

Roles of microRNA-140 in stem cell-associated early stage breast cancer

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Abstract

An increasing body of evidence supports a stepwise model for progression of breast cancer from ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC). Due to the high level of DCIS heterogeneity, we cannot currently predict which patients are at highest risk for disease recurrence or progression. The mechanisms of progression are still largely unknown, however cancer stem cell populations in DCIS lesions may serve as malignant precursor cells intimately involved in progression. While genetic and epigenetic alterations found in DCIS are often shared by IDC, mRNA and miRNA expression profiles are significantly altered. Therapeutic targeting of cancer stem cell pathways and differentially expressed miRNA could have significant clinical benefit. As tumor grade increases, miRNA-140 is progressively downregulated. miR-140 plays an important tumor suppressive role in the Wnt, SOX2 and SOX9 stem cell regulator pathways. Downregulation of miR-140 removes inhibition of these pathways, leading to higher cancer stem cell populations and breast cancer progression. miR-140 downregulation is mediated through both an estrogen response element in the miR-140 promoter region and differential methylation

of CpG islands. These mechanisms are novel targets for epigenetic therapy to activate tumor suppressor signaling *via* miR-140. Additionally, we briefly explored the emerging role of exosomes in mediating intercellular miR-140 signaling. The purpose of this review is to examine the cancer stem cell signaling pathways involved in breast cancer progression, and the role of dysregulation of miR-140 in regulating DCIS to IDC transition.

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Key words: Breast cancer; Ductal carcinoma *in situ*; Invasive ductal carcinoma; Cancer stem cells; MicroRNA-140

Core tip: MiR-140 is an important tumor suppressor. By inhibiting stem cell growth through interaction with the Wnt, SOX2 and SOX9 pathways, breast cancer initiation, progression and growth are reduced. miR-140 is progressively downregulated as breast cancer grade decreases, through both estrogen binding and differential methylation in the miR-140 promoter region. By targeting these mechanisms using epigenetic therapy miR-140 tumor suppressor signaling can be reactivated.

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INTRODUCTION

Breast cancer is a heterogeneous disease comprised of several histologic and molecular subtypes. Transformation from normal mammalian epithelial cells to aggressive malignancy is due to the accumulation of numerous genetic and epigenetic changes. Early breast cancer such as

ductal carcinoma *in situ* (DCIS) exhibit similar patterns of gene and protein expression to invasive ductal carcinoma (IDC), suggesting a stepwise model of non-obligate precursor^[1]. Following benign proliferative changes to the ductal lumen, atypical ductal hyperplasia (ADH), DCIS and IDC are more likely to occur^[2]. Molecular signatures for development and progression of breast cancer are poorly established, due to limited data for early lesions. Classification systems based on histological features and proliferation rate are useful in patient management to some extent, and are used to assign DCIS a grade of low, intermediate or high. The distinction between low grade DCIS and ADH is somewhat subjective, as they maintain many molecular and genetic similarities. High grade DCIS is much more likely to progress to IDC and is associated with increased likelihood of recurrence^[1]. Currently there is no way clinicians can predict if a DCIS lesion will progress to IDC, which would improve therapeutic management.

DCIS treatment is able to prevent progression from early stage breast cancer, but therapeutic options are lacking. DCIS lesions are heterogeneous with treatment success varying for the different molecular subtypes. Lumpectomy and radiation therapy remain the standard of care in most cases of DCIS. Estrogen receptor positive DCIS patients benefit from Tamoxifen treatment, but no molecularly targeted treatment is available for basal lesions^[2].

In contrast to the shared genetic and epigenetic alterations of IDC and DCIS, mRNA/miRNA expression profiles are significantly altered. Deep sequencing of DCIS and IDC lesions has identified differential miRNA signatures that may be involved in the acquisition of an invasive phenotype. miR-140-3p downregulation was observed for all investigated groups of IDC and DCIS patients, leading our lab to investigate potential tumor suppressive roles^[3].

Here we will review the underlying mechanisms behind microRNA-140 dysregulation in breast cancer. We will discuss the role of cancer stem cells in the DCIS to IDC transition and the importance of microRNAs in regulating breast cancer stem cells. Briefly, we will discuss the emerging role of exosomal miRNAs as intercellular signaling molecules. Finally, we will discuss possible therapeutic avenues for modulating miRNA signaling in breast cancer and highlight the potential for epigenetic therapies to activate tumor suppressor miRNAs.

MICRORNA BIOGENESIS

MiRNAs are short noncoding RNA molecules, approximately 22 nucleotides in length, which bind primarily to the 3' untranslated region (UTR) of messenger RNAs. The primary function of miRNAs is to regulate gene expression. miRNAs function through targeting mRNA for degradation or translational inhibition. mRNA is targeted through a semi-complementary seed sequence (6-9 bp) in miRNAs, which guides binding to the miRNA response

elements. Each seed sequence potentially matches hundreds of mRNA molecules, giving the miRNA many potential targets^[4]. Most mammalian miRNA genes are found in well-defined transcriptional units, and can be in either intronic or exonic regions in non-coding transcriptional regions, or as intronic miRNAs in coding regions^[5].

The primary miRNA transcript (pri-miRNA) genes are transcribed predominantly by RNA polymerase II, although other isoforms may be involved. Pri-miRNA is cleaved at the 5' and 3' ends by the Microprocessor complex, comprised of ribonuclease III Droscha and RNA-binding protein DGCR8, forming the pre-miRNA. The approximately 70 nucleotide stem-loop pre-miRNA is transported out of the nucleus by exportin-5 and Ran-GTP. In the cytosol the RISC loading complex, composed of RNase III Dicer, Argonaute-2, and double-stranded RNA-binding domain proteins Tar RNA binding protein (TRBP) and protein activator of PKR (PACT), facilitates pre-miRNA processing and RISC assembly^[6]. Dicer cleaves the pre-miRNA near the hairpin loop, forming a 20-23 nucleotide long miRNA duplex. The miRNA duplex incorporates into the RNA induced silencing complex (RISC), where it is unwound, isolating the guide strand while the complementary strand (miRNA*) is degraded by RISC^[6,7].

MiRNA dysregulation often occurs through modification of key enzymes associated with biogenesis. Specifically, loss of Dicer expression has been observed in many cancers, including breast cancer^[8]. This results in decreased miRNA expression, and is associated with breast cancer progression^[9]. Dysregulation occurs through a wide variety of genetic and epigenetic mechanisms, deletion or amplification of the miRNA genes, transcriptional activation and suppression, as well as epigenetic dysregulation, *i.e.*, methylation of CpG islands^[10].

MIR-140 IN CHONDROCYTES

MiR-140 was first identified as regulating cartilage development in chondrocytes^[11]. The primary transcript of miR-140 is found in intron 16 of the E3 ubiquitin protein ligase WWP2 gene on chromosome 16, and mature miR-140 is co-expressed with Wwp2-c. MiR-140 expression is induced by SOX9 binding to intron 10 of the WWP2 gene^[12], inhibition of SOX9 by Wnt/ β -catenin signaling has been demonstrated to suppress miR-140 in certain cell lines^[13].

MiR-140 promotes chondrocyte proliferation by targeting of transcription factor Sp1, leading to cell cycle inhibition^[12]. MiR-140 has also been found to suppress HDAC4, promoting cartilage differentiation^[14]. Additionally, miR-140 plays an important role in protecting against diseases of cartilage destruction through regulation of protease Adamts-5^[11]. MiR-140 has also been identified in other tissues, including breast, brain, lung, ovary and testis. A potential tumor-suppressive role has been identified, as miR-140 is down regulated in ovarian, lung, colon, osteosarcoma and breast carcinomas^[13].

In the majority of miRNA species, the 5-prime miRNA is annotated as the guide strand, while the complementary 3-prime miRNA* is degraded. Rakoczy *et al.*^[15] found that in testis and chondrocytes, miR-140-3p is more highly expressed than miR-140-5p, and likely has its own function. Our lab has observed this in breast tissue. MiR-140-3p and miR-140-5p have different seed sequences, and thus have a different set of target genes, many of which may not yet be known^[15]. The miRNA guide strand is chosen based on thermodynamic stability, with the strand that has relatively unstable base pairs at the 5' end remaining^[5]. Uracil-bias at the 5'-end of the highly expressed strand, cysteine-bias at the 3'-end of the low expressed strand and an excess of purines in the low expressed strand have also been identified as determinants of strand selection^[16]. However, the mechanism of strand selection is still unknown.

BREAST CANCER STEM CELLS

Cancer stem cells (CSCs) were first discovered in hematopoietic malignancies. They are believed to comprise a small subpopulation of cancer cells that have the ability to self renew and differentiate into heterogeneous tumor lineages. CSCs have an important role in resistance to chemotherapy and disease recurrence, a dangerous combination that allows them to survive treatment and regenerate the tumor leading to treatment failure^[17]. Overexpressed ABC transporters mediate the resistance of CSCs to most current chemotherapeutics^[18]. In order to cure cancer, therapeutics must be developed in conjunction with debulking therapies that can specifically eliminate cancer stem cells.

Isolation and characterization of CSC

There are a number of assays used to isolate and characterize cancer stem cells, the gold standard being the ability of a small number of cells obtained by serial dilution to initiate a tumor in NOD/SCID mice. Fluorescence-activated cell sorting (FACS) can be used to study cell surface markers associated with the cancer stem cell population. Further assays test common attributes of stem cells. Aldehyde Dehydrogenase 1 (ALDH1) activity is detectable by the Aldefluor assay. The presence of a "side-population" in FACs when cells are treated with Hoechst 33342 dye is an indicator of increased ABC transporters, which expel Hoechst 33342.

Stem cell surface markers were first identified in human acute myeloid leukemia. The CD34+/CD38- subpopulation is able initiate tumors histologically similar to the parent tumor from a low cell count in NOD/SCID mice^[19]. Using a similar approach, cancer stem cells were identified in breast cancer as a CD44+/CD24- lineage. A small number of cells from this lineage are able to initiate xenografts and differentiate into heterogeneous tumors. This population also shares the extensive proliferative capacity and ability to self renew identified in hematopoietic cancer stem cell populations^[20].

DCIS stem cells

Previous studies have shown that cancer stem cells exist in DCIS lesions and may determine the malignant potential of the cancer. Unsorted cell populations from human DCIS lesions were able to form mammospheres under non-adherent conditions, as well as initiate tumors in NOD/SCID mice^[21]. We identified a cancer stem cell population within basal-like DCIS identified by ALDH1+ and CD49f+/CD24- cells. This group possesses enhanced migration and self-renewal capacity, and initiates fast growing tumors in nude mice. It is possible that this population is involved in progression of DCIS lesions to IDC and serves as a malignant precursor cell^[22]. We investigated stem cell signaling in both DCIS and triple negative invasive breast cancer models, focusing on stem cell regulators SOX2 and SOX9.

CSC signaling

There are a number of pathways associated with de-regulated self-renewal in cancer stem cells, including the Notch, Sonic hedgehog, Wnt, and Pluripotency factor pathways^[18]. Dysregulation in these signaling pathways is common in breast cancer. The Notch pathway is involved in breast development, and dysregulation is an early event in DCIS. Notch is up regulated in breast cancer stem cells^[23], and may be involved in DCIS stem cell mediated progression to IDC. The Wnt pathway is involved in regulation of stem cell proliferation. Deregulation of Wnt signaling and proliferation predisposes to cancer^[24]. Overexpression of Wnt is correlated with increased mammary tumor formation^[25], an event mediated by cancer stem cells. Sonic hedgehog is also involved in regulating self-renewal of mammary stem cells as well as inhibiting differentiation, potentially through the Notch signaling pathway^[26]. Hijacking of embryonic pluripotency factors (OCT4, SOX2, KLF4) has also been reported in cancer stem cells. Sry-related HMG box 2 (SOX2) has been reported to be an oncogene in early stage breast cancers^[27]. Furthermore, we have identified a critical role for the related HMG-box protein SOX9 in DCIS stem cells^[28].

SOX2 and SOX9

SOX9 transcription factor is an important stem cell regulator and works cooperatively with Slug to promote tumorigenesis and cancer initiation. Slug is an epithelial-mesenchymal transition transcription factor, upregulated in mammary stem cell populations. When coexpressed with SOX9, differentiated mammary epithelial cells are converted into mammary stem cells^[29]. SOX9 is overexpressed in a number of breast malignancies, and is necessary for mammosphere formation of basal DCIS cell lines. SOX9 expression increases with DCIS grade^[28]. In basal like, IDC cell lines, expression of both Slug and SOX9 is necessary for tumor initiation; SOX9 is also necessary for maintaining tumorigenicity^[29]. This may demonstrate a relationship between risk of progression from DCIS to IDC and an increase in cancer stem cell population.

SOX2, OCT4 and NANOG form a complex that binds promoters of numerous differentiation factors. Dysregulation of any member of this complex leads to aberrant self-renewal, a primary characteristic of cancer stem cells^[27]. Overexpression of SOX2 is a common mechanism of aberrant self-renewal signaling, and is required for tumor-initiation. Stable knockdown of SOX2 in MCF-7 breast cancer cells results in a significant decrease in the CD44 high/CD24 low stem cell population. SOX2 overexpression increased this population, as well as increasing mammosphere formation, the ability of breast cancer stem cells to grow in a non-adherent culture^[27].

A major risk factor for breast cancer is estrogen exposure. Mammary tumor formation is mediated through a combination of toxic estrogen metabolites and estrogen receptor signaling affecting survival and proliferation^[30]. Estrogen has been shown to increase the frequency of the CD44+/CD24- subpopulation through ER α association with the OCT4 promoter, potentially affecting self-renewal signaling through the OCT4/SOX2/NANOG complex^[27]. In ER positive breast cancer cells we have found that ER signaling can indirectly regulate SOX2 levels, one mechanism through which ER signaling may impact stem cell signaling in breast cancer.

MIR-140 IN THE DCIS TO IDC TRANSITION

To further interrogate the DCIS to IDC transition, we performed microarray profiling of DCIS lesions and matched normal tissue and compared our results to published deep sequencing datasets. We identified miR-140 loss as a reproducible marker of DCIS lesions. The level of miR-140 downregulation increases as DCIS grade increases and progresses to invasive ductal carcinoma (IDC), demonstrating a potential role in disease progression.

Role of miR-140 in DCIS stem cells

For patients with ER positive DCIS, adjuvant tamoxifen treatment significantly reduces recurrence and disease progression. However, for patients with basal like DCIS there are no available molecularly targeted therapeutics. In addition, basal like DCIS is a particularly aggressive form of DCIS (often also classified as comedo-type DCIS) frequently detected with concomitant IDC lesions. As such, we chose to continue our investigation into the tumor suppressor roles of miR-140 in a model of basal-like DCIS. Knockdown of miR-140 in 3D cell culture resulted in increased proliferation, as well as a decrease in acinar apoptosis, indicating a role for miR-140 in differentiation or stem cell signaling in mammary stem cells. Further investigation into the potential role of miR-140 in DCIS stem cells revealed dramatic loss of miR-140 in DCIS stem cells compared to normal mammary stem cells. We identified a CpG island in the miR-140 promoter with differential methylation, and validated its function using epigenetic therapy. This demonstrated that downregulation might be mediated through epigenetic mechanisms.

Predicted miR-140 targets SOX9 and ALDH1 are dramatically upregulated in DCIS stem cells compared to parental cell lines with miR-140 expression. Targeting of both by miR-140 was validated using luciferase reporters for either the SOX9 or ALDH1 3'-UTRs. Restoration of miR-140 in DCIS cells significantly reduced mammosphere formation, suggesting miR-140 negatively regulates DCIS stem cell renewal. Similarly, SOX9 overexpression/knockdown resulted in mammosphere formation suggesting that a miR-140/SOX9 pathway may be an important regulator of DCIS stem cells. DCIS tumor growth in nude mice was significantly reduced when miR-140 was overexpressed. When stem-like mammosphere cells were used to initiate xenografts, tumor growth and initiation was much faster than whole cell population. miR-140 overexpression was again able to almost completely eliminate growth of DCIS tumors^[28].

Role of miR-140 in IDC stem cells

In order to interrogate the role miR-140 plays in breast cancer, we investigated miR-140 expression in estrogen receptor positive invasive breast tumor cells. We found that miR-140 expression is inversely related with SOX2 expression. Tissue staining of ER α + IDC revealed a significant increase in SOX2 expression, and qRT-PCR revealed a dramatic downregulation in miR-140 expression. A luciferase reporter assay for the 3'-UTR of SOX2 showed that miR-140 directly targets and inhibits SOX2 expression, and mammosphere assays demonstrated that miR-140 targeting regulates stem cell signaling in tumors. While examining the molecular mechanisms regulating miR-140 expression we identified predicted estrogen response elements (ERE) in the miR-140 promoter region. Due to the previous reports linking ER α and self-renewal signaling, we investigated a potential ER α miR-140 relationship. In non-tumorigenic cells engineered to express ER α , E2 treatment significantly inhibited miR-140 expression, while also stimulating SOX2 expression. We examined the miR-140 promoter using a luciferase reporter and found that E2-mediated miR-140 downregulation was decreased when the ERE at -79/50 in the miR-140 promoter was mutated. Binding of ER α to the miR-140 promoter was validated using ChIP. In the absence of estrogen, miR-140 expression had very little effect on cancer stem cell frequency. There was a significant decrease in the CD44+/CD24- population when miR-140 was overexpressed following estrogen stimulation, indicating miR-140 plays an important role in the regulation of estrogen stimulated tumor-initiation cells, potentially through inhibition of SOX2^[27].

EXOSOMES

Exosomes are spherical membrane vesicles between 50-100 nm, secreted by the majority of cells. Multivesicular bodies fuse with the cellular membrane, releasing exosomes into the extracellular matrix^[31]. They contain a variety of protein, RNA, products of signaling pathways

and miRNAs, some common to all exosomes and some cell specific^[32]. The common set of proteins consists of the tetraspanin family (CD9, CD63, CD82), members of the endosomal sorting complexes required for transport (ESCRT) complex (TSG101, ALix) and heat shock proteins (Hsp60, Hsp70, Hsp90)^[33]. Several of these proteins are used for exosome detection in Western blotting or FACS, including CD63 and CD9^[34,35].

Exosome function in tumorigenesis

There are three known functions of exosomes in tumorigenesis; restructuring of microenvironment, modulation of tumor immune response and direct modification of tumor cells *via* delivery of protein or genetic material^[31,36]. Tumor development is dependent on the relationship between cancer cells and the surrounding microenvironment^[37]. Secreted factors promote angiogenesis and invasion, aiding in tumor growth and progression. Communication between cancer cells and the microenvironment is likely mediated in part by exosomes, both secreted by cancer cells and the microenvironment itself. Stromal secreted exosomes promote breast cancer motility and metastasis^[38]. Tumor secreted exosomes can promote endothelial tubule formation^[39], as well as secrete matrix metalloproteinases, aiding in invasion^[40]. Molecular changes in tumor stroma are an important part of breast cancer initiation and progression^[37].

Exosomes can suppress immune response by promoting T regulatory cell expansion and inducing apoptosis of effector T cells^[41]. In tumor cells exosomes mediate upregulation of anti-apoptotic genes and anchorage independent growth^[42], and are believed to be involved in resistance to drug and radiation resistance^[32]. Exosomes transfer their contents to receiving cells *via* internalization of the exosome. Heparan sulfate proteoglycans are necessary receptors of cancer cell derived exosomes, and are necessary for exosome uptake and delivery of macromolecular contents^[43].

A precise method for identifying tumor secreted exosomes is not yet available. Tumor secreted exosomes are differentiated by analysis of their contents. Proteins and miRNA found in exosomes closely match those in the parent cell. In some cases, FACS can be conducted using antibody for tumor specific protein in exosomes, such as HER2/neu^[44]. Marker proteins that are often over-expressed in tumors are found in exosomes, including EpCAM, CD24, L1CAM, CD44 and EGFR. The utility of these markers for identification of tumor-secreted exosomes is under investigation^[45].

Exosomal miRNAs

Breast cancer heterogeneity is reflected in tumor-secreted exosomes. While miRNA sequencing of secreted breast cancer exosomes is still in its infancy, exosomal miRNA expression from other diseases exhibit a high level of correlation to parental cells^[46]. Exosomes have been successfully isolated from many sources in the body, including blood plasma, serum and urine^[32]. Due to their ubiquity

and disease specific expression, there is significant potential for exosomal use as biomarkers of disease state or progression^[36].

MiRNA array shows differential expression of miR-140 between DCIS stem-like and DCIS whole cell populations. Similarly, miR-140 is downregulated in exosomes derived from DCIS stem-like cells compared to exosomes derived from DCIS whole cell population. Exosomal levels of miR-140 from stem cell populations can be rescued by treatment with sulforaphane. Treatment of invasive basal like breast cancer cells and DCIS cells with miR-140 containing exosomes resulted in an increased level of miR-140 in both cell lines, demonstrating the potential of exosomal secretion to impact miR-140 signaling in nearby cells. Treatment with sulforaphane may block paracrine signaling by increasing miR-140 secretion in the tumor microenvironment^[22].

TRANSLATIONAL POTENTIAL

MiR-140 represents a potential target to prevent cancer initiation and progression. Promoter region hypermethylation is a common mechanism for miRNA dysregulation, and is also observed in early stage breast cancers. A CpG island exists within the miR-140 locus, and has a higher level of methylation in DCIS cells compared to nontumorigenic mammary epithelial cells. This methylation region is a potential therapeutic target to restore miR-140 expression^[28].

Targeting stem cells in ER α positive IDC

We demonstrated the presence of an ER α /miR-140/SOX2 signaling axis, through which ER α binds the miR-140 promoter region, halting transcription and preventing miR-140 targeting of SOX2 mRNA. Targeting ER α signaling may rescue miR-140 inhibition of SOX2, preventing stem cell signaling and promoting tumor cell differentiation. While this strategy could prove effective for ER α positive tumors, other avenues must be pursued to target miR-140 in basal-like breast cancers^[27].

Targeting DCIS stem cells

Treatment of DCIS cells with 5-aza-2-deoxycytidine (DNA methyltransferase inhibitor) or sulforaphane (inhibitor of histone deacetylase and DNA methyltransferase) restored miR-140 expression^[47,48]. Sulforaphane treatment significantly inhibited DCIS tumor growth *in vivo*, as well as restoring miR-140 expression and down regulating SOX9 and ALDH1. Treatment of triple negative, basal-like invasive breast cancer with sulforaphane had the same effect, upregulation of miR-140 and decreased cancer stem cell frequency. Cancer stem cell xenografts of MDA-MB-231 showed dramatically decreased growth when treated with sulforaphane^[28].

Targeting stem cell signaling in nearby cancer cells through exosomal miR-140

Sulforaphane treatment of DCIS stem-like cells resulted

in increased exosomal miR-140. This indicates that in addition to restoring miR-140 expression in treated stem cells, sulforaphane may block stem cell signaling in nearby cells through exosomal delivery of miR-140^[22].

CONCLUSION

Stem cells present in the DCIS population may serve a critical role in progression and recurrence of breast cancer. Through interaction with SOX2 and SOX9, miR-140 serves as a tumor suppressor in both DCIS and IDC, preventing stem cell signaling and tumor initiation. When miR-140 is downregulated there is an increase in stem cell populations and breast cancer progression, initiation and growth. We have identified two primary downregulation mechanisms. In IDC, we found estrogen binding in the miR-140 promoter, and epigenetic regulation through CpG island methylation in DCIS. By targeting these mechanisms, miR-140 signaling is recovered and the stem cell population decreased, reducing tumor growth and progression. Targeting of the DCIS stem cell population may be critical to preventing progression to invasive ductal carcinoma. Epigenetic therapy rescuing miR-140 suggests a novel therapeutic strategy for both DCIS and IDC lesions, and would be especially important for patients with tamoxifen insensitive ER α - DCIS lesions.

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