susceptibility gene *BRCA1*. To determine the influence of miR-203 and miR-335 on *BRCA1*, the endogenous expression was assessed following transfection of MCF7 cells with mature microRNAs. Since the regulation of *BRCA1* is a secondary target effect of the microRNA activity, expression was analyzed up to 72 h after transfection. Due to the apoptosis-inducing effect of miR-335, the expression level was measured after 24 h and 48 h following transfection. Whereas overexpression of miR-203 led to an approximately 50% decreased expression of *BRCA1* (Fig 16A), miR-335 caused a profound upregulation of the cancer susceptibility gene, reaching a maximum of approximately 3-fold (Fig. 16B)

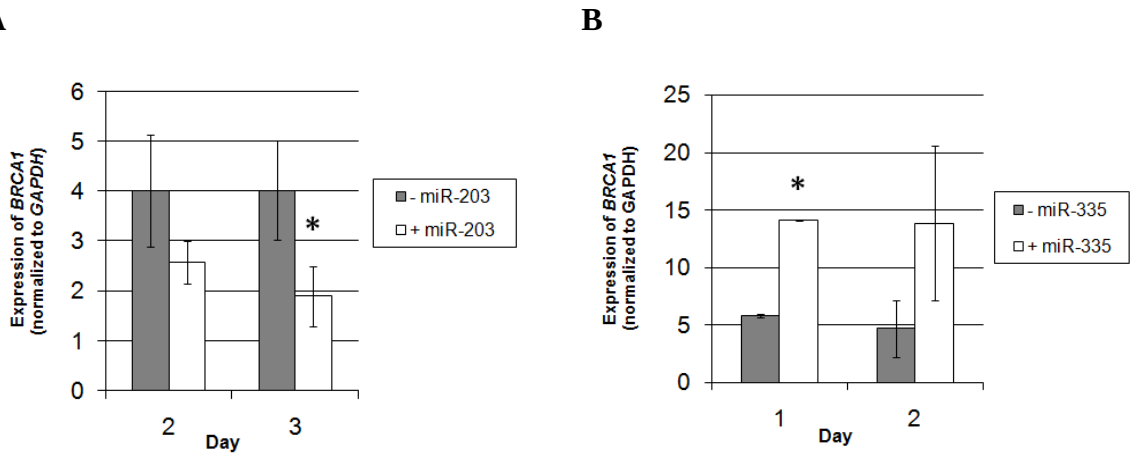


Fig. 16: The microRNAs miR-203 and miR-335 had an impact on the expression of *BRCA1*. The endogenous expression level of *BRCA1* was quantified by qRT-PCR at indicated time points following overexpression of the microRNA miR-203 (A) and miR-335 (B). The results represent the absolute expression levels normalized to *GAPDH*. P-values < 0.05 (compared with control transfections) are indicated (*).

5.5 MiR-203 and miR-335 influenced cellular behavior and fate

Since the previously identified targets of the microRNA miR-203 and miR-335 were all involved in important cellular mechanisms like growth, apoptosis and the regulation of the tumor susceptibility gene *BRCA1*, the effects of overexpressing both microRNAs in MCF7 or the *BRCA1* deficient cell line HCC1937 cells were analyzed. Two days after transfection with mature microRNAs, both cell lines were phenotypically changed compared to the control-miR transfections.

5.5.1 MiR-203 induced apoptosis and decelerated growth

MCF7 cells overexpressing miR-203 rounded up, without losing attachment to the well surface. Detailed analysis revealed a reduction of cell viability to 63% and 71%, on day three following overexpression of miR-203 in MCF7 or HCC1937 cells, respectively (Fig. 17A). In the case of HCC1937 cells, this could be traced back to an increased rate of

apoptosis (186%), whereas the caspase activity in MCF7 cells only slightly increased to 124% (Fig. 17B). Analysis of the cell cycle status of MCF7 cells overexpressing miR-203 revealed a significantly ($p > 0.05$) increased amount of cells in the G0/G1 phase (65%) compared to the control (57%) (Fig. 17C), suggesting a reduced proliferation rate due to a decelerated cell cycle.

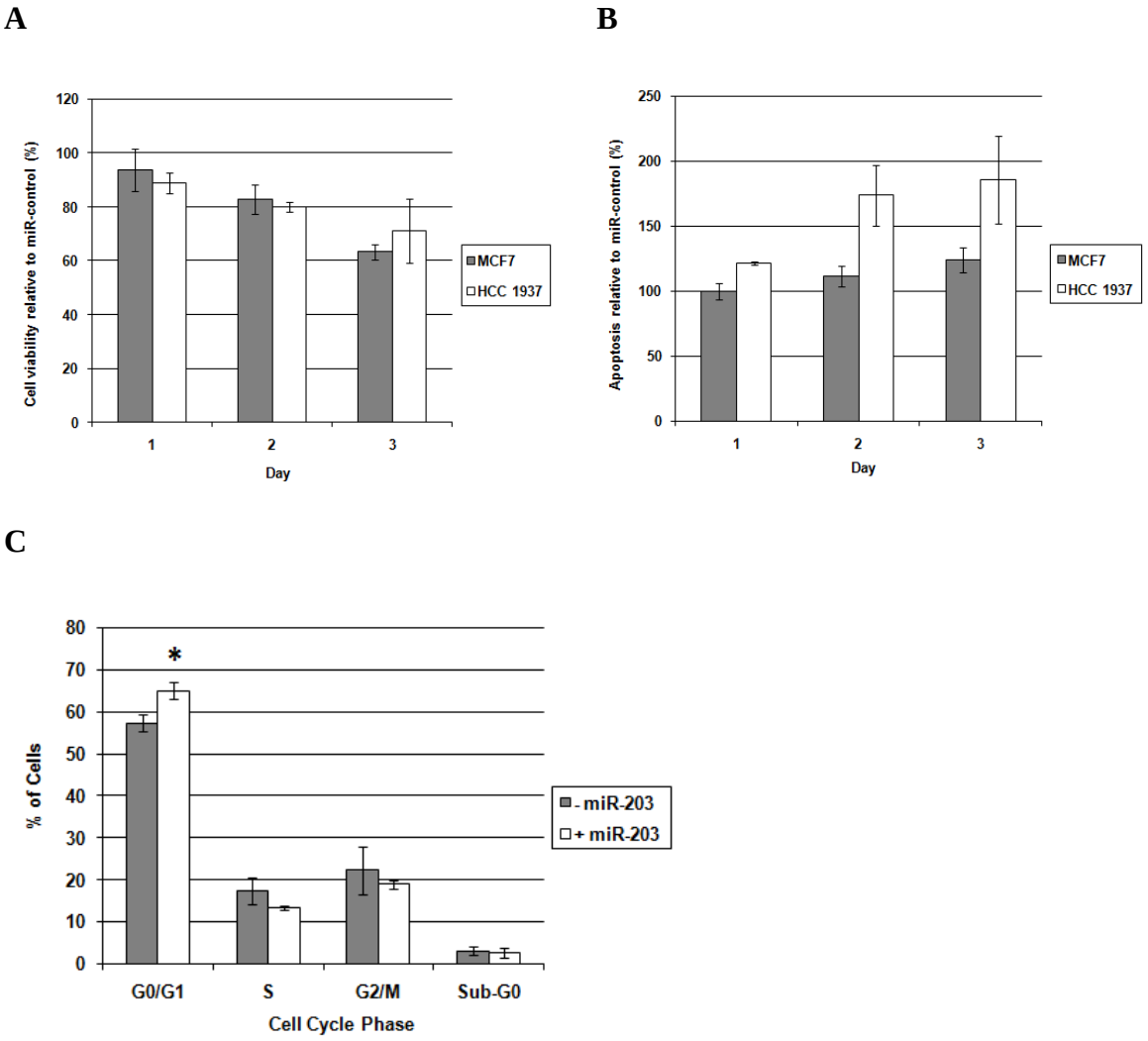


Fig. 17: Overexpression of miR-203 resulted in decreased cell viability, increased apoptosis or decelerated cell cycle. (A) The viability of MCF7 and HCC1937 cells was determined up to three days following overexpression of miR-203. The data were assessed using the WST-1 assay and are displayed relative to control-miR transfected cells. (B) The induction of apoptosis was determined by quantification of the caspase 3 and 7 activities up to three days after transfection of mature miR-203 in MCF7 and HCC1937 cells. Data are normalized to the cell number and are displayed relative to control-miR transfected

cells. (C) The cell cycle status was determined in MCF7 cells overexpressing miR-203 after 48 h. The cell cycle phases and the apoptotic fraction (Sub-G0) of transfected cells were determined by FACS analysis following propidium iodide staining and are displayed relative to control-miR transfected cells. Significance was determined by comparison to control transfected cells. P-values < 0.05 are indicated (*).

5.5.2 MiR-335 induced apoptosis in cancer cells

Cells overexpressing miR-335 showed distinct signs of cell death (Fig. 18). Overexpressing miR-335 reduced the viability to 35% for MCF7 and to 53% for HCC1937 cells on day three following transfection (Fig. 19A). This was accompanied by a maximum caspase activity of 350% and 298% respectively compared to control-miR treated cells (Fig. 19B). These results were verified by FACS analysis following propidium iodide staining, showing a significantly higher amount of fragmented DNA (22% in sub-G0 fraction) of MCF7 cells overexpressing miR-335 compared to control transfected cells (4%) (Fig. 19C). These results were accompanied by a simultaneously decreasing G2/M fraction (23% vs. 9%).

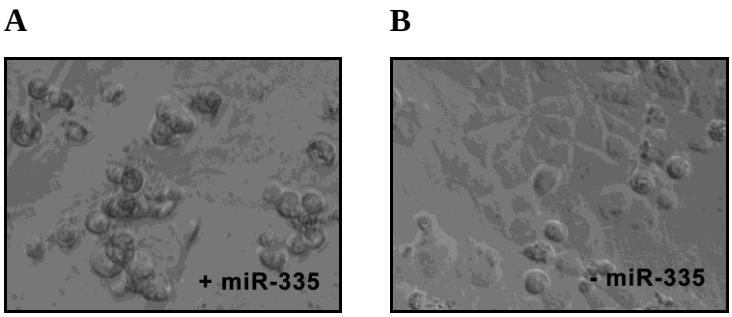


Fig. 18: Overexpression of miR-335 phenotypically changed the MCF7 cells. Displayed are MCF7 cells 48 h following transfection of mature microRNA miR-335 (A) or control-miR (B), visualized by light microscope with a 20-fold magnification.

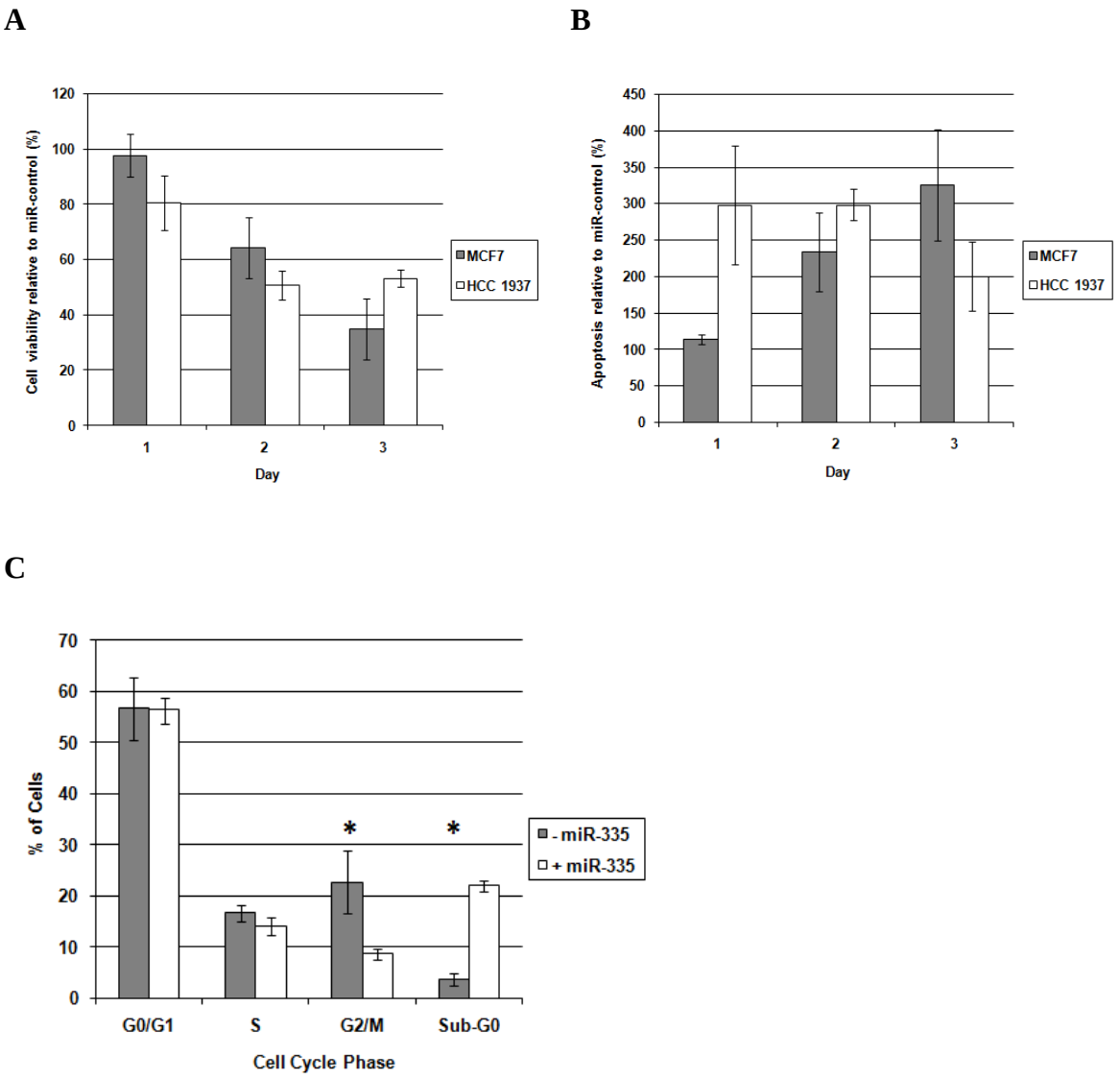


Fig. 19: Overexpression of miR-335 resulted in decreased cell viability, increased apoptosis and DNA fragmentation. (A) The viability of MCF7 and HCC1937 cells was determined up to three days following overexpression of miR-335. Data were assessed using the WST-1 assay and are displayed relative to control-miR transfected cells. (B) The induction of apoptosis was determined by quantification of the caspase 3 and 7 activities up to three days after transfection of mature miR-335 in MCF7 and HCC1937 cells. Data are normalized to the cell number and are displayed relative to control-miR transfected cells. (C) The cell cycle status was determined in MCF7 cells overexpressing miR-335 after 48 h. The cell cycle phases and the apoptotic fraction (Sub-G0) of transfected cells were determined by FACS analysis following propidium iodide staining and are displayed relative to control-miR transfected cells. Significance was determined by comparison to control transfected cells. P-values < 0.05 are indicated (*).

5.6 MicroRNA miR-203 and miR-335 expressions were altered in human sporadic breast cancer

To validate the relevance of the microRNAs for breast cancer development or progression, primary breast cancer samples were analyzed for the expression of both microRNAs. 27 ductal invasive breast tumor samples from postmenopausal breast cancer patients were used in this study and considered as sporadic. Normal breast tissues from seven women undergoing breast-reducing surgery served as controls. To enhance the specificity of the study, ductal cells from normal breast tissues and tumor cells from breast cancer samples with a tumor fraction lower than 80% were isolated using laser capture microdissection prior to RNA extraction. The microRNA expression levels were determined by qRT-PCR. Comparing tumor samples and normal breast tissues, expression data for the miR-203 could be divided into two subgroups, with the first showing a significantly lower microRNA expression (-58%) compared to the normal tissue and the second a 5-fold increased median transcript level (Fig. 20A). The median miR-335 expression was significantly decreased in the cancer specimens by approximately 3-fold (Fig. 20B).

A **B**

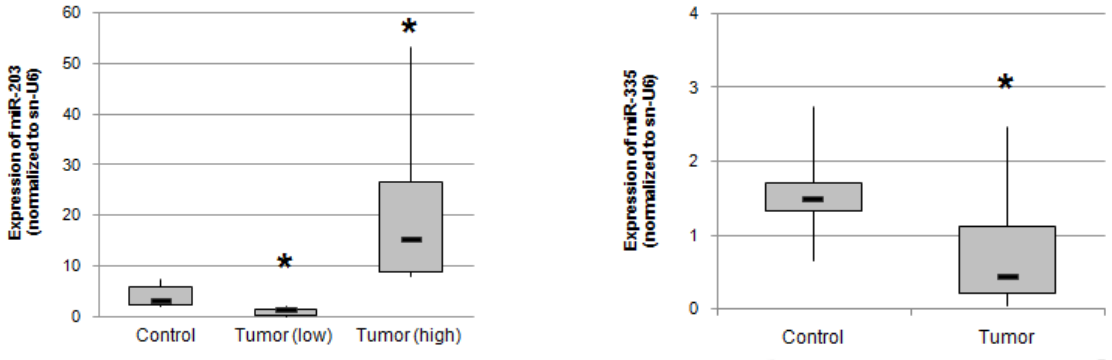


Fig. 20: Analysis of sporadic breast cancer revealed an altered expression of miR-203 and miR-335. Expression of microRNA miR-203 (A) and miR-335 (B) in normal breast tissue (n=7) and sporadic human breast cancer samples (n=27) were analyzed by qRT-PCR and normalized to sn-U6. Results are displayed as a box-plot. P-values < 0.05 (compared with control specimens) are indicated (*).

The expression data of both microRNAs were further analyzed for a potential correlation with certain clinical features. The presence or absence of the estrogen and progesterone receptor is routinely detected in cancer samples. The expression level of Her-2 was scored ranging from 0 to 3. The metastatic potential of the tumor was studied by checking the distinct lymph nodes of the patients. These data, when available, were correlated to the expression levels of miR-203 and miR-335. For miR-203, the features were compared between tumors representing the high (n = 10) or low (n = 13) expressing subgroup (Tab. 3). Here, tumors expressing low levels of miR-203 revealed a tendency to form lymph node metastasis (11/13, 85%) and to express the ER (10/14, 71%). Tumors with increased levels of miR-203 showed a reduced expression of the progesterone receptor (2/7, 22%) and higher levels (approximately 2-fold) of Her-2 compared to the other subgroup. No correlation could be detected between the microRNA expression and the age of the patient.

To correlate the expression of miR-335 to the clinical features, tumors with low expression of the microRNA (n = 20) were compared with tumors showing similar expression levels as the control specimen (n = 7) (Tab. 4). Cancer samples showing high levels of miR-335 were associated with a positive expression of the estrogen receptor (7/7, 100%), whereas the tumors expressing miR-335 at decreased levels had a positive ER signal only in 50% of the specimens. Tumors with low amounts of the miR-335 transcript revealed slightly decreasing amounts of the progesterone receptor. The other features (age, Her-2 status and occurrence of metastasis) showed no correlation to the microRNA expression.

Tab. 3: Expression levels of miR-203 correlated with diverse clinical features. The expression level of miR-203 was assigned to the routinely detected features of age, occurrence of lymph node metastasis, estrogen (ER) and progesterone (PR) receptor and Her-2 status.

miR-203	Age	Metastase	ER	PR	Her-2
Tumor (down)	66,0	11/13 (85%)	10/14 (71 %)	7/14 (50%)	0,7
Tumor (up)	64,9	3/8 (38%)	4/8 (50%)	2/9 (22%)	1,3

Tab. 4: Low levels of miR-335 were associated with decreased progesterone and estrogen receptor status. The expression level of miR-335 was assigned to the routinely detected features of age, occurrence of lymph node metastasis, estrogen (ER) and progesterone (PR) receptor and Her-2 status.

miR-335	Age	Metastase	ER	PR	Her-2
Tumor (down)	66,0	11/18 (61%)	9/18 (50%)	6/19 (32%)	0,8
Tumor (control)	67,2	4/6 (66%)	7/7 (100%)	4/7 (57%)	1,1

Following quantification of *BRCA1* expression, the transcript amounts were correlated to the level of miR-203 and miR-335. For miR-335, a significant positive correlation (0.35; $p < 0.05$) between both transcript levels was found (Fig. 21), supporting a regulatory pathway of *BRCA1* that involves miR-335. In contrast, no correlation could be detected between the expression levels of *BRCA1* and the level of miR-203 (data not shown).

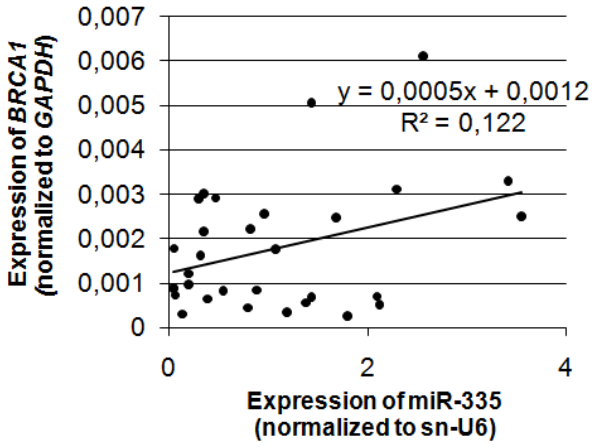


Fig. 21: Expression levels of miR-335 and *BRCA1* correlated positively. Both expression levels were determined for primary breast samples (n=30) by qRT-PCR and compared using Pearson`s correlation. The tested parameters revealed a significant positive correlation (0.35; $p < 0.05$), displayed by a regression line.

5.7 ID4 revealed a crucial function in the microRNA-dependent network

To identify the role of ID4 in the regulatory network involving microRNAs, growth factors and suppressor genes, functional assays were performed in a cell culture model. Therefore, simultaneous overexpression of the microRNAs and ID4 was performed in MCF7 cells. The expression plasmid used, solely contained the coding sequence for ID4 without its 3´ UTR to avoid any influence by the microRNA activity. Transfection setups using miR-control oligonucleotides and an empty expression plasmid served as control experiments. As previously shown, overexpression of the microRNA in the absence of ID4 led to a dramatic decrease in cell viability (compare Fig. 19). On day three after transfection, the relative viability of miR-335-transfected cells decreased to 57% (Fig. 22). Overexpression of ID4 in the absence of active microRNAs had rather a promoting effect on cell proliferation. Co-expressing ID4 along with the microRNA revealed a complete interruption of the microRNA-mediated effects. Co-expression of ID4 and miR-335 led to a relative viability of 106 % compared to the controls.

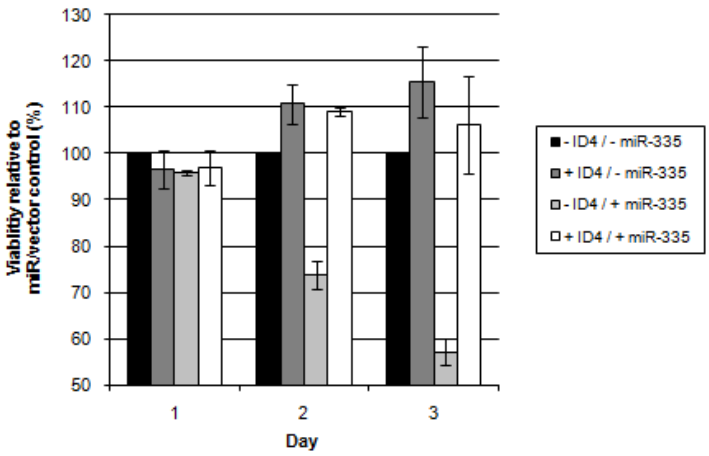


Fig. 22: Overexpression of ID4 rescued the microRNA-mediated effect. The viability of MCF7 cells overexpressing miR-335 in the absence or presence of ID4 was assessed by WST-1 assay for three days and is displayed relative to control-miR/control-vector transfected cells.

Since ID4 could rescue the effect of miR-335, its function on the cellular fate was further analyzed. In an attempt to mimic the microRNA-mediated knockdown of ID4, an siRNA-

mediated knockdown was performed. Again, MCF7 cells were used to assess the relevance of the abolished ID4 expression in a viability assay. To enhance the efficiency of the siRNA-mediated knockdown, a surface marker was co-transfected in the reaction. Marker-positive cells were enriched using a magnetic bead-based selection system and subcultured for eight days. The specificity of the selection was controlled by FACS analysis of the final cell suspension. The knockdown efficiency was assessed by determining the ID4 expression in selected cells. Two siRNAs (#1 and #3) revealed a significant knockdown of the ID4 transcript level by 69% and 52%, respectively (Fig. 23A). Furthermore, the knockdown led to a decreased cell proliferation of MCF7 cells of 36% (#1) and 28% (#3) (Fig. 23B). Notably, the siRNA with the higher knockdown efficiency led to a more profound effect on cancer cell growth. No phenotypical changes of the transfected cells could be detected.

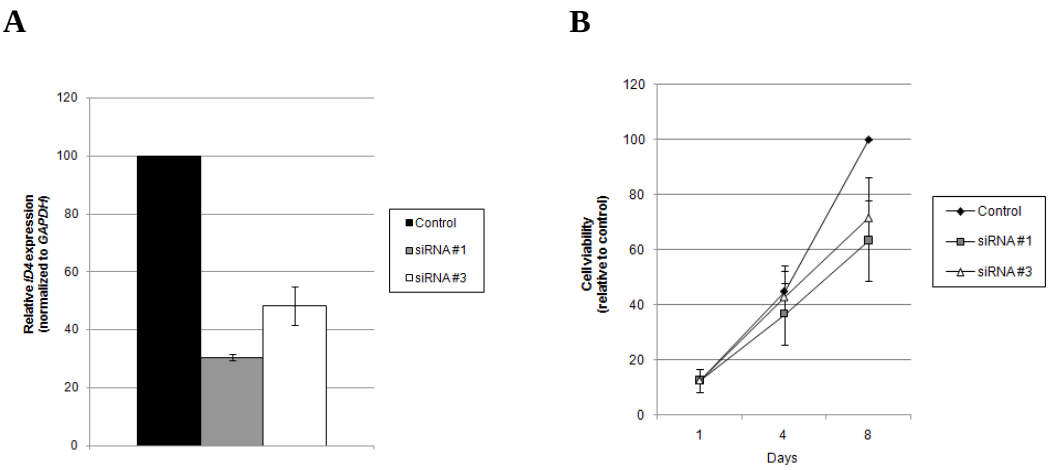


Fig. 23: The knockdown of ID4 resulted in decreased cancer cell proliferation. (A) The siRNA-mediated knockdown of ID4 was supported by a bead-based selection system to enhance the efficiency, which was analyzed by qRT-PCR on day two after transfection. The results are displayed relative to control-siRNA transfected cells. (B) The impact of the knockdown on cell proliferation was determined by the WST-1 assay up to eight days after selection. The data are presented relative to control-siRNA transfected cells.

As previously proven for the microRNAs, the relevance of the ID4 function for breast cancer development was studied in primary breast cancer samples. Furthermore, the influence of miR-335 on ID4 was determined by correlating the expression levels, extending the study from the cell culture model to primary tumor specimens. The ID4

expression was determined on both the transcript and protein levels. The amount of *ID4* transcript was assessed by qRT-PCR after RNA isolation from fresh frozen tumor samples enriched by microdissection. The protein level was determined in a previous work by S. Außenhofer using immunohistochemistry performed on formalin-fixed paraffin-embedded samples (188). The protein data are displayed in this study to underline the importance of post-transcriptional mechanisms for the regulation of ID4. According to the previously identified microRNA-mediated regulation of ID4, human breast cancer samples showed a significant decrease in *ID4* transcripts ($p < 0.05$), while the ID4 protein expression increased significantly ($p < 0.05$), when compared to normal breast epithelial cells (Fig. 24A and 24B). The biological relevance of the microRNA miR-335 on the *ID4* RNA expression was validated by detecting a significant ($p < 0.05$) inverse correlation (-0.42) between both expression levels in the cancer samples (Fig. 25). Since the ID4 protein expression data were assessed for different tumor samples, no direct correlation analysis could be performed.

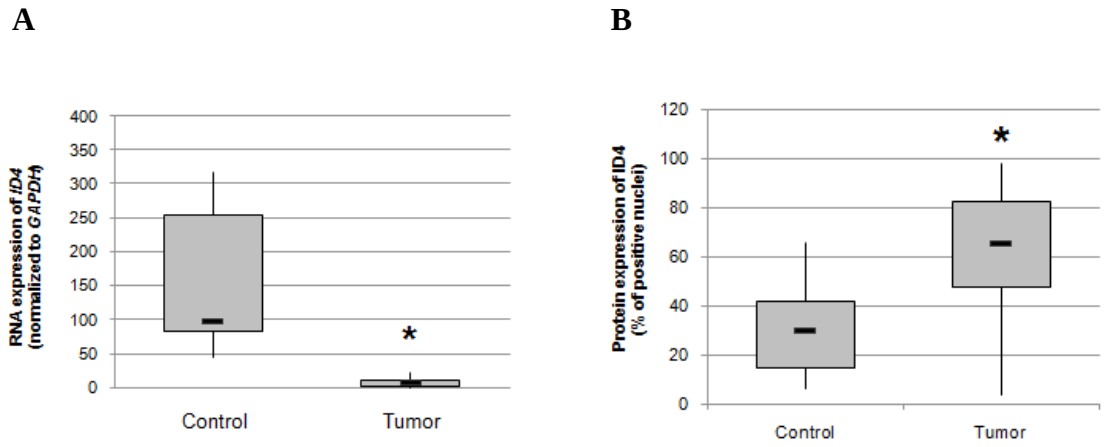


Fig. 24: High discrepancy of ID4 expression on the RNA and protein levels. (A) *ID4* mRNA expression analysis in normal control tissues (n=8) and tumor samples (n=36) assessed by qRT-PCR. Relative expression was determined by normalizing *ID4* expression to *GAPDH* and is displayed as a box plot. * $p < 0.05$ (compared with control specimens). (B) Immunohistochemical staining of normal control breast tissues (n=15) or tumor samples (n=30) using anti-human ID4 antibody. The ID4 protein expression was determined by counting the expression of 100 nuclei and is displayed as a box plot. P-values < 0.05 (compared with control specimens) are indicated (*).

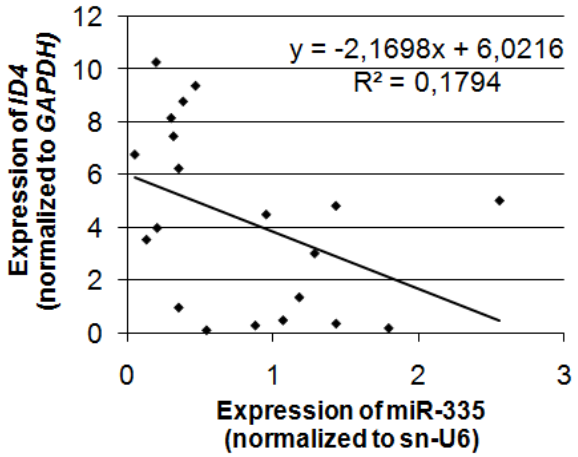


Fig. 25: The expression level of miR-335 inversely correlated with the transcript level of *ID4*. Both expression levels were determined for sporadic breast cancer samples by qRT-PCR and compared using Pearson`s correlation. The two features revealed a significant negative correlation (-0.42; $p < 0.05$) displayed by a regression line.

5.8 The promoter regions of *miR-203* and *miR-335* harbored different regulatory elements

To determine transcriptional regulators of both microRNAs, the sequence 2000 bp upstream of the transcription start was analyzed using PROMO software (189). Both microRNAs revealed multiple regulatory elements, such as binding sites for SP1 and ERα (Fig. 26, 33A and 33B). To determine regions prone to epigenetic modification the CpG island searcher software was used (190). Here, *miR-203* revealed a CpG island spanning its proximal promoter region (Fig. 26).

5.8.1 The transcription factor SP1 regulated the expression of miR-203

Bioinformatic analysis of the promoter region of microRNA *miR-203* detected a binding motif for the transcription factor SP1 (5`-GGGGCGGGG-3`) 489 bp upstream to the transcription start (Fig. 26). This element is embedded in a CpG island spanning from position -685 to -55 in the promoter region.

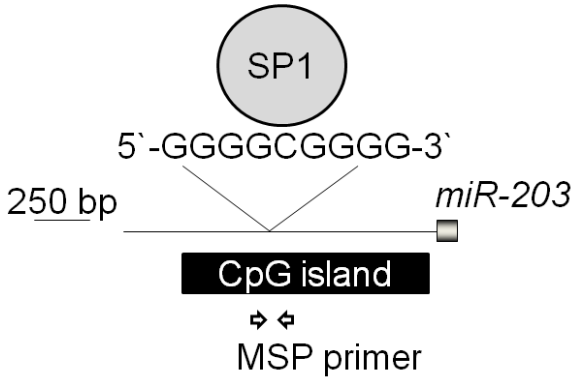


Fig. 26: Regulatory elements mapped to the proximal region of *miR-203*. The specific binding element for the transcription factor SP1 was located upstream of the transcription start of microRNA *miR-203*. A 630 bp fragment spanning the SP1 binding site was predicted to be sensitive to epigenetic modification through CpG methylation. A methylation-specific PCR with indicated primer locations (arrows) was performed to identify base modifications within the regulatory region of *miR-203*.

To validate the relevance of the predicted regulating elements in the promoter region of *miR-203*, cancer cell lines with differing expression levels of miR-203 (scoring according to 5.1), but similar protein amounts of SP1 were identified (Fig. 27). Interestingly, SP1 seemed to be ubiquitously expressed within any cell line with only slight variations, whereas the miR-203 expression is highly diverse. Cell lines presenting higher levels of SP1 showed the tendency to express increased amounts of miR-203. To evaluate the influence of epigenetic modifications in the form of CpG methylation, four cell lines were selected with high, moderate or low expression of miR-203 but similar SP1 levels and analyzed for their methylation status by a methylation-specific PCR (MSP) (Fig. 28). Here, the expression level of miR-203 correlated positively with the event of CpG methylation. MCF7 and HeLa cells showing high or moderate expression of the microRNA revealed a hypomethylated promoter region, whereas the CpG islands of cell lines with low expression levels (PA-1 or MiaPaCa2) were hypermethylated.

To identify the impact of the methylation status on miR-203 expression, the cell lines were treated with the demethylating reagent 5-Aza-2´-deoxycytidine (5-Aza). In MiaPaCa2 and PA-1 cells, the hypermethylation could be partly reversed following treatment with 2 µM 5-Aza for 3 days (Fig. 28). In parallel, the level of miR-203 was increased 94-fold or 33-fold for MiaPaCa2 and PA-1 cells, respectively, suggesting that the methylation status

may influence the microRNA expression (Fig. 29). In both cell lines harboring a hypomethylated promoter region of *miR-203* (MCF-7, HeLa), treatment with 5-Aza had no effect on CpG methylation and only a weak influence (approximately 2-fold) on the expression level of the microRNA.

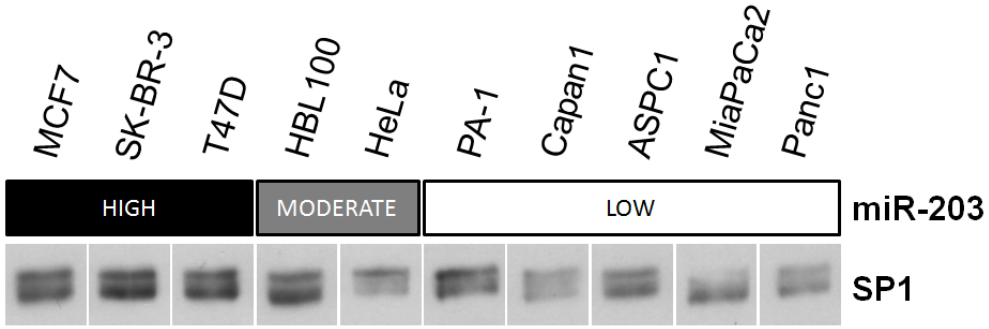


Fig. 27: The transcription factor SP1 was ubiquitously expressed in all cell lines. The endogenous protein amount of SP1 was identified in total cell lysates for the indicated cell lines by Western blotting. Displayed here is the specific band for SP1. All samples were loaded at similar total protein amounts. The expression of miR-203 is assigned to three subgroups representing high, moderate or low levels of the microRNA transcript.

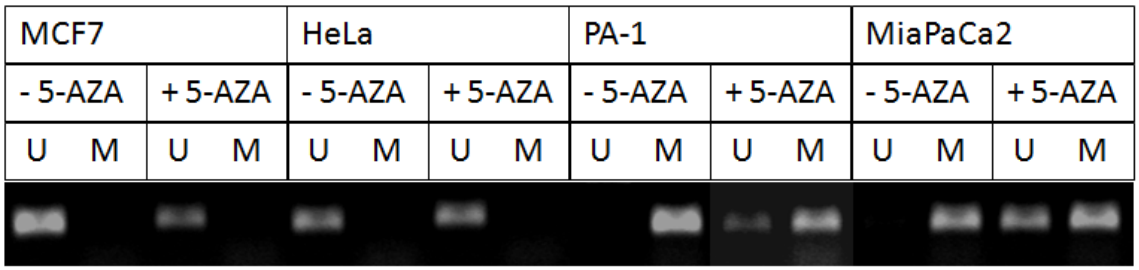


Fig. 28: The promoter of *miR-203* revealed a varying methylation status in different cell lines. The genomic DNA of the cell lines MCF7, HeLa, PA-1 and MiaPaCa2 was analyzed for CpG methylation in the promoter region of *miR-203*. MSP analyses were performed for untreated (- 5-AZA) cells and cells treated (+ 5-AZA) with the demethylating reagent 5-Aza. Methylation- (M) and unmethylation- (U) specific PCR reactions are separated in individual columns as indicated.

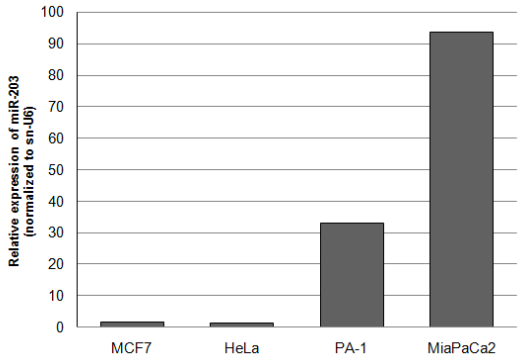


Fig. 29: Expression of miR-203 was dependent on the methylation status. The expression level of miR-203 was assessed by qRT-PCR in MCF7, HeLa, PA-1 and MiaPaCa2 cells following treatment with 2 µM 5-Aza for three days. The amounts of the mature microRNA are normalized to sn-U6 and displayed relative to untreated control cells.

According to previous results, the cell line MCF7 was chosen to clarify significance of the SP1-mediated regulation of miR-203. The breast cancer cells revealed an unmethylated promoter region and high levels of miR-203 and therefore was an adequate model in which to study the influence of the transcription factor, predicted to bind to the proximal promoter region. The impact of varying levels of SP1 on the transcription efficiency of miR-203 was assayed using an siRNA-mediated knockdown approach. The expression of miR-203 was assessed two days following transfection by qRT-PCR. Transfection of MCF7 cells with a specific siRNA directed against SP1 forced a reduced expression of 52%, presenting an adequate approach to study the effects of the transcription factor (Fig. 30A). According to the proposed model, siRNA-mediated knockdown diminished the expression of miR-203 by 23%, revealing a positive correlation between the expression of the microRNA and its transcription factor (Fig. 30B).

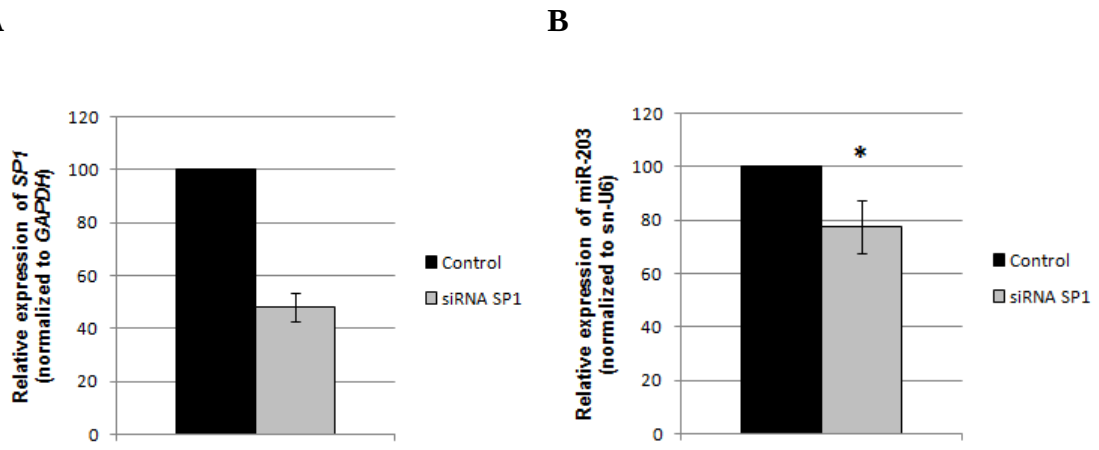


Fig. 30: The transcription factor SP1 regulated the expression of *miR-203*. (A) The transcript amounts of SP1 were determined following siRNA-mediated knockdown (+/- siRNA SP1) of the gene. Expression levels were analyzed two days after transfection by qRT-PCR, normalized to *GAPDH* and displayed relative to control transfected cells. (B) Expression of miR-203 was determined 48 h after knockdown (+/- siRNA SP1) of the transcription factor SP1. Expression was quantified using qRT-PCR, normalized to sn-U6 and is presented relative to control transfected cells. P-values < 0.05 (compared with control transfections) are indicated (*).

To establish the correlation of SP1 and miR-203 and to prove its biological relevance, primary tumor samples were analyzed for the expression of the transcriptional activator SP1. Since SP1 was previously identified as a target of post-transcriptional regulation, the expression level was detected on the transcript and protein levels. Comparing the *SP1* mRNA levels in cancer samples and normal breast tissue, no difference could be detected between the two cohorts (Fig. 31A). Protein analysis of total tumor cell lysates by Western blotting revealed samples with increased protein levels of SP1 in cancer specimens compared to control samples (Fig. 31B). To clarify the connection between SP1 as a transcriptional activator of miR-203, both expression levels were correlated. Here, no significant correlation could be detected (data not shown). Although, no correlation between the protein amount of SP1 and its post-transcriptional regulator miR-335 could be identified, there was a 1.6-fold higher median expression level of the microRNA in samples with low SP1 expression (n = 12), compared to samples with a high amount of SP1 (n = 10).

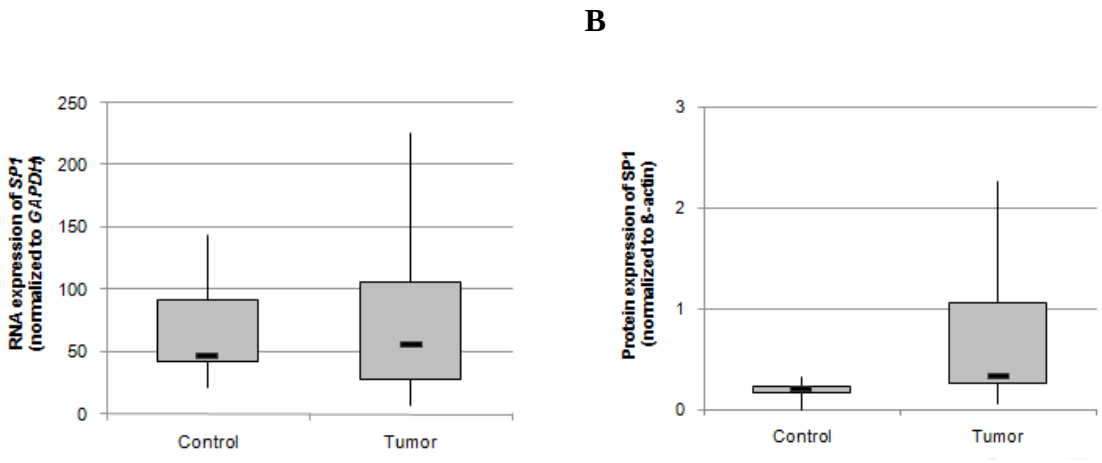


Fig. 31: Protein expression of SP1 was increased in sporadic breast cancer. (A) Primary sporadic breast cancer samples were analyzed for their expression level of *SP1* on the mRNA level. The amount of *SP1* transcript was determined by qRT-PCR and normalized to *GAPDH*. Results are displayed as a box plot for normal control breast tissues (n = 8) or tumor samples (n = 35). (B) Primary sporadic breast cancer samples were analyzed for their expression level of SP1 on the protein level. The amount of protein was determined by Western blotting and normalized to ß-actin. Results are displayed as a box plot for normal control breast tissues (n = 7) or tumor samples (n = 12).

Finding no direct correlation between the expression of miR-203 and its potential transcription factor SP1, the identified epigenetic regulation mechanism was analyzed in primary breast tumor samples. The regulating effect mediated by epigenetic modifications was analyzed by MSP. Therefore, a number of samples showing high (n = 3) or low (n = 11) miR-203 expression (according to 5.6) and normal breast epithelial cells (n = 2) were studied for CpG methylation in the promoter region. Here, one tumor sample (CA #1) with normal SP1 protein level (compared to the healthy control) showed a heterozygous methylation of the promoter region of *miR-203* (Fig. 32). Conversely, this was accompanied by decreased levels of microRNA miR-203 expression compared to the control sample. The other tumors (e.g. CA #2,3) with reduced or high expression of the microRNA, as well as the normal tissue, showed no detectable methylation of the *miR-203* promoter region, indicating the absence of a methylation mediated regulation of the microRNA in these samples.

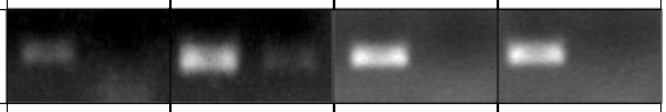
	Control		CA #1		CA #2		CA #3	
	U	M	U	M	U	M	U	M
MSP								
SP1	0.22		0.22		0.14		0.18	
miR-203	2.63		0.62		0.03		1.30	

Fig. 32: Epigenetic modifications altered miR-203 expression in breast tumors. Selected tumors were analyzed for the promoter methylation status by MSP. Displayed here is the MSP result of three tumors (CA) compared to one representative healthy control. The expression values for SP1 protein and the miR-203 for all samples are displayed as normalized absolute values. U = unmethylated, M = methylated

5.8.2 The expression of miR-203 and miR-335 was induced by estrogen

Bioinformatic analysis of the promoter region of microRNA *miR-203* identified two binding sites (-1192, -1039) for ERα (Fig. 33A). The promoter region of *miR-335* contains seven predicted binding sites for the estrogen receptor α located 5´ to the transcription start (-1727, -1458, -1431, -885, -552, -149) (Fig. 33B). The influence of estrogen for the expression of both microRNAs was assessed following estrogen stimulation of serum-starved MCF7 cells for 24 h. Both microRNAs demonstrated an increase of expression to 231% (miR-203) and 191% (miR-335) after estrogen-stimulation (Fig. 34).

A

B

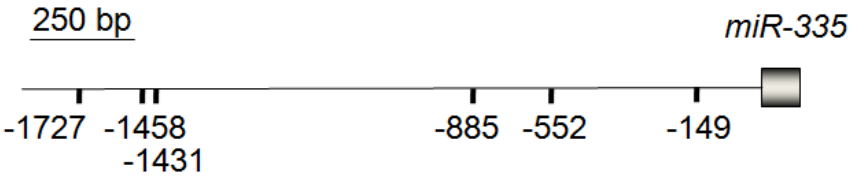


Fig. 33: The promoter regions of *miR-203* and *miR-335* harbored potential ERα binding sites. Schematic overview of the promoter region and the coding sequence of *miR-203* (A) and *miR-335* (B). The putative binding sites of ERα are indicated with the exact location relative to transcription start site.

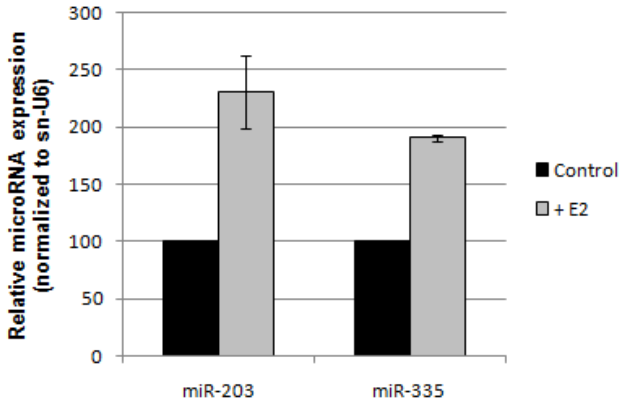


Fig. 34: The expression of miR-203 and miR-335 is regulated by estrogen. Quantitative analysis of microRNA expression by qRT-PCR in serum-starved MCF7 cells 24 h after induction with 10 nM ß-estradiol (E2). Data were normalized to sn-U6 expression and are given relative to control ethanol-treated cells.

6 Discussion

A biological network is more than the sum of its members. Analyzing the structure of a networking process, it is necessary to determine its members, their specific functions, connections and interactions. Subsequently, the single factors form a theoretical framework, predicting the processes and interactions within the machinery. However, the determination of the framework is only the beginning of a speculative input-output scenario without the demand of accuracy. As an example, associated with the network analyzed in this study, the interaction of the estrogen and the aryl hydrogen receptor perfectly illustrates the difficulties in analyzing closely connected cascades. Following binding of its specific ligand TCDD, AhR binds to liganded ERα. This interaction can exhibit an activating or repressing effect on the target gene expression depending on the current context. The activation of the *CAD* gene depends on the interaction of ERα with SP1 (191). Following ligand binding, AhR translocates to the nucleus, interacts with ERα, thereby disrupting the activator complex for *CAD* and inhibiting its expression. In this context, the AhR/ERα interaction represses gene transcription, whereas for another gene (*CYP1A1*), the same complex was shown to be essential for gene activation (192). In the regulation of *BRCA1*, unliganded AhR forms an activating complex with ERα, whereas ligand binding disrupts this formation, thereby leading to gene repression (80). This example demonstrates different outcomes following the interaction of only two proteins and their ligands. Considering the complex network regulating *BRCA1*, influences of single events are difficult to predict. Regarding the multiple factors involved in the regulation, it is likely to be prone to various aberrations causing destabilization of the homeostasis. *Vice versa*, diverse feedback mechanisms might protect the network and ensure its integrity.

Considering the high number of cancer types associated with the single members of the network, multiple aberrations or misregulation of key factors might lead to cancer promotion or tumor progression. As detected for inherited breast cancers, disrupting the function of BRCA1 highly predisposes to cancer formation (19). BRCA1 represents a key factor in the network, since disruption of its function leads to a number of tumor-promoting effects. First, BRCA1 performs a crucial function in the DNA repair mechanism via homologous recombination and decelerates the cell cycle of proliferating cells (5). Thus, BRCA1 favors an error-free replication cycle, guaranteeing genomic stability and

functions as a gate-keeper forcing apoptosis or cell cycle arrest of damaged cells (193). Second, BRCA1 indirectly serves as an anti-proliferative factor due to its repressive interaction with ERα (89) and SP1 (88), as well as its inhibition of IGF1R expression, blocking highly mitogenic pathways. Disrupting the function of BRCA1 therefore leads to destabilization of the genome by inhibiting a major checkpoint in DNA repair and to acceleration of cell growth by induction of mitogenic pathways. Germline mutations of *BRCA1* result in a high predisposition to develop breast cancer due to an enhanced probability of a complete loss of function of the tumor suppressor through a second disruptive event. Although only few mutations of *BRCA1* were detected in sporadic breast cancer (4), a misregulation of the gene was also confirmed in sporadic tumors (22). Hypermethylation of the promoter is one mechanism identified, but it only covers a small number of cases (27). Misregulation of activating factors therefore is a proposed model to explain the reduction of the tumor susceptibility gene. Regarding the high number of activators, a more specialized effect might be assigned to negative regulators of *BRCA1*. Accordingly, the only yet identified dominant inhibitor ID4 might act as a key molecule for the regulation of *BRCA1* and the development of sporadic breast cancer (46). Considering the controversial results obtained from ID4 expression studies (35-44), a detailed analysis is also part of this study.

Searching for other superordinated components of the regulating complex of *BRCA1* and for breast cancer development, this study extended the traditional cohort of controlling molecules to the recent research field of small regulatory RNAs. Since microRNAs were identified as negative post-transcriptional regulators for multiple predicted target molecules, the effect of microRNAs was interesting for the question addressed in this study.

A number of microRNAs were previously detected to be deregulated in sporadic breast cancers. A study comparing the expression profile of normal breast tissue with diverse breast tumor samples revealed miR-21 and miR-155 to be upregulated, whereas miR-10b, miR-125b and miR-145b were suppressed (177). This and other expression studies increased the insight into the initiation and progression of sporadic breast cancer and led to the identification of novel “oncomirs”, some specific to breast cancer, others previously described for different cancer types. Considering the tissue-specific background of the cancer origin, breast cancer “oncomirs” occasionally revealed varying functions compared to other tumor types. Here, one example is the microRNA gene cluster *mir-17-92*. As previously described, gene amplification and overexpression are associated with a number

of lymphomas and solid cancers. In contrast, in breast cancer specimens, loss-of-heterozygosity was observed for the chromosomal region 13q31 harboring the cluster. As a potential target mediating the effect specific for breast cancer, the transcriptional co-activator AIB1 was identified (194). AIB1 is involved in the regulatory functions of ERα and E2F (195). Reduced expression of AIB1 decreases estrogen-dependent and -independent cancer cell proliferation, suggesting AIB1 to be a potent mediator for tumor growth signals. Here, AIB1 represents one part of the tissue-specific background that mediates the function of the *mir-17-92* cluster.

Another microRNA that can be closely connected to hormone-dependent tissues like breast and ovarian cancer is miR-206. Initial expression studies revealed its increased expression in a subset of ER-negative tumor samples (177). Follow-up studies could validate the estrogen receptor as a direct target of miR-206 (196). The same study describes an estrogen-dependent miR-206 expression, suggesting a feedback loop in the regulatory pathway of the microRNA. Clinical significance was established showing an inverse correlation of miR-206 and the estrogen receptor in primary cancer samples (197).

The microRNAs previously described are predominantly downregulated in sporadic breast cancer. Their assigned targets are frequently overexpressed in tumor samples and exhibit growth-promoting functions, indicating a tumor-suppressive effect of the associated microRNAs. In contrast, expression analysis of tumor samples and metastasis revealed other microRNAs with oncogenic function by suppressing potential tumor suppressor genes. For example, microRNA miR-21 is overexpressed in a range of tumors including breast tumors (198). Its function is associated with tumor growth and apoptosis. These oncogenic features could be elevated by studies validating the tumor suppressor genes TPM1 (199) and PDCD4 (200) as primary targets of miR-21. TPM1 suppresses anchored-independent growth and is repressed in cancer cells derived from breast cancers. PDCD4 is a pro-apoptotic regulator suppressed in invasive carcinomas compared to normal tissues. The simultaneous repression of both factors by miR-21 and the previously described involvement in the p53 pathway suggests that this microRNA is important for cancer formation not only in breast tissue.

In breast cancer, the development of metastasis is a crucial prognostic marker. Here, microRNAs were proven to contribute to the spreading of cancer cells. Mir-10a, initially shown to be downregulated in breast cancer specimens, seems to exhibit prometastatic features in a later stage of tumor progression. Functional analysis of miR-10a identified its promotion of tumor invasion and formation of metastasis following overexpression (154).

As a primary target, the transcription factor HOXD10 was identified, which is part of a signaling cascade leading to the activation of RHOC, a pro-metastatic gene. Additional microRNAs linked to the formation of metastasis were detected by comparing highly metastatic tumor cells to unselected controls. Microarray analysis revealed a reduced expression of miR-335, miR-206 and miR-126 in metastatic cells (201). Conversely, restoration of the microRNA expression converted the phenotype.

This study focuses on the impact of an altered microRNA expression on breast cancer development by deregulating the network controlling the *BRCA1*. As previously shown for the regulation of the oncogene *Ras*, a single microRNA can control entire pathways by regulating multiple key factors of the cascade (187). Hence, one might hypothesize that one or more microRNAs can exhibit superior regulating function by controlling multiple or key molecules in the initiation complex of *BRCA1* or the tumor suppressor itself. A very straight-forward study, screening mutations in microRNAs regulating key molecules for breast cancer tumorigenesis, identified post-transcriptional regulation of *BRCA1* by miR-17 and gives a first example of microRNA regulation in this context (202). The aim of this study was to establish microRNAs controlling multiple factors in the regulatory complex of *BRCA1*. As key components of the complex associated with cancer formation, the hormone receptors ERα, AhR and IGF1R, the transcription factor SP1 and the dominant inhibitor ID4 were analyzed in this study.

In previous studies, some of these factors were already identified as targets of microRNA deregulation with a connection to cancer formation. The estrogen receptor was shown to be regulated by a number of microRNAs in different types of cancer. Here, miR-221 and miR-222 were determined to be overexpressed in breast tumors and cell lines with reduced ERα expression and were further validated to directly bind to the 3´ UTR of the receptor (203). Conversely, an overexpression of the microRNAs resulted in resistance to tamoxifen treatment, while blocking the microRNAs had the opposite effect. Another study revealed miR-18a as a regulator of ERα in HCC samples (204). An increasing level of miR-18a inversely correlated with reduced amounts of the receptor in tumor tissues. Furthermore, Adams and colleagues determined miR-206 to negatively control the estrogen receptor in breast cancer cell lines (196). These results were extended by studying the microRNA expression in breast tumor samples (197). Here, miR-206 and ERα revealed an inverse correlation, suggesting functionality of the microRNA for tumor formation. Interestingly, Sun and colleagues determined miR-22 as a negative regulator of ERα and SP1 (205), underlining the hypothesized superior function of microRNAs analyzed in this study. For

the remaining components of the *BRCA1* regulating complex, no direct interaction with a microRNA has yet been described. However, molecules up- and downstream of the factors are affected. MicroRNA miR-27b was identified to control the expression of CYP1B1, an oncogenic enzyme activated by AhR (206). In concordance with this, an increased CYP1B1 level revealed an inverse correlation with a reduced amount of miR-27b in breast cancer samples. SP1 also represents a target of indirect microRNA regulation, since miR-27a alters the expression of the SP1 repressor ZBTB10 (207).

In this study, the influence of microRNAs on the expression of multiple targets in the upstream cascade regulating *BRCA1* was analyzed. Therefore, prediction algorithms were used to identify microRNAs likely to bind to the 3´ UTRs of *SP1*, *AhR*, *ESR1*, *IGF1R*, and *ID4*. This bioinformatic analysis detected miR-203 targeting all five molecules involved in the study. Furthermore, miR-335 is predicted to control four out of the five targets, favoring a good probability that both microRNAs hold a key position in the network. Both microRNAs were previously connected to diverse cancer types including sporadic breast cancer. Controversially, published expression studies of miR-203 indicate different functional consequences of an altered miR-203 expression profile depending on the tissue context. Whereas overexpression of miR-203 was detected in bladder cancer (208) and colon adenocarcinomas (209), another study revealed a reduced expression in oral squamous cell carcinoma cell lines (210). Studies supporting the tumor-suppressive function of the microRNA detected an increased cell growth of lung cancer cells following miR-203 inhibition (211) and a reduced proliferation rate after its overexpression in hematopoietic cancer cells (212). The latter was associated with a reduction of oncogenic ABL1 or BCR/ABL1. For skin cell differentiation, miR-203 was shown to repress “stemness” of basal cells by inhibiting p63, a crucial factor for stem-cell maintenance (213). In summary, miR-203 function is predominantly associated with tumor-suppressive features, as it reduces cell proliferation and expression of oncogenes and favors differentiation. Since it is overexpressed or repressed in different cancer types, a tissue-dependent function cannot be excluded.

Previous studies analyzing the function of miR-335 also detected a suppressive effect of this microRNA for tumor formation. Interestingly, this was predominantly observed in cancer cells derived from hormone-dependent tissues. Here, miR-335 lacking tumors are prone to exhibit metastasis (214) or drug resistance (215), hence promoting a more unfavorable prognosis. In concordance with this, miR-335 revealed anti-mitogenic and pro-apoptotic functions mediated by repressing Jagged-1 in brain cells (216). The

expression of the Notch signaling ligand Jagged-1 correlates negatively with disease-free and overall survival in breast cancer samples (217, 218). Although no analysis compared normal and tumor samples, there is strong evidence linking miR-335 to severe phenotypes of breast tumors. Since its locus is frequently lost in breast cancer samples (219), miR-335 is suspected to function as a tumor and metastasis suppressor microRNA.

The present study identified target molecules of miR-203 and miR-335 mediating the predominantly tumor-suppressive phenotype. In overexpression experiments, the importance of the microRNAs for the expression of the *BRCA1* regulators SP1, AhR, ERα, IGF1R and ID4 was determined. In concordance with this, functional analysis demonstrated the effect of the microRNAs on cancer cell behavior and cell fate. Furthermore, the microRNA expression was directly connected to *BRCA1* regulation. To detect biological relevance, the expression profiles of miR-203 and miR335 were assessed in sporadic breast cancer samples, predetermined for their expression status of *BRCA1*, *SP1* and *ID4*. In an attempt to reveal the origin of microRNA expression alterations, regulating elements in the promoter region were identified and validated. Finally, the exclusive influence of ID4 on the regulatory network of *BRCA1* and on the function of microRNAs was clarified. Therefore, a novel technique was established, identifying the direct interaction of microRNAs with their associated mRNA. This new method enables the identification of microRNA activity on targets previously undetectable by standard techniques such as ID4.

At the start, the basic hypothesis of the study was verified by transferring the bioinformatically predicted model to a cellular system. Therefore, an adequate cancer cell line model was identified that presents a suitable environment for this study. Requirements for the cell lines were the expression of all transcription factors, microRNAs and *BRCA1* at a detectable level. Since the prediction algorithms do not account for different time- and tissue-dependent expression patterns of the microRNA and its target, one can exclude false positive predictions by detecting the expression status of the molecules in the system analyzed. Predicted interaction partners that are not expressed in the same tissue are unlikely to be biologically relevant. Diverse non-breast cancer cell lines were included, since *BRCA1* misregulation was also described for other cancer types (220). To determine the amount of transcribed microRNAs, stem-loop RT-PCR was performed (221). This approach transmits specificity already in the reverse transcription reaction using specific primers binding to the mature microRNA. In a probe-based real-time PCR, specific microRNA levels were detected by excluding genomic DNA and precursor

oligonucleotides. MicroRNA miR-335 revealed an ubiquitous expression in the majority of the cell lines analyzed. Notably, the pancreatic cell lines exhibited a lower level compared to breast cancer cells. A similar result was achieved by detecting the amount of mature miR-203. MicroRNA miR-203 expression was absent or weak in all pancreatic cells, while the breast cancer cell lines revealed moderate or high levels of miR-203. Functional relevance of both miR-335 and miR-203 is therefore possible in breast cancer tissues, whereas at least for miR-203 a functionality is unlikely in the pancreas. For further studies, the breast cancer cell line MCF7 was chosen, which expresses miR-203 and miR-335, harbors detectable protein levels of the transcription factors, and was established for BRCA1 and ID4 experiments in the past (46, 80).

To determine the regulating effect and the impact of misregulated microRNAs on SP1, AhR, ERα, and IGF1R, the proteins were analyzed following overexpression in MCF7 cells. Therefore, oligonucleotides mimicking the function of mature microRNAs were transfected. The advantage of this approach is the independence from the microRNA processing machinery, disrupted in various cancers and cancer cell lines. In concordance with their predicted functions, miR-203 and miR-335 both downregulated the expression of the estrogen, the aryl hydrocarbon and the insulin-like growth factor 1 receptor. A specific suppressive function of miR-335 was observed for SP1, whereas miR-203 led to rather increased levels of the receptor. The results are in agreement with the prediction scores, showing low probabilities for miR-203 to regulate SP1. The effect of miR-335 on AhR is either due to an unpredicted binding activity or to a secondary target effect caused by the overexpression of the microRNAs, which may also explain the upregulation of SP1. Regarding the high complexity of the network, secondary target effects were expected, not only for the *BRCA1* expression, but also through feedback mechanisms for single members of the cascade.

For ID4 no antibody is available to detect the protein expression by Western blotting. The lack of a specific and sensitive antibody against the protein of interest is an important weakness in the analysis of the microRNA activity on a target of interest. Currently, the most common method to prove the activity of specific microRNAs on any target of interest is a luciferase system using potential regulatory sequences downstream to the reporter gene (222). Transfection of these constructs together with a simultaneous overexpression or knockdown of a predicted microRNA reveals its functionality on the target sequence. Since this model works with artificial constructs, naturally occurring modifications, e.g. mRNA folding, are not considered (223). Concerning the high number of predicted

microRNA:target interactions, this system is also unsuitable for a broad and complex screening setup. In this context, new systems combining biochemistry and bioinformatics have recently been developed. In an attempt to screen for microRNA targets on the protein level, Baek and colleagues used quantitative mass spectroscopy following manipulation of single microRNAs (224). This technique extends the standard protein detection methods to a high throughput system for the identification of microRNA targets. However, this technique requires expensive equipment and profound expertise in mass spectrometry, that may not be available in each laboratory investigating microRNA:mRNA interactions. Another approach detecting direct interactions of the analyzed molecules is based on the isolation of the ribonucleoprotein (RNP) complex harboring the microRNA and its associated mRNA (225, 226). Argonaute, as the major protein in this aggregate, is immunoprecipitated followed by a quantification and bioinformatic analysis of enriched molecules. Here, interactions are predicted based on statistics, but lack a direct detection system. A more straightforward technique by Lund et al. is based on microRNA labeling, directly detecting the interaction of the two molecules in the RNP (227). Following precipitation of one specifically tagged microRNA, quantitative analysis can detect mRNAs co-purified by this method. Furthermore, they extended the use of the technique to a high-throughput screening of isolated target molecules (128). Since this approach is appropriate to detect effector molecules for specific microRNAs, a detection of different microRNAs regulating one specific gene is not possible.

In the present study, we established a reverse technique, termed microRNA co-precipitation, which allows the detection of active microRNA response elements (MREs) in a single gene of interest. Using biotin-tagged nucleotides, a labeled mRNA is introduced and rescued from a cellular system. MicroRNAs bound over RNPs are co-precipitated and can be quantified by standard techniques. This novel approach is suitable for virtually all genes that can be transcribed *in vitro* and is therefore valuable for a broad range of researchers screening for microRNAs regulating their specific genes of interest.

The microRNA co-precipitation technique uses the advantage of simple mathematical mechanisms. Having an equation with two variables represented by microRNAs and mRNAs, the method assigns one of them, thus leading to a simple identification of the other. Using biotin-labeling and specific precipitation techniques, the variable “mRNA” is transferred to a constant. Consequently, the second variable “microRNA” can be determined using quantitative real-time PCR.

This novel method was established in a proof-of-principle experiment using the well described regulation of microRNA Let-7 on the oncogene *NRAS* (187, 166). Following validation of the principle, the technique was performed to detect microRNA interactions in the 3´ UTR of *ID4*.

Prior to the proof-of-principle, the influence of the biotin-labeling on the transcription and translation efficiency was determined using the transcript of the reporter gene *GFP*. Analysis of the integrity of the mRNA and the signal intensity following transfection of the transcript revealed no influence of the biotin-labeled UTPs.

To establish the co-precipitation technique, mRNA-labeling was applied to the oncogene *NRAS* harboring three binding sites for Let-7 in its 3´ UTR. The method enriched Let-7 in significant amounts above the background, which was defined by a control transcript and the binding of unspecific small RNAs, such as sn-U6. Stringent washing steps were sufficient to enrich the analyzed molecules, but unspecific binding could not be avoided. Interestingly, the isolated *NRAS* transcripts as well as the amount of unspecific background were suitable for normalization. Here, normalization to sn-U6 provides advantages to the purified transcripts, since differences in length and integrity, in terms of breakage products, can influence the results of the precipitation. The unspecific background is equally distributed to all isolated fragments and mirrors the actual amount of purified molecules.

This technique combines the advantages of a number of established methods like protein co-immunoprecipitation and biotin-labeling. All steps are performed in a cellular system using constructs with original gene sequences. The secondary structures probably imitate the original ones, creating a natural system to avoid false positive results. Furthermore, a control construct lacking the 3´ UTR and therefore the region harboring the most active MREs serves as an adequate control for normalization. Notably, much more attention has to be paid to unspecific bindings for the microRNA co-precipitation compared to the reverse approach using labeled microRNA. Using intensive blocking and stringent washing conditions, the established technique is suitable to specifically detect active MREs in untranslated regions of selected genes. Using standard techniques, it is applicable in any laboratory setting to validate the binding of microRNAs to virtually all genes. It is valuable for a broad range of researchers screening for microRNAs regulating their gene of interest. Furthermore, this technique is also appropriate for high-throughput platforms such as microRNA microarrays to identify a high number of valid microRNA:mRNA interactions in a single step. Since this modification is not limited to prediction algorithms, it might

further improve our knowledge of the criteria crucial for the mRNA:microRNA interaction.

The established technique was then performed to identify predicted interactions between microRNAs and the mRNA of *ID4*. Here, microRNA miR-335 was particularly enriched by co-precipitation with the full-length *ID4* transcript, whereas miR-203 had signal intensities comparable to the two control RNAs. MicroRNA miR-335 reached statistical significance in comparison to both controls, clearly suggesting a functional interaction with the *ID4* 3´ UTR. Since this was the first time the novel technique was applied to detect predicted, but as yet unvalidated microRNA:mRNA interactions, the results were validated using an independent method. Therefore, the lack of detectable endogenous protein amounts was bypassed by ectopic overexpression of *ID4*. According to the previous results, inhibition of endogenous miR-335 by specific oligonucleotides resulted in an increased translation rate and protein amount using full length ID4 mRNA.

To further validate a direct influence of miR-203 and miR-335 on the expression of *ESR1*, *AhR*, *IGF1R*, *SP1* and *ID4* reporter gene assays were performed. Here, a direct regulation of miR-335 of *ESR1*, *SP1* and *IGF1R* could be detected. The previously identified impact of miR-335 on *AhR* probably represents a secondary target effect in the tightly connected regulatory cascade of *BRCA1*, since no MREs are predicted in its 3´ UTR. The results obtained by protein analysis and co-immunoprecipitation of *ID4*, as well as the correlation in primary breast cancer tissue and the phenotype rescue, strikingly suggest a direct effect of miR-335 on *ID4*. As reporter assays present a highly artificial detection system, the absence of miR-335 activity on the *ID4* luciferase construct might reflect a false negative result, for example due to altered mRNA folding.

For miR-203, reporter assays determined a direct influence of the microRNA on the expression of *AhR*, *IGF1R* and *SP1*. No regulatory effect was detected for *ESR1* and *ID4*. These data reflect the profound impact of secondary target effects in the tightly regulated cascade upstream of *BRCA1*, as the results obtained for SP1 and ERα on protein level correlate inversely to the reporter assays.

In summary, the overexpression, as well as precipitation experiments and the reporter system proved that miR-203 and miR-335 influence components of the regulatory complex of *BRCA1* either in collaboration or with distinct functions. The effect of the microRNAs on their distinct targets is predominantly direct. However, secondary events also participate in the regulation. The results suggest that misregulation of the microRNAs has a downstream effect on key regulators of *BRCA1*, which also directly participate in

tumorigenesis. Therefore, the microRNAs themselves might exhibit tumor-promoting or suppressing functions.

To underline this hypothesis, the influence of the microRNAs on *BRCA1* expression was determined. Surprisingly, altering the levels of miR-203 and miR-335 caused opposite effects on *BRCA1*. While an overexpression of miR-203 led to a decreased level of the tumor suppressor, miR-335 activated the expression of *BRCA1*. The negative effect of miR-203 can be traced back to the depletion of activating factors, such as ERα, AhR and IGF1R. As miR-335 also inhibits the expression of stimulating factors of *BRCA1*, a similar effect of both microRNAs could be expected. Despite the reduction of activators following overexpressed miR-335, *BRCA1* is induced. This may be mediated through the inhibition of the dominant negative regulator ID4. These results suggest ID4 and hence miR-335 as key molecules with a superior function for the regulation of *BRCA1*. However, detecting no direct regulation of ID4 by miR-335 in the luciferase reporter system, we cannot rule out that other, yet undefined, repressors of *BRCA1* might be involved in the cascade.

In consequence, miR-335 strikingly reveals a tumor-suppressing function by suppressing mitogenic signaling and activating control mechanisms ensuring genomic integrity. Accordingly, a reduction of miR-335 creates an environment with highly oncogenic features.

To further address this question, a cancer cell model was set up to identify the influence of an altered expression of miR-203 and miR-335 on cancer cell behavior and fate. Interestingly, upregulation of both microRNAs in MCF7 cells led to a reduced cell proliferation with distinct triggers. Overexpression of miR-203 caused a decreasing proliferation rate due to a deceleration of the cell cycle, visualized by an increasing number of cells in the G0/G1 phase. Only weak simultaneous activation of apoptotic mechanisms was detected. The effect of miR-203 on cell cycle progression is probably due to a depletion of mitogenic signals, usually driving the cell through the replication cycle. Since overexpression of miR-203 results in downregulation of ERα, AhR and IGF1R, major mitogenic signal transducers are missing, resulting in a deceleration of the cell cycle. In concordance with this, individual knockdown of these growth-promoting factors has previously been shown to inhibit cell cycle progression (73, 83, 228).

In contrast to the miR-203 mediated effect, overexpression of miR-335 resulted in a striking phenotypic change, characterized by a profound reduction of cell viability and an increase of apoptosis. Cell cycle analysis demonstrated that the decreased proliferation rate is not due to a cell cycle arrest, since the number of cells in the G0/G1-phase remained

stable. Apoptosis is usually induced during the transition from the S- to the M-phase. Comparing the identified targets of miR-203 and miR-335, the latter has distinct functions controlling SP1 and ID4. Since a knockdown of these factors did not reveal a dramatic phenotypic change or an increased apoptosis, further targets might be responsible for this effect of miR-335. Other studies could connect miR-335 to the anti-apoptotic factors SOX4 (214) and Jagged-1 (216). A specific knockdown of the factors results in decreased proliferation and an induction of apoptosis (229, 230). MicroRNA miR-335 might therefore suppress tumor formation by repressing mitogenic factors such as SP1, ERα, AhR, IGF1R and ID4 and transducing pro-apoptotic signals via SOX4 and Jagged-1.

Previous studies also identified BRCA1 as a potent inducer of apoptosis (6). To test whether the pro-apoptotic effect of the microRNA is mediated by the tumor suppressor, overexpression experiments were performed in *BRCA1*-deficient cells. MicroRNA miR-335 overexpression resulted in an earlier apoptotic response compared to wild-type *BRCA1* cells. In contrast to the previous results, miR-203 was able to induce apoptosis in deficient cells, but at a lower magnitude compared to miR-335. First, these results suggest a *BRCA1*-independent effect of the microRNA and second, an increased sensitivity to microRNA-mediated apoptotic signals in *BRCA1*-deficient cells compared to cells expressing active BRCA1. The increased sensitivity to apoptosis-stimulating signals might be due to the insufficiency of survival signals in a genomically unstable background. *BRCA1* deficiency creates an environment that favors both tumor progression and cell death. On the one hand, tumors lacking *BRCA1* show impaired DNA repair that promotes the probability of oncogenic mutations. On the other hand, it leads to a very unstable genomic condition raising sensitivity to DNA damage-inducing agents followed by the induction of cell death (231, 232). In *BRCA1*-deficient cells, depletion of survival and mitogenic signals by microRNAs might have a similar effect leading to an increasing rate of cell death. *Vice versa*, the inhibition of the microRNAs in tumor cells lacking *BRCA1* could lead to a tumor-promoting environment by accelerating cell growth in the absence of a potent DNA damage control mechanism. Regarding the influence of the microRNAs on the initiation complex of *BRCA1*, this might be a self-promoting, tumor-inducing mechanism, since a single microRNA can induce growth-promoting signals and suppress control mechanisms.

To validate the biological relevance of the insights derived from these cell culture experiments, the study was extended to primary breast cells. Thus, a number of sporadic

breast cancer samples and tissues from healthy control donors were analyzed for the expression levels of miR-203 and miR-335. To improve the insight into the regulatory network controlling *BRCA1*, the expression of the tumor suppressor was determined in parallel. As all samples are derived from patients examined at the Hannover Medical School, they are routinely analyzed for the expression of the estrogen and progesterone receptor, Her-2 and the appearance of lymph node metastasis. These features were also integrated into this study by correlating them to the microRNA expression levels.

Compared to the healthy controls, the cancer samples revealed an aberrant expression profile for miR-203, lacking a clear tendency. In relation to normal breast epithelial cells, one group of cancer samples can be classified as miR-203 overexpressing samples, with another demonstrating a clear repression of the microRNA. Analyzing both subgroups, they exhibited a significant difference to the control. These subgroups might represent tumors which are triggered by different alterations. It has been shown before that miR-203 can function as an oncogene and a tumor suppressor gene, depending on the background presented by the host cell (208-212). For breast cancer, similar results were obtained for the estrogen receptor alpha. On the one hand, activation of the receptor leads to formation of breast cancer (56), whereas on the other hand receptor depletion is a poor prognostic factor for cancer progression (58). Likewise, miR-203, which is functionally related to ERα, might have distinct functions at different stages during cancer development. Furthermore, as yet unknown targets of the microRNA can exhibit different effects depending on the cell and tissue status. In tumors expressing low levels of miR-203, a growth-promoting effect can be suspected by activation of ERα, AhR, and IGF1R. All could previously be connected to breast cancer development due to their function as mitogenic signal transducers. In primary breast cancer samples, a reduction in the miR-203 level was accompanied by an increase in the number of ERα-positive samples underlining this theory. Interestingly, a reduced expression of miR-203 correlates with an increased appearance of lymph node metastasis. This supports the idea that as yet undefined targets extend the functionality of miR-203 for breast cancer development and progression.

Analyzing the expression level of miR-335 in breast cancer specimens revealed clearer results. Compared to healthy breast tissues, tumor samples presented a significantly lower miR-335 expression. In concordance with the results obtained from the cell culture experiments, the expression levels correlated positively with the amount of *BRCA1* transcripts. These data further supported the idea of miR-335 acting as a positive regulator of *BRCA1*. Hence, its downregulation in sporadic breast cancer might cause repression of

the tumor suppressor and promotion of tumor formation. Therefore, miR-335 might be one missing link responsible for the downregulation of *BRCA1* in sporadic human breast cancers. In contrast to the expression data of miR-203, miR-335 expression does not inversely correlate with the ERα status. Taking into account that ERα is regulated by at least five validated microRNAs (miR-221, miR-222, miR-18a, miR-206, miR-203 and miR-335), a direct correlation with one single regulator cannot be expected. Extending our attention to the functional effects associated with miR-335, a correlation with metastasis-promoting features was identified (201). In concordance with the findings by Tavazoie and colleagues describing a connection of miR-335 with formation of metastasis, tumors expressing low levels of the microRNA are associated with a decrease of ERα-positive samples. Reduction of the estrogen receptor favors a poor prognosis, in part by supporting the formation of metastasis (58). These results promote the association of a reduced miR-335 expression with a more aggressive tumor type and underline the data of our study.

The data obtained from the analysis of primary breast cancer samples strongly suggest that miR-335 functions as a tumor suppressor microRNA. First, miR-335 is downregulated in sporadic breast cancers. Second, the expression of miR-335 positively correlates with the activation of the tumor suppressor gene *BRCA1* in sporadic breast samples and in a cancer cell model. Third, miR-335 negatively regulates the expression of the known *BRCA1* repressor ID4. Fourth, overexpression of miR-335 is associated with a significant increase in apoptosis. Fifth, miR-335 suppresses the expression of pro-mitotic signal transducers such as ERα, AhR, IGF1R and SP1. Sixth, miR-335 reduction is accompanied by reduced levels of ERα, supporting pro-metastatic features of the microRNA. MicroRNA miR-335 therefore inhibits tumor formation and progression on two independent ways multiplying each other. On the one hand it functions anti-mitogenically by suppressing ERα, SP1, IGF1R and AhR and pro-apoptotically by controlling SOX4 and Jagged-1. On the other hand, it ensures genomic stability by activation of the key factor *BRCA1*, through repression of its dominant repressor ID4. These functions also multiply each other in opposite directions, when miR-335 is downregulated in tumors, leading to accelerated tumor growth, genomic instability and cancer progression.

Having proven the importance of miR-203 and miR-335 for human sporadic breast cancer, the underlying mechanisms leading to an altered expression of the microRNAs can next be questioned. Here, miR-203 and miR-335 represent two very different types of microRNAs.

While the first is located in an intergenic region on chromosome 14, the latter is a member of the intronic microRNAs, termed “mirtrons”. Therefore, miR-203 is regulated by an independent promoter, whereas miR-335 is co-transcribed with its host gene *MEST/PEG1* and is activated following splicing of the primary mRNA. Recently, the interaction of intronic microRNA promoter regions with the DNA polymerase II was detected, suggesting also an independent transcriptional control of the “mirtrons” (145).

Detailed analysis of the promoter region of *miR-203* revealed a binding motif for the transcription factor SP1. Interestingly, this interaction site is overlaid by a region of epigenetic regulation through cytosine methylation. This CpG island spans the major part of the proximal promoter region including the SP1 binding motif. To clarify the influence of SP1 on the expression of miR-203, the protein amount of the transcription factor was analyzed in various cancer cell lines and correlated with the expression of miR-203. The results revealed only a weak correlation between the two expression levels. While the miR-203 level showed high variance between the cell lines, the amount of SP1 was about equal in all cell lines. These data suggested an epigenetic influence on the regulation of miR-203. Promoter methylation is a frequent event in microRNA regulation (233) and was previously shown to control the expression of miR-203 in other cancer types (179, 210). In agreement with the expression level of the microRNA, there was complete methylation of the promoter region in cancer cell lines with low or absent expression of miR-203. Conversely, tumor cells with increased levels of the microRNA revealed no methylation of the CpG island in the proximal region of the gene. Accordingly, the expression of miR-203 was induced following the treatment with a demethylating reagent in methylated cell lines. This supports the idea that binding of activating factors to the promoter of *miR-203* is prevented by methylation of crucial binding motifs. To determine whether SP1 is a potent activator of miR-203, an unmethylated cancer cell line was chosen for functional assays. Here, specific knockdown of SP1 led to a decrease of miR-203 expression. To transfer these insights from the cell line system to primary breast cells, the expression of SP1 was analyzed in a range of sporadic breast tumors, predetermined for their expression level of miR-203. In contrast to the direct correlation of the microRNA and its activator in the cell culture model, no connection could be identified in primary breast cancers. While SP1 is ubiquitously expressed on the transcript level in all tumor samples and healthy controls, the protein amount was increased in cancer cells. This finding is in line with other studies describing an increased expression of the transcription factor in other cancer types (99, 234). The lack of a direct correlation between SP1 and the expression of miR-203 suggest

that additional factors are involved in the regulation of the microRNA. Having identified promoter methylation to be relevant in cell cultures, primary breast samples were studied for their methylation status. The analysis of a number of tumors gave one specimen with a hypermethylated microRNA promoter. These data suggest a biological relevance of the epigenetic modification. Conversely, the tumor samples exhibit a decreased expression of miR-203, while the SP1 protein amount was not altered compared to the healthy controls. Although at a decreased level, miR-203 expression was detectable in this hypermethylated tumor sample, which is explained by the heterozygous occurrence of the methylating event.

In addition, the promoter region of miR-203 harbored direct binding motifs for the estrogen receptor α. Cell culture experiments in this study underline the activating effect of ERα on the expression of the microRNA.

In summary, the expression of the microRNA miR-203 is controlled by the transcription factors SP1 and ERα, as well as by epigenetic modifications in terms of cytosine methylation. Taking into account that the SP1 level does not correlate with miR-203 expression and that hypermethylation seems to be a rare event in primary cancers, additional factors such as ERα are likely to be involved in the regulation of the microRNA.

As an intronic microRNA, the expression of miR-335 is predominantly directly linked to its host gene *MEST/PEG1* (235) located on chromosome 7q31. Interestingly, this locus has previously been shown to be deleted by LOH in 41% of breast cancer samples (219). LOH of this region was associated with a higher frequency of metastasis and shorter overall survival. This event might also explain the significant downregulation of miR-335 in breast cancer samples analyzed in this study and underlines the importance of miR-335 for breast cancer progression. The miR-335 host gene *MEST/PEG1* encodes for three different transcript variants. The coding region of the microRNA is located in the second intron included in all variants. Pederson and colleagues described a frequent loss of imprinting (LOI) of the gene in cancer cells (236).

As intronic microRNA expression is also controlled by host gene-independent promoters, the 5´ region of miR-335 was analyzed for putative regulatory binding sites. Interestingly, the promoter region of miR-335 revealed direct binding motifs for ERα, for which the impact on the microRNA expression was proven in this study. Since both miR-203 and miR-335 were established as repressors of the hormone receptor, these results indicate a feedback-loop to control the network. This might be a common self-regulatory mechanism

of ERα, since BRCA1, which itself is activated by estrogens, was shown to negatively regulate the function of the receptor (89).

Comparing the identified targets of miR-203 and miR-335 suggests that the latter performs a dominant function in the regulation of *BRCA1* by controlling the expression of ID4. The superior regulating function of miR-335 might therefore be mediated and dependent on the dominant-negative repressor. Since the effect of miR-335 on the expression of *BRCA1* was already proven in primary tissues, the expression level of *ID4* was analyzed in the identical sample cohort. Despite the fact that both expression levels decreased in tumor specimens compared to normal tissues, a significant inverse correlation between the transcript level of *ID4* and miR-335 was detected in the cohort of tumor samples. Analyzing the transcript level to detect a post-transcriptional regulation is a common approach for high-throughput screening, however, microRNAs predominantly function as translational inhibitors. The resulting hits consist of secondary effects, but also of primary targets of the microRNAs (237). This is not due to a direct cleavage of the miRISC, but is thought to be a result of a shortening of the poly(A)-tail (122) and of a complex storage in the P-bodies, which are rich in RNA-degrading enzymes (238). Therefore, the identified inverse correlation between miR-335 and *ID4* mRNA in breast cancer samples along with previously described results strongly suggests *ID4* as a primary target of the microRNA *in vitro* and *in vivo*.

Interestingly but not surprisingly, a striking discrepancy between the transcript and protein level of ID4 was detected. While the cancer samples analyzed in this work exhibited a dramatic decrease in *ID4* mRNA, a previous study could identify an increase in the ID4 protein amount. This is in concordance with the miR-335 expression analysis showing lower levels of the microRNA in breast cancer samples compared to normal tissue controls. The dramatic reduced expression of *ID4* on the transcript level might be the result of a feedback-mechanism mediated by suppressed BRCA1, which acts as an activator of *ID4* (49). Despite the repression on the transcript level of *ID4*, the reduction of miR-335 leads to an increase of ID4 protein expression. Together, these data suggest that miR-335 not only controls ID4 in the cell culture model, but also regulates its expression in the breast tissue.

Furthermore, ID4 seems to be involved in the microRNA function independent of *BRCA1*. This becomes strikingly clear when miR-335 is overexpressed parallel to ID4. A

simultaneous expression resulted in an abolished function of the microRNA. In detail, the pro-apoptotic effect of miR-335 that was shown to be *BRCA1*-independent was completely compensated, when ID4 was co-expressed. This implies that ID4 is located downstream in a linear signal cascade activated by miR-335. Overexpression of the microRNA downregulates ID4, thereby transducing pro-apoptotic signals. This cascade is disrupted when ID4 is overexpressed in parallel. Further experiments could exclude an exclusive role of ID4 in this cascade, since a knockdown of the repressor resulted in decreased proliferation, without induction of apoptosis. Despite that fact, ID4 appears as a key factor in the regulatory cascade of miR-335 by inducing a strong survival signal to the cells and repressing the expression of *BRCA1*.

In consequence, the overexpression of ID4 in primary cancer samples transduces strong survival signals. It inhibits the decelerating function of *BRCA1* on cell proliferation (binding ERα, SP1, suppression of *IGF1R*) on the one hand and its assurance of genomic stability on the other, driving the cell more rapidly through tumor progression. Reduction of miR-335 further increases the tumorigenic potential not only by promoting the expression of ID4, but also by increasing translation efficiency of the mitogenic factors SP1, ERα and AhR.

The event of *BRCA1* disruption by inactivating mutations as is the case for inherited breast cancer serves as a good model for the development of sporadic breast cancers. Although a mutational loss of function is not the event of *BRCA1* inactivation in the sporadic form, one can assume that *BRCA1* also plays an important role in sporadic breast cancer. BRCA1 generally functions as a key component for the maintenance of genomic integrity. However its deregulation is predominantly associated with hormone-controlled tissue types such as the breast and the ovary, intimating that there are distinct functions or regulatory events in these cells. BRCA1 function and regulation is surrounded by an intense regulated network involving various co-factors. It is notable that crucial key factors regulating *BRCA1* and transmitting its function are hormone-dependent, suggesting a function and regulation in a hormone-dependant manner. Here, the estrogen receptor alpha exhibits a key position since it activates *BRCA1* expression and also interacts with the protein. The ligand-bound receptor has high mitogenic activity, which is blocked following binding to BRCA1, thereby forming a balanced network to ensure tissue integrity. In the inherited form of breast cancer, this equation is shifted to the growth-promoting function of ERα, since the repressive effect of BRCA1 is missing. A similar model can be assumed for

sporadic breast cancer, replacing the mutation by a deregulated control mechanism. Extending the model from the estrogen receptor to the actual number of involved components, there are several intersections where aberrations can disrupt this balanced status.

This work describes the relation between these factors and adds novel components to the complex. Here, two microRNAs were established with superior functions forming a cross-talking network in the regulation of *BRCA1* and growth signal transduction (Fig. 35A). The microRNAs miR-203 and miR-335 were shown to share identical target molecules, but also to exhibit distinct functions regarding *BRCA1* regulation and cancer cell behavior. The miR-335 possesses a superior function by activating *BRCA1* through repression of ID4, inducing apoptosis and repressing mitogenic signaling. Therefore, this work suggests that miR-335 acts as a tumor suppressor microRNA in breast epithelial cells. Thus, its downregulation promotes tumor formation in this tissue, as proven in primary samples. Repression of miR-335 triggers two major cascades leading to a growth-promoting and anti-apoptotic environment (Fig. 35B). On the one hand, its downregulation results in lack of repression of ERα, AhR, IGF1R and Sp1, thereby supporting proliferation. On the other, it induces the *BRCA1* repressor ID4 leading to suppression of a key factor for genomic stability.

Characterization of miR-203 still remains a difficult task, since its expression is associated with both oncogenic and tumor-suppressive features. This is underlined by its expression profile in primary samples. It is certain that miR-203 is a component of the regulatory cascade of miR-335 since the first is regulated by SP1, which is controlled by the latter. However, future studies will have to further characterize the significance of miR-203 for breast cancer development and progression.

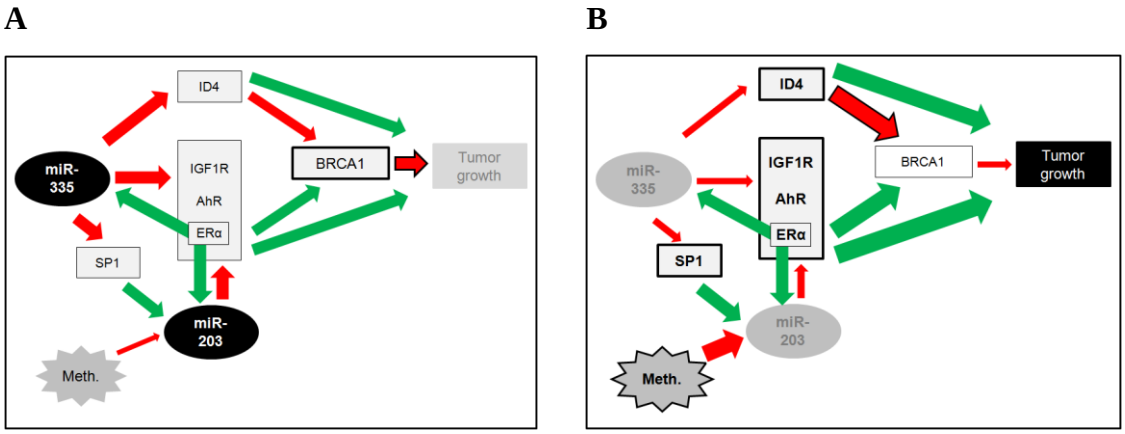


Fig. 35: An altered expression of miR-203 and miR-335 promotes tumor formation. Model of *BRCA1* regulation and tumor promotion in normal cells (A) and its deregulation in breast tumors (B). The green arrows indicate an activating effect, whereas the red arrows illustrate a repressing event. The size of the arrows represents the intensity of the effects.

This is one of the first studies identifying single microRNAs as key molecules for the regulation of an entire pathway related to breast cancer. Considering the importance of other microRNAs for key factors such as p53 or c-MYC, one can speculate that microRNAs are crucial components for cancer diagnosis and therapy in the future. The microRNAs identified here might therefore participate as prognostic markers or as effectors for a targeted therapy in future trials.

7 Future perspectives

Regarding the successful identification of microRNAs involved in breast cancer tumorigenesis and the importance for the regulatory cascade of *BRCA1*, the future perspectives have to be divided into two subgroups. On the one hand, the microRNAs miR-203 and especially miR-335, identified in this study, have to be studied in more detail, regarding their functionality *in vivo* and their involvement in breast cancer predisposition. On the other hand, other microRNAs might participate in the regulatory pathway of *BRCA1* to increase the complexity of the cascade and thereby enhancing its sensitivity.

The identified miR-203 and miR-335 present profound tumor-suppressive features, in terms of the regulation of apoptotic signaling and the stabilization of the genome via *BRCA1*. Consequently, they might be a promising candidate to prevent tumor formation *in vivo*. To address their importance for breast cancer and for potential therapeutic strategies, the effect of an altered miR-203 and miR-335 expression on normal breast cells has to be determined. Here, overexpression or knockdown of both microRNAs in human mammary epithelial cells (HMECs) might extend the previous experiments to a non-tumor model and give a first impression of its value in a specific tumor treatment.

Subsequently, a mouse model might give further insights of the relevance of reduced miR-203 and miR-335 expression for breast cancer development, progression and the aggressiveness. Here, in a xenograft model, subcutaneously injected cells with downregulated miR-203 and miR-335 expression are supposed to gain a growth advantage and to reveal an enhanced potential to form metastasis. Conversely, re-expression of the microRNA should reduce the tumor formation. In addition, these experiments could determine whether expression of miR-203 and miR-335 can prevent the formation of tumors, and whether they are also able to reduce preexisting tumors. Inducible induction of microRNA expression can be achieved by stably transfecting cell lines with suitable expression vectors as shown by Kumar and colleagues in a lung cancer model (133).

To get a deeper insight of the impact of both microRNAs for sporadic breast cancer formation and their potential as clinical marker, the expression level in a greater cohort of primary tumors with profound knowledge on biological markers, survival rates, and response to therapies has to be evaluated. These data might further establish miR-203 and miR-335 as diagnostic tool and prognostic marker.

Furthermore, germline mutations in miR-203 and miR-335 might predispose to breast cancer development. Here, families with breast cancer history showing no mutations of *BRCA1* and *BRCA2* might harbor mutations in the microRNAs, representing an additional marker for breast cancer predisposition. Disruption of the second allele of miR-203 or miR-335 might be an early event with multiple tumorigenic effects. Germline mutations of miR-15/16 were previously associated with predisposition for leukemia (181). A knockout mouse model might clarify, if miR-203 and miR-335 are essential for a normal development and if disruption of the microRNA is sufficient to force tumor formation. Here, overexpression of breast cancer specific oncogenes, such as HER-2 or estrogen supplementation in parallel might result in additive or synergistic effects.

These and further studies might strengthen the importance of miR-203 and miR-335 for breast tumor formation and distinguish their potential for cancer therapy.

In addition to miR-203 and miR-335, a number of other microRNAs might participate in breast cancer formation and progression. Here, microRNAs that have multiple targets in the cascade of *BRCA1* might also be suppressed in sporadic breast cancer or mutated in the inherited form. *Vice versa*, microRNAs that bind directly to the 3´ UTR of *BRCA1* might be overexpressed.

Summarizing, miR-203 and miR-335 have the potential to be valuable for breast cancer prognosis and might be a specific therapeutic target in the future. In general, microRNAs were shown to participate in crucial cellular mechanism as superordinated regulators and will therefore be of great interest in future cancer diagnostics and targeted therapy.

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9 List of abbreviations

ATCC	American Type Culture Collection
AhRE	Aromatic hydrocarbon response element
BASC	BRCA1-associated surveillance complex
BAT	BRCA1-associated transcriptional complex
Bp	Base pairs
CA	Carcinoma
cDNA	complementary DNA
C. elegans	Caenorhabditis elegans
ChIP	Chromatin immunoprecipitation
Ct	Threshold Cycle
CTD	C-terminal domain
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
e.g.	Exempli gratia (for example)
ERE	Estrogen response element
FACS	Fluorescence activated cell sorter
FF	Forward
Fig.	Figure
G0/1-phase	Gap phase 0/1
HCC	Hepatocellular carcinoma
HLH	Helix-loop-helix domain
HMAC	Human mammary epithelial cell
IHC	Immunohistochemistry
hnRNP	Heterogeneous ribonucleoprotein particles
LOH	Loss of heterozygocity
LOI	Loss of imprinting
M-Phase	Mitotic phase
miR	MicroRNA
miRISC	MicroRNA-induced silencing complex
miRNP	MicroRNA ribonucleoproteins
mRNA	Messenger RNA
MSP	Methylation specific PCR
nt	Nucleotids
PAGE	Polyacrylamide gel electrophoresis
P-bodies	Processing bodies
PCR	Polymerase chain reaction
PIWI	RNaseH-like P-element induced wimpy testis domain
Pol.II	DNA polymerase II
qRT-PCR	Quantitative real-time PCR
RING	Really interesting new gene
RNAi	RNA interference
RNP	Ribonucleoprotein particles
RV	Reverse
SD	Standard deviation
S-phase	Synthesis phase
siRNA	Small interference RNA
ssDNA	Single stranded DNA

Tab.	Table
UTR	Untranslated region
XRE	xenobiotics response element

9.1 List of abbreviations (chemicals)

5-AZA	5-Aza-2´-deoxycytidine
Bio-16-UTP	Biotin-16-uridine-5'-triphosphate
BSA	Bovine serum albumin
CO2	Carbone dixide
DMEM	Dulbecco's Modified Eagle Medium
E2	ß-estradiol
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
MEM	Modified Eagle Medium
MRE	MicroRNA response element
NaAc	Sodium acetate
NaCl	Sodium chloride
NFDM	Non fat dry milk
NP40	Nonidet P-40
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonylfluorid
RIPA	Radio immunoprecipitation assay buffer
RPMI	Roswell Park Memorial Institute Medium
SDS	Sodium dodecyl sulfate
SSC	Saline-Sodium Citrate
Tris	Tris(hydroxymethyl)aminomethane
WST	water soluble tetrazolium

9.2 List of abbreviations (genes and proteins)

ABL1	Abelson murine leukemia viral oncogene homolog 1
AGO	Argonaute
AhR	Aryl-hydrocarbon receptor
AIB1	Amplified in breast cancer-1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ARNT	Aryl hydrocarbon receptor nuclear translocator
BACH1	BTB and CNC homology 1
BARD1	BRCA1-associated RING domain gene 1
BAX	BCL2-associated X protein
BCR	Breakpoint cluster region
BRCA1/2	Breast cancer 1/2
CAD	Carbamoylphosphate synthetase 2/aspartate transcarbamylase/dihydroorotase

CDK	Cyclin dependant kinase
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
DGCR8	DiGeorge syndrome critical region gene 8
E2F	E2F transcription factor
E47	E2A immunoglobulin enhancer binding factors E12/E47
EIF4E	Eukaryotic translation initiation factor 4E
ERα/β	Estrogen receptor α/β
ESR1	Estrogen receptor 1
FAS	TNF receptor superfamily, member 6
FOS	FBJ murine osteosarcoma viral oncogene homolog
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GW182	Eukaryotic translation initiation factor 2C, 1
GFP	Green fluorescence protein
HER-2	Erythroblastic leukemia viral oncogene homolog 2
HOXD10	Homeobox D10
ID4	Inhibitor of DNA binding 4
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor I receptor
JUN	Jun oncogene
KRAS	Kirsten rat sarcoma viral oncogene homolog
Let-7	Lethal 7
Lin-28	Abnormal cell lineage 28
LNGFR	Low affinity nerve growth factor receptor
MAPK	Mitogen-activated protein kinase
MEST	Mesoderm specific transcript homolog
MYC	Myelocytomatosis viral oncogene homolog
MyoE	Myosin IE
NFkappa	Nuclear factor kappa
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
P300	E1A binding protein p300
P450	Cytochrome P450, family 2, subfamily B, polypeptide 6
P53	Tumor protein p53
P63	Tumor protein p63
PABP1	Poly(A) binding protein, cytoplasmic 1
PEG	Mesoderm specific transcript homolog
PDCD4	Programmed cell death 4
PR	Progesterone receptor
RAD51	DNA repair protein RAD51 homolog 1
RAS	Rat sarcoma
RB	Retinoblastoma 1
RHOC	Ras homolog gene family, member C
sn-U6	U6 small nuclear 1
SOX4	SRY (sex determining region Y)-box 4
SP1	Specificity protein 1
TCBP	T-cluster binding protein
TGFβ	Transforming growth factor beta
TPM1	Tropomyosin 1
TRBP	TAR RNA binding protein
TWIST1	Twist homolog 1
ZBTB10	Zinc finger and BTB domain containing 10

10 Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation mit dem Titel „Identification of microRNAs miR-203 and miR-335 forming a network of regulation in breast cancer development“ selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den

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(Unterschrift)

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11 Curriculum Vitae

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