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REVIEW

miR-200c: a versatile watchdog in cancer progression, EMT, and drug resistance

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Abstract MicroRNAs (miRNAs) are 20–22-nucleotide small endogenous non-coding RNAs which regulate gene expression at post-transcriptional level. In the last two decades, identification of almost 2600 miRNAs in human and their potential to be modulated opened a new avenue to target almost all hallmarks of cancer. miRNAs have been classified as tumor suppressors or oncogenes depending on the phenotype they induce, the targets they modulate, and the tissue where they function. miR-200c, an illustrious tumor suppressor, is one of the highly studied miRNAs in terms of development, stemness, proliferation, epithelial-mesenchymal transition (EMT), therapy resistance, and metastasis. In this review, we first focus on the regulation of miR-200c expression and its role in regulating EMT in a ZEB1/E-cadherin axis-dependent and ZEB1/E-cadherin axis-independent manner. We then describe the role of miR-200c in therapy resistance in terms of multidrug resistance, chemoresistance, targeted therapy resistance, and radiotherapy resistance in various cancer types. We highlight the importance of miR-200c at the intersection of EMT and chemoresistance. Furthermore, we show how miR-200c coordinates several important signaling cascades such as TGF-β signaling, PI3K/Akt signaling, Notch signaling, VEGF signaling, and NF-κB signaling. Finally, we discuss miR-200c as a potential prognostic/diagnostic biomarker in several diseases, but mainly focusing on cancer and its potential application in future therapeutics.

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Keywords miR-200c · Epithelial-mesenchymal transition (EMT) · Drug resistance · TGF-β · ZEB1/2 · Cell signaling · Biomarker

Introduction

MicroRNAs (miRNAs), small non-coding RNAs that control gene expression at post-transcriptional level, were discovered only two decades ago, and their importance in cell biology is exponentially increasing since then [1, 2]. Lin-4 gene, rather than being translated into a protein, transcribed into a pair of small RNAs (later termed as miRNAs) which imperfectly base-paired to complementary sequences in the 3′-UTR of different messenger RNAs (mRNAs), thus found to be involved in post-transcriptional gene regulation. Later, the discovery of the second miRNA, let-7 [3], and its sequence conservation across species from flies to human triggered seminal follow-up works to unveil the basic concepts of miRNA biogenesis and function [4–6]. Over the period of the last 15 years, a total of more than 24,000 miRNA loci have been discovered in 206 different species highlighting the potential key regulatory functions of miRNAs in several organisms [7]. To date, miRNAs have been estimated to regulate more than 60 % of human genome [8] and dysregulated miRNA expression has been implicated in regulating various diseases and developmental disorders. Moreover, the packed structure of miRNAs led to their less vulnerability to degradation even in serum environment [9] ensuring their suitability for diagnostic and therapeutic purposes [10–12]. miR-200c was first shown to be dysregulated in several cancer cell lines [13]. Since then, miRNAs from miR-200 family, in general, but miR-200c, in particular, have been explored to have an impact on a large variety of biological processes and have been demonstrated to play crucial roles in epithelial-mesenchymal

transition (EMT), cell invasion, proliferation, metastasis, apoptosis, autophagy, and therapy resistance in several cancer types [14, 15]. There are more than 400 publications related to miR-200c depicting the importance of this miRNA; thus, the vast information about miR-200c deserves a compilation. In this review, we focus on the molecular functioning of miR-200c with special emphasis on regulation of EMT, cell invasion, therapy resistance, role in signaling pathways, and its potential as a biomarker in diverse diseases including cancer.

Regulation of miR-200c expression

miR-200 family is composed of two clusters: the miR-200a/b/429 cluster containing miR-200a, miR-200b, and miR-429 on chromosome 1p36 and the miR-200c/141 cluster containing miR-200c and miR-141 on chromosome 12p13 (Fig. 1). In addition to four enhancer boxes (E-box 1–4, CAGGTG), miR-200c promoter has two specific sites (Z-box 1 and 2, CAGGTA) restricted for the binding of ZEB transcription factors [16]. ZEB1 alone or bound to different co-repressors such as CtBPs and BRG1 suppresses miR-200c expression [17, 18]. On the other hand, P300/PCAF complex can activate miR-200c promoter via lysine acetylation of ZEB1 resulting in the release of ZEB1 suppression on miR-200c/141 transcription [19]. A study conducted on vMH neural progenitor cells revealed Sox2 and E2F3 as additional transcription factors regulating transcription from the miR-200c/141 gene cluster. Specific binding sites conserved across species (human, mouse, and rat) have been reported for Sox2 and E2F at the distal (in the −1.4 to −1.3 kb region with respect to the transcriptional start +1 of miR-200c/141 cluster) and proximal promoter regions (−500 to −400 bp), respectively [20]. c-Myb transcription factor also activates miR-200c gene through the binding sites located in −1653 to −1647 bp and −988 to −982 bp regions of the promoter [21]. Furthermore, the p53/p63/p73 family of transcription factors has been reported to positively regulate miR-200c expression in ovarian cancer cells [22]. In mouse embryonic fibroblasts, stemness-related transcription factor, Oct4, in combination with Sox2, has been implicated to serve as an activator of miR-200c gene expression [23].

Epigenetic gene regulatory mechanisms, such as DNA methylation, are also capable of playing a predominant role in regulating the expression of miR-200c (Fig. 1). For instance, in breast cancer cells, miR-200c/141 cluster often undergoes aberrant silencing due to methylation of CpG island located in its promoter region [24]. In advanced stage bladder cancer and poorly differentiated breast cancer cell lines, miR-200 expression, along with miR-205, is found to be silenced either due to promoter hypermethylation or due to direct binding of Twist transcriptional repressor on the promoter [25]. Interestingly, miR-200c, itself, can indirectly act as an epigenetic regulator. For example, ectopic expression of miR-200b or miR-200c in mesenchymal breast cancer cells resulted in partial upregulation of E-cadherin level due to the degradation of ZEB1-histone deacetylase repressor complexes and increase in the acetylation of histone H3 at the E-cadherin promoter [26]. Overall, in addition to transcriptional regulation of miR-200c, epigenetic mechanisms also play a critical role in tuning miR-200c expression and in regulating its downstream effects.

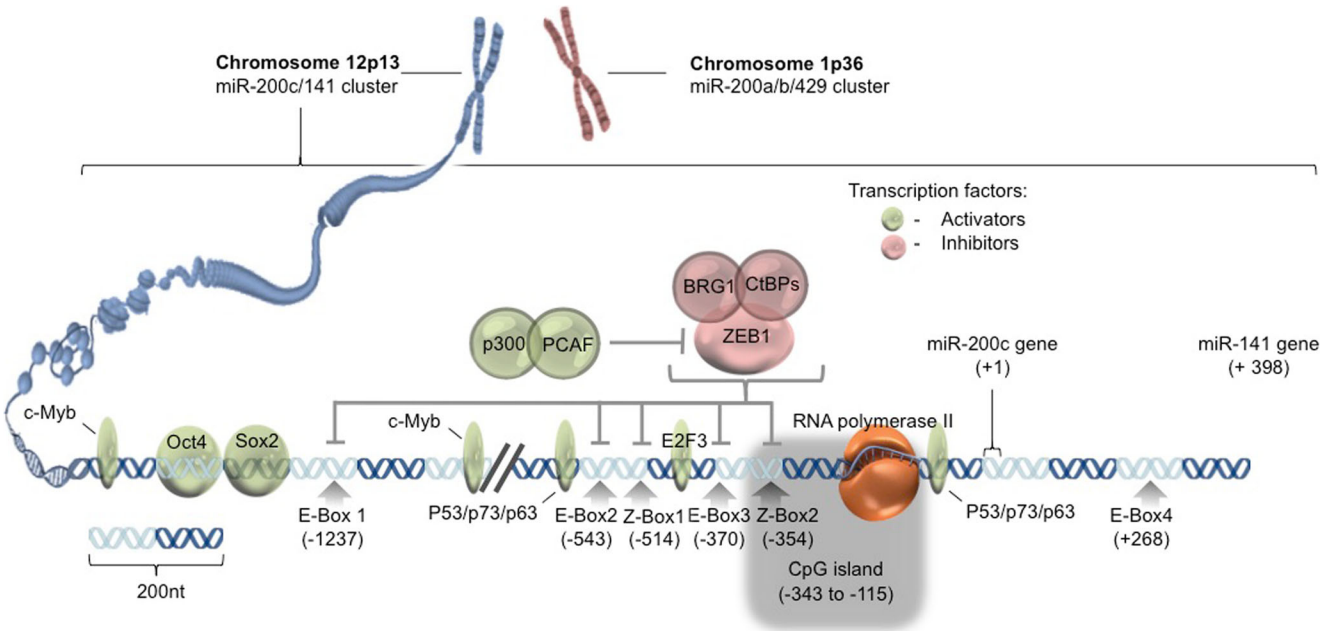

Fig. 1 Regulation of miR-200c expression. Graphical depiction of chromosomal position of miR-200c/141 gene cluster, promoter and enhancer sequences, and transcription factor binding sites

Role of miR-200c in epithelial-mesenchymal transition

EMT, defined as transformation of immotile epithelial cells to motile and invasive mesenchymal cells by losing their cell-to-cell adhesion properties [27], is a crucial step in metastatic cascade. One of the hallmarks of EMT is functional loss of cell surface marker E-cadherin and gain of mesenchymal characteristics via elevating the levels of mesenchymal proteins such as vimentin, fibronectin 1 (FN1), and N-cadherin [28]. Transcription factors associated with EMT, notably ZEB1 and ZEB2, suppress E-cadherin expression and thus trigger the transformation of epithelial cells into mesenchymal state and subsequently initiate the early steps of metastasis [29, 30].

miR-200c inhibits EMT via regulating ZEB1/2/E-cadherin axis

miR-200 family members including miR-200c directly target and repress ZEB1 and ZEB2 expression [31] (Fig. 2), and their frequent loss with concomitant increase in ZEB1 and ZEB2 expression has been observed in different cancers promoting EMT by downregulating E-cadherin [16, 32, 33]. Conversely, ectopic expression of E-cadherin in mesenchymal cells promotes the reverse process and mesenchymal-epithelial transition, confirming bona fide effects [31, 34]. For instance, via regulating miR-200c in a feedback loop, ZEB1 contributes to harbor stabilized EMT in invading cancer cells [16, 35]. Interestingly, miR-200c-ZEB1 interaction-mediated EMT is also associated with acquisition of cancer stemness as miR-200c suppresses cancer stem cell (CSC)-like features by directly targeting ZEB1 as well as stem cell factors like Bmi1, Sox2, and Klf4 [33, 36]. ZEB1 expression has been shown to be high in embryonic stem (ES) cells, and the expression of miR-200 family members is positively correlated with ESC differentiation [33, 35, 37].

Furthermore, the other factors involved in regulation of miR-200c also hold significant importance in initiating metastatic cascade. For instance, tumor suppressor transcription factor, p53, has been demonstrated to suppress EMT and EMT-related cancer stemness via provoking transcriptional activation of miR-200c and associated downregulation of ZEB transcription factors [38, 39]. In addition, the C subunit of oncogenic mucin 1 (MUC1-C) protein, which induces ZEB1 expression in a nuclear factor kappa B (NF-κB) p65-dependent manner, can

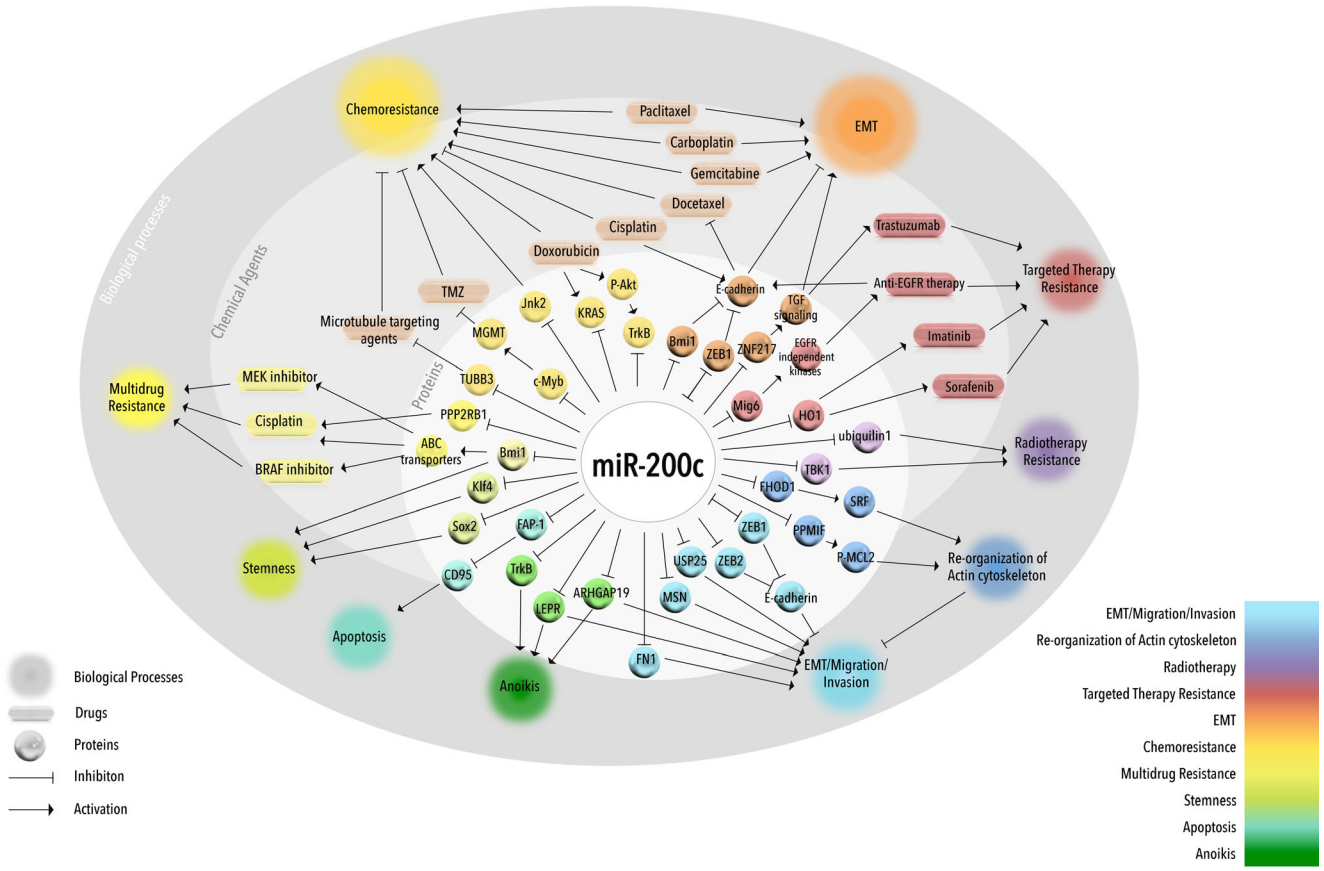

Fig. 2 miR-200c regulates EMT and drug resistance. Graphical representation of miR-200c-mediated regulation of EMT and drug resistance via direct or indirect targeting of several proteins involved in EMT and multiple drug resistance mechanisms

transcriptionally suppress miR-200c by forming a repression complex with ZEB1 and thus can regulate subsequent induction of EMT [40] (Fig. 2).

miR-200c inhibits EMT via regulating targets other than ZEB1/2

Despite its importance, E-cadherin restoration is not always enough for the inhibition of migration and invasion potential of cancer cells; however, it has been shown that miR-200c expression, alone, is enough to inhibit EMT, migration, and invasion which suggests the involvement of other direct targets of miR-200c as EMT regulators [41]. A recent study has suggested miR-200c-mediated repression of protein kinase A subunits as the underlying mechanism of miR-200c-mediated inhibition of migration in breast cancer cells [42]. This effect of miR-200c has also been linked to its targeting of ubiquitin-specific peptidase 25 (USP25) in non-small-cell lung cancer (NSCLC) [43]. In addition, we have demonstrated that miR-200c directly targets actin cytoskeleton regulatory proteins, FHOD1 and PPM1F, and represses migration and invasion of breast cancer cells in a ZEB1/2/E-cadherin-independent manner [41]. Importantly, several molecules linked to cell motility, such as FN1, moesin, neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB), leptin receptor (LEPR), and Rho GTPase-activating protein 19 (ARHGAP19), are among the direct targets of miR-200c, and in aggressive breast and endometrial cancer cells which lack miR-200c, aberrant expression of these targets contributes to the EMT phenotype [44] (Fig. 2). Moreover, not only ZEB2 but also other proteins in ZEB2 and Snail1 transcriptional repressor complexes such as Crtap, FHOD1, Smad2, Map3k1, Tob1, Ywhag/14-3-3γ, Ywhab/14-3-3β, Smad5, Zfp36, Xbp1 and Mapk12 are also among the direct targets of miR-200c [45].

Cyclin-dependent kinase inhibitor, p21, is also capable of blocking EMT [46] whereas loss of p21 induces EMT and downregulates several miRNAs, including miR-200c, in a colorectal cancer cell line. Interestingly, overexpression of miR-200c, alone, in these p21-deficient cells inhibited EMT, migration, and invasion [47]. miR-200 family miRNAs are usually repressed in cells subjected to EMT progression. For example, cells, which underwent EMT in response to transforming growth factor beta (TGF-β) or to ectopic expression of protein tyrosine phosphatase, Pez, show decreased levels of miR-200 family miRNAs [48]. Using a mouse model system, another study has also shown that TGF-β-mediated EMT progression is indeed correlated with downregulation of miR-200 family miRNAs in mammary epithelial cells [34]. Overall, these studies suggest that both the regulation and regulatory effects of miR-200c are critical determinants in initiating EMT and pursuing the subsequent steps in metastatic cascade.

Role of miR-200c in therapy resistance

In addition to regulation of EMT and metastasis, miR-200 family, in general, and miR-200c, in particular, also take part in regulating therapy resistance. For instance, a recent study with tissue samples from esophageal cancer patients receiving neoadjuvant chemotherapy has clearly shown an inverse correlation between miR-200c expression and sensitivity toward chemotherapeutic agents. The underlying mechanism is proposed to be enhanced activation of the PI3K/Akt pathway, which is preceded by downregulation of a subunit of protein phophatase 2A, PPP2R1B, a negative regulator of Akt phosphorylation. PPP2R1B is a direct target of miR-200c; therefore, its downregulation through upregulated miR-200c results in the activation of the PI3K/Akt pathway and subsequent resistance against cisplatin [49] (Fig. 2). In addition, higher levels of circulating miR-200c have been observed in patients of castration-resistant prostate cancer (CRPC), particularly, in those showing resistance to docetaxel. Despite this seemingly negative correlation between miR-200c levels and drug sensitivity in patients, most of the in vitro studies suggest an opposite link and present miR-200c as an inducer of sensitivity against anti-cancer agents. This discrepancy may be due to the influence of non-tumor cells on the levels of circulating miR-200c as well as the regulatory effects of the tumor microenvironment on cancer cells at the primary tumor site [50]. Several targets of miR-200c are known to play important roles in mediating multidrug resistance, chemo-sensitivity and chemo-resistance, and targeted therapy resistance as well as response to radiotherapy.

miR-200c and multidrug resistance

The role of miR-200c in multidrug resistance was first demonstrated in relation to ATP-binding cassette (ABC) transporters (Fig. 2), the largest transmembrane protein family, responsible for resistance against multiple drugs. In general, overexpression of ABC transporters creates increased drug efflux, resulting in a decreased intracellular drug deposition and a subsequent acquisition of resistance against the given therapy [51]. miR-200c has been shown to play an active role in limiting ABC transporter-mediated multidrug resistance in melanoma as its ectopic expression in these cells was able to downregulate ABC transporters: ABCG2, ABCG5, and multi-drug resistance gene (MDR1). The underlying effect is attributed to direct targeting of Bmi1 by miR-200c, and knocking down Bmi1 in these cells results in lower ABC transporter levels and higher sensitivity to a DNA-damaging agent cisplatin, as well as to a BRAF and a MEK inhibitor (PLX4720 and U0126, respectively) [52]. Being a member of polycomb group proteins, Bmi1 is reported to be upregulated in various cancers and is associated with a more aggressive

disease progression and cancer stemness [53]. From these findings, miR-200c seems to be an important player during multidrug resistance considering the pivotal role of ABC transporters in mediating chemoresistance in a wide variety of cancers.

miR-200c and chemotherapy resistance

Overexpression of miR-200c has been shown to enhance the response to several chemotherapeutic agents in various cancer types. In melanoma cells, miR-200c overexpression results in increased sensitivity to cisplatin [52] (Fig. 2). In endometrial and ovarian cancers, miR-200c increases taxane sensitivity by targeting class III B-tubulin gene (TUBB3), which is known to be a mediator of resistance against microtubule-targeting agents [54, 55]. A recent study, encompassing a panel of ovarian adenocarcinoma cell lines, has also shown the inhibitory action of miR-200c on TUBB3 expression, which also depends on the nuclear localization of an RNA-binding protein, HuR [56]. Another study revealed that decitabine treatment-dependent upregulation of miR-200c and miR-141 promotes epithelial-like characteristics in highly invasive oxaliplatin-resistant colorectal cancer cells [57].

On the other hand, loss of miR-200c has been shown to be associated with acquisition of resistance against various chemotherapeutic agents in different cancer types. Doxorubicin treatment of a breast cancer cell line, BT474, results in decreased miR-200c level, which ultimately leads to an increase in KRAS, a direct target of miR-200c. This study suggests miR-200c as an important checkpoint for the development of chemoresistance mediated through activation of an important oncogenic pathway [58]. In recurrent and metastatic colorectal cancer, miR-200c expression is suppressed, and its overexpression attenuates the JNK signaling pathway resulting in an increased sensitivity of resistant cells to several chemotherapeutic agents and a subsequent decrease in their metastatic potential in both in vitro and in vivo models. Furthermore, miR-200c level is inversely correlated with JNK2, ABCB1, and MMP-9, which are the downstream targets of the JNK pathway, and this negative correlation may be used as a predictive marker of therapeutic outcome in these patients [59]. In two different breast cancer cell lines, resistance against doxorubicin is correlated with marked inhibition of miR-200c where TrkB has been suggested as the key target of miR-200c in regulating this resistance [60]. TrkB plays important roles in differentiation, in proliferation, and mostly, in cell survival mediated through PLC, MAPK, or PI3K/Akt signaling in neurons [61]. Notably, TrkB has also been found to be associated with drug resistance in neuroblastoma [62, 63] and head and neck squamous cell carcinoma [64]. Interestingly, TrkB downregulation after ectopic expression of miR-200c in doxorubicin-resistant breast cancer cells sensitizes the cells toward doxorubicin treatment. Furthermore, TrkB expression has been correlated with the phosphorylation status of Akt in miR-200c-treated doxorubicin-resistant cells where low p-Akt level has been observed with a concomitant decrease in TrkB protein level [60]. All in all, these studies propose possible mechanisms to explain the multi-factorial nature of a given miRNA (here: miR-200c) in mediating the response of cancer cells against different types of chemotherapy.

miR-200c and targeted therapy resistance

Besides regulating chemotherapy resistance, miR-200c also takes part in mediating targeted therapy resistance (Fig. 2). In a microarray-based study on tamoxifen-sensitive MCF-7 and tamoxifen-resistant LY2 breast cancer cell lines, several novel miRNAs including miR-200c were found to be differentially expressed between sensitive and resistant cells [65]. In a later study by the same group, it has been shown that ectopic expression of miR-200c in endocrine-resistant LY2 cells has significant effect on enhanced sensitivity to fulvestrant, but not to tamoxifen [66]. Targeting the MAPK pathway is a current treatment option for metastatic melanoma patients; however, nearly 50 % of the patients receiving BRAF and MEK inhibitors develop resistance within 6–7 months following treatment initiation. Overexpression of miR-200c also significantly enhanced the anti-proliferative effects of both PLX4720, a specific BRAF inhibitor, and trametinib, a selective inhibitor of MEK1/2 kinase, in melanoma cells [67] suggesting a potential anti-MAPK therapy sensitizer role for miR-200c.

Reduced miR-200c and subsequent increase in its target genes such as Bmi1, ZEB2, TUBB3, ABCG5, and MDR1 have also been shown to promote acquisition of BRAF inhibitor resistance in melanoma. Therefore, these genes could be potential therapeutic targets against development of BRAF inhibitor resistance [68]. In sorafenib- and imatinib-resistant renal cell carcinoma cells, heme oxygenase 1 (HO1) has been found to be a direct target of miR-200c. This enzyme is induced during oxidative stress, and its overexpression following a decrease in miR-200c level in resistant cells is found to be partially responsible for drug resistance by inhibiting apoptosis and autophagy [69]. Exploring druggable targets of miR-200c responsible for conferring drug resistance may help in shaping the strategies to improve the efficacy of chemotherapy and targeted therapy.

miR-200c and radiotherapy resistance

The first line of evidence regarding association between miR-200c and radiotherapy resistance came from a study on breast cancer cells where low levels of miR-200c expression were correlated with radio-tolerance, and ectopic miR-200c expression enhanced the (IR)-induced apoptosis by directly targeting

TANK-binding kinase 1 (TBK1) [70] (Fig. 2). Recently, another target of miR-200c, ubiquilin 1, has been shown to positively regulate radio-resistance by enhancing IR-induced autophagy in MDA-MB-231 breast cancer cells. Ectopic expression of miR-200c reversed the phenotype and sensitized the cells to radiation [71]. Furthermore, nanoparticle-assisted delivery of miR-200c has been shown to inhibit CSC-like properties and to augment radiosensitivity in three different gastric cancer cell lines [72]. Altogether, miR-200c-assisted/conjugated therapies (chemotherapy, targeted therapy, and radiotherapy) may have greater potential to overcome radiotherapy resistance and to limit escape mechanisms in multiple cancer types.

miR-200c at the intersection of EMT and drug resistance

Over the last decade, several lines of evidence show that EMT might render the cells resistant against the implemented anticancer therapy supporting the notion that EMT and therapy resistance go hand in hand [73]. In addition, involvement of miRNAs in combined regulation of drug resistance and EMT is evident [74]. Likewise, miR-200c, a negative regulator of EMT, has shown great potential to overcome both chemotherapy and targeted therapy resistance through reversing the EMT process or promoting epithelial-like state (Fig. 2).

miR-200c, EMT, and chemotherapy resistance

Platinum-based chemotherapeutic agents, e.g., cisplatin, are being used as first line treatment for NSCLC, but resistance to these agents is inevitable. In highly aggressive cisplatin-resistant NSCLC cells, miR-200c is lost due to promoter hypermethylation, and forced introduction of miR-200c induces loss of mesenchymal features, which reverses the resistance against cisplatin [75]. In addition, upregulation of several EMT markers including ZEB1/ZEB2 and downregulation of epithelial marker, E-cadherin, are identified as the characteristic features of docetaxel-resistant prostate cancer cells, and the underlying mechanism again correlates with decreased miR-200c expression [76] (Fig. 2). Furthermore, gemcitabine-resistant pancreatic cancer cells showed EMT features along with low miR-200a, miR-200b, and miR-200c levels. Upregulation of these miRNAs upon treatment with two natural agents, 3,3′-diindolylmethane and isoflavone, reversed the EMT phenotype [77]. Moreover, the anticancer effects of an anti-diabetic drug, metformin, have been associated with downregulation of several CSC markers, such as CD44 and EPCAM in pancreatic cancer, which, in turn, linked with re-expression of several miRNAs including miR-200c in gemcitabine-sensitive and gemcitabine -resistant cells [78]. Similarly in pancreatic CSCs, decrease in chemoresistance has been attributed to overexpression of miR-200c [79]. In addition, miR-200c together with other miRNAs from the miR-200 family has been shown to sensitize ovarian cancer cell lines with strong EMT phenotype to anti-cancer drugs paclitaxel and carboplatin [80].

Although miR-200c-mediated attenuation of chemoresistance is mainly associated with reversal of the EMT process, underlying mechanisms are not restricted to the canonical miR-200c/ZEB1/E-cadherin axis but involve other miR-200c targets as well. For instance, in glioblastoma cells, ectopic ZEB1 expression decreased the sensitivity to chemotherapeutic agent, temozolomide (TMZ), in vitro while ZEB1 knockdown resulted in opposite effects in both in vitro and in vivo. ZEB1 was found to be co-localizing with a major chemoresistance enzyme, MGMT, at the edges of glioblastoma xenograft tumors where cells are more invasive and chemoresistant. In principle, increased ZEB1 level in these cells blocks miR-200c expression and subsequently upregulates c-Myb transcription factor (a direct target of miR-200c) which, in turn, activates MGMT transcription resulting in attenuation of anti-proliferative effects of TMZ [81]. Interestingly, a recent study on breast cancer lung metastasis has shown that EMT is dispensable for lung metastasis, but miR-200-mediated reversal of EMT helps in sensitizing metastatic cells to chemotherapeutic drug cyclophosphamide [82]. These results indicate the importance of miR-200c as a key element in determining the fate of chemoresistant cancer cell via regulating EMT.

miR-200c, EMT, and targeted therapy resistance

Association of miR-200c-mediated inhibition of EMT with targeted therapy resistance has been established through several studies. In particular, resistance against anti-EGFR therapies (e.g., cetuximab, gefitinib, erlotinib, etc.) has been demonstrated to be tightly linked with acquisition of EMT phenotypes proceeded by the loss of miR-200c in various cancer types. For instance, in urothelial carcinoma cells, showing mesenchymal-like features and exhibiting resistance against anti-EGFR therapies, a complete reversal of resistance could be acquired with the introduction of miR-200c and subsequent downregulation of ZEB1/2 and upregulation of E-cadherin [83]. In gefitinib-resistant EGFR-mutant lung cancer cell lines, low miR-200c expression due to DNA hypermethylation was found to be correlated with EMT features characterized by increased ZEB1 and decreased E-cadherin levels in these cells [84]. Likewise, reversal of cetuximab resistance by miR-200c is accompanied by the loss of EMT features in highly invasive and aggressive NSCLC cells [75]. Similarly, reversal of erlotinib resistance in EGFR-mutant NSCLC xenografts treated with a potent natural agent, silibilin, is associated with the loss of EMT-related features including low miR-200c and E-cadherin and high ZEB1, Snail, and N-

cadherin expression [85]. However, in a similar study with erlotinib-resistant lung adenocarcinoma cells, overexpressed miR-200c regulates expression of EMT network proteins resulting in a slight increase in erlotinib sensitivity. Furthermore, a TGF-β1-responsive element is identified at the promoter region of miR-200c, which suggests the suppression of miR-200c in a Smad3/4-dependent manner at the downstream of activated TGF-β signaling. Interestingly, TGF-β1 is also not able to fully induce EMT and to cause significant changes in erlotinib response in these cells [86]. Therefore, further understanding of the effects of TGF-β signaling on miR-200c-mediated EMT and anti-EGFR resistance could provide more details about the importance of the tumor microenvironment in controlling miRNA expression and response to targeted therapies.

Recently, a study in the context of the TGF-β/miR200c axis explored that prolonged exposure to TGF-β not only facilitates EMT but also makes cells undergo EMT-associated kinase switch, which results in EGFR inhibitor resistance characterized by decrease in miR-200c levels and upregulation of one of its direct targets, Mig6/ERRFI-1. Mig6 is a negative regulator of the EGFR family, and its overexpression increases the internalization of EGFR and thereby reduces its localization to the surface. Despite having lower EGFR activation, cells overexpressing Mig6 show higher Akt phosphorylation through activation of EGFR-independent tyrosine kinases and thereby increase their survival. Furthermore, elevated Mig6/miR-200c ratio is found to be associated with erlotinib resistance in a panel of 25 tumor cell lines and in patient-derived xenografts (PDXs) of NSCLC and pancreatic cancer [87]. Introduction of miR-200c to bladder cancer cells results in complete reversal of resistance against anti-EGFR therapies mainly through Mig6 downregulation, which in turn leads to increased surface levels of EGF receptors, thus making the anti-EGFR therapy more effective in inhibiting the signal [83]. Recently, a feedback loop between miR-200c and TGF-β pathway was discovered in which miR-200c can regulate TGF-β signaling by directly targeting ZNF217, a transcriptional activator of autocrine TGF-β. Elevated TGF-β signaling leads to increased invasiveness as well as trastuzumab resistance (Fig. 2). In trastuzumab-resistant breast cancer cells, miR-200c was found to be the most significantly downregulated miRNA, and its ectopic expression decreases invasiveness and increases trastuzumab sensitivity through downregulation of ZNF217. Besides ZNF217, ZEB1 expression was also shown to be significantly upregulated in trastuzumab-resistant cells. Double knockdown of ZEB1 and ZNF217 decreased invasiveness and partially restored trastuzumab sensitivity. Furthermore, miR-200c treatment of xenografts of a trastuzumab-resistant breast cancer cell line leads to re-gain of trastuzumab sensitivity [88]. In the same scenario, another level of regulation comes from a long non-coding RNA, Lnc-ATB, which is expressed at the downstream of TGF-β signaling and competitively binds to miR-200c conferring ZEB1 and ZNF217 (upregulation)-mediated trastuzumab resistance [89]. In summary, these studies clearly provide the cue that miR-200c could efficiently control the response of cancer cells to anti-cancer therapies through EMT-dependent and EMT-independent pathways.

miR-200c regulation of signaling pathways

Over the years, it has been well established that miR-200c plays critical roles in regulating multiple biological processes and signaling cascades varying from MAPK signaling, to Notch signaling, to TLR signaling (Fig. 3). In the context of developmental program, miR-200c has been found to accumulate in epithelial buds in developing submandibular glands and control FGFR-mediated epithelial proliferation by targeting vldlr and its ligand reelin which regulate FGFR signaling in these cells [90]. Furthermore, miR-200c-mediated activation of BMP signaling via upregulation of amelogenin and E-cadherin and via downregulation of noggins triggers tooth development and renewal through dental epithelial cell differentiation in mice [91].

IGF-induced PI3K/Akt and MAPK signaling can enhance EMT and metastasis by downregulating miR-200c levels in gastric cancer, which can be restored by Cbl-b-mediated ubiquitination of IGF-IR [92] (Fig. 3). Recent data showed that miR-200c negatively regulates KRAS as well, resulting in inhibition of both PI3K/Akt and MAPK pathways in breast and lung cancer cells [58, 93]. TGF-β-induced expression of ZEB transcription factors downregulates miR-200c resulting in reduced EGFR activity due to upregulation of Mig6 and confers EMT [87]. On the contrary, TGF-β-induced upregulation of miR-200c has been shown in diabetic nephropathy where miR-200c targets FOG2, which is a negative regulator of the PI3K/Akt pathway [94]. miR-200c also suppresses TGF-β signaling by targeting ZNF217 in breast cancer cells, an indirect mechanism to target ZEB-induced metastasis [88].

In addition to the well-characterized role of miR-200 family miRNAs in the regulation of EMT, miR-200c has been widely reported to take part in controlling tumor progression either by regulating or being regulated by different signaling mechanisms. Regarding cell death signaling, miR-200c sensitizes breast cancer cells to aniokis by targeting NF-κB-induced TrkB and NTF3 [44, 95] and enhances Fas-associated apoptosis by targeting FAP-1 in different cancer cell types [96] (Fig. 3). Additionally, in triple negative breast cancer (TNBC), miR-200c has been shown to enhance apoptosis by directly targeting X-linked inhibitor of apoptosis (XIAP) which serves as E3 ubiquitin ligase and degrades caspases essential for apoptosis [97]. In undifferentiated human ESCs, miR-200c is upregulated, and its knockdown downregulates Nanog expression and enhances

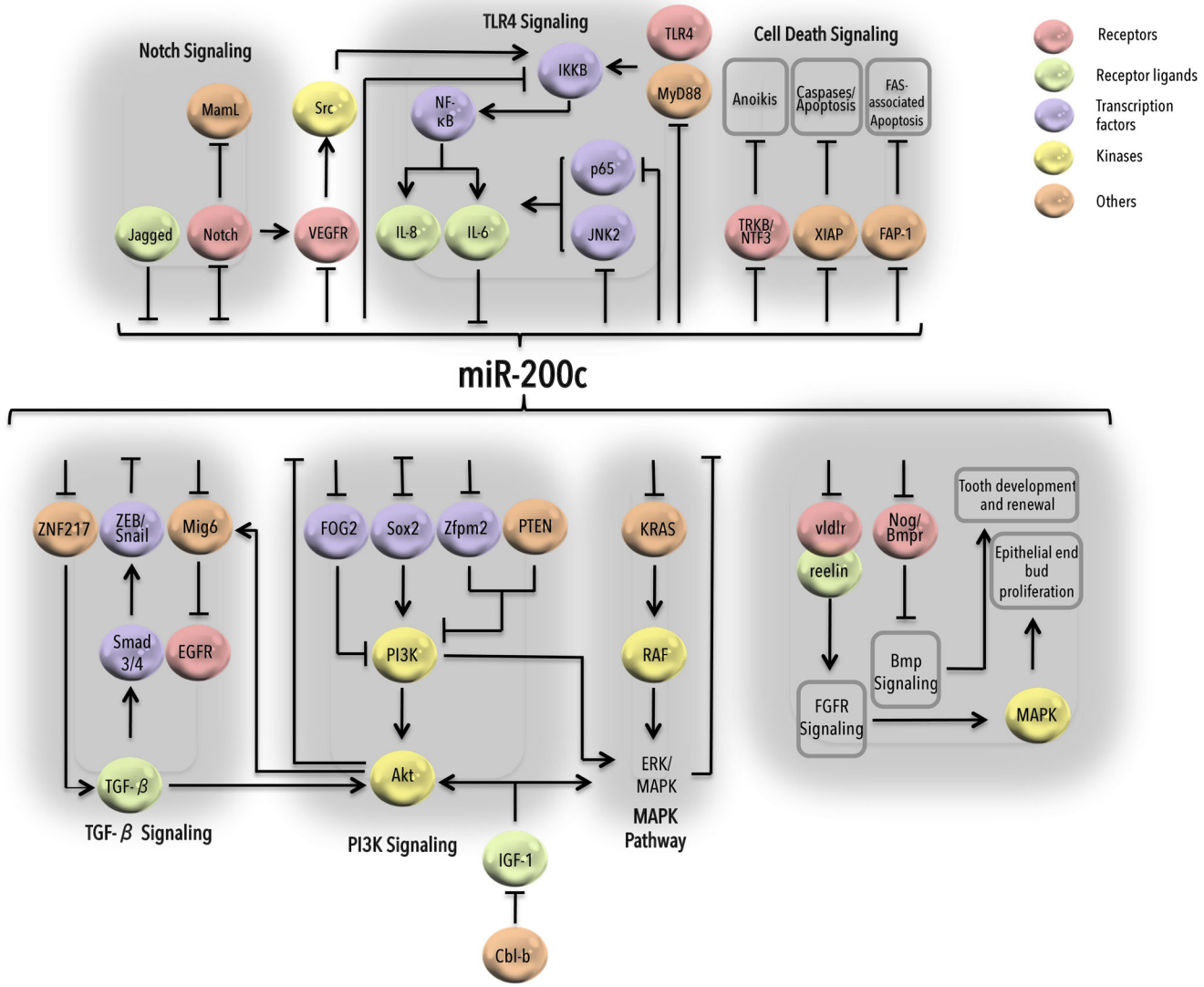

Fig. 3 miR-200c in signaling pathways. Graphical representation of involvement of miR-200c in multiple signaling cascades and their cross-talk

GATA4 levels resulting in apoptosis [98]. Furthermore, Oct4 and Sox2 can bind to miR-200c promoter and enhance MET and induce pluripotent stem cell development by suppressing ZEB2 [23]. miR-200c was found to be downregulated in normal mammary progenitor cells and breast CSCs. When overexpressed, it targets Bmi1, a known regulator of stem cell self-renewal, and suppresses the mammary duct formation ability of mammary stem cells and tumor growth driven by breast CSC [36]. In colorectal cancer, miR-200c regulates stemness by targeting Sox2 in a double negative feedback loop, and loss of miR-200c resulted in enhanced expression of stem cell markers (CD166, CD133, and beta-catenin) downstream of Sox2. In the same study, miR-200c-Sox2 feedback loop has also been shown to regulate the PI3K/Akt pathway and control the proliferative and metastatic potential of colorectal cancer via phosphorylation of PI3K by Sox2 [99]. On the contrary, in pancreatic and ovarian cancer, miR-200c has been shown to be directly targeting Zfpm2 and indirectly reducing Pten levels, both of which are negative regulators of the PI3K/Akt pathway [100, 101].

Notch signaling determines accurate growth and development of various tissues and organs, and its aberrant activation may lead to cancer. Overexpressing miR-200c in metastatic prostate cancer impedes proliferative potential of cancer cells by directly targeting Jagged1 receptors involved in Notch signaling [102] whereas overexpression of Notch1 decreases miR-200c levels in pancreatic cancer [103] (Fig. 3). Notch also activates truncated isoform of Flt1/VEGFR-1 (which is downregulated in the presence of miR-200c [104] and of importance in angiogenesis-associated VEGF signaling) leading to activation of proto-oncogene Src, which cross talks with and downregulates miR-200c in an NF-κB/IL-6-dependent manner [105]. miR-200c regulates TLR4/MyD88-dependent activation of NF-κB by directly targeting MyD88.

Interestingly, TAB2, TRAF6, and IRAK1, at the upstream of NF-κB activation in TL4/MyD88 and TNF-α signaling, are also among the potential targets of miR-200c depicting multi-step targeting of signaling components by miRNAs [106]. In breast cancer cells, MUC1-C induces EMT by upregulating ZEB1 in an NF-κB/p65-dependent manner and downregulating miR-200c by making a complex with upregulated ZEB1 [40]. IL-6-mediated suppression of miR-200c led towards activation of its downstream targets JNK2 and p65 (required for constitutive activation of IL-6) whereas loss of IL-6 in a mouse model impaired tumorigenicity due to activation of miR-200c [107]. Overexpressing miR-200c also controls inflammation-associated IL-8 in leiomyoma (smooth muscle tumor) by targeting IKKB at the upstream of NF-κB, thus decreasing p65 binding at IL-8 promoter [108]. Overall, in addition to playing important roles in EMT and metastasis, miR-200c has been adapted to regulate multiple normal growth, development, cancer, and other diseases.

miR-200c as a biomarker

Similar to proteins, expression level of individual or group of miRNAs has been suggested to be utilized as biomarkers to predict disease progression, diagnosis, and treatment response. In this scenario, dysregulated expression of miR-200c in multiple cancer-related mechanisms, which we have discussed here, is enough to support its use as a biomarker, at least in cancer. miR-200c can both be (i) an intracellular marker in tissues (cancer and normal) and (ii) an extracellular marker in body fluids (blood, urine) as free molecules or secreted in exosomes (Table 1).

As an intracellular marker, significantly downregulated miR-200c together with downregulation of miR-335 and miR-218 and upregulation of miR-122, miR-155, and miR-210 has been anticipated to be used as a potential biomarker of pathogenesis for clear cell renal cell cancer [120]. In addition, miR-200c has been suggested as predictor of survival and biomarker of relapse in stage I epithelial ovarian cancer patients [119]. In Caucasian and Asian intrahepatic cholangiocellular carcinoma (ICC) patients, miR-200c downregulation resulted in enhanced EMT which led towards increased cell motility and invasion capacity of ICC as well as induced hepatic stem cell-like gene expression pattern, such as NCAM1 upregulation [111]. In bladder cancer, low levels of miR-200c along with miR-141 and miR-30b are associated with poor prognosis [123]. In TNBC, reduced expression of miR-200c along with miR-205 has been found to be significantly associated with lymph node metastasis [124]. Recently, it has been revealed that miR-200c levels are inversely correlated with advanced clinical stages and lymph node metastasis of ovarian cancer [47]. In intraductal papillary mucinous neoplasm (IPMN), the high-grade tumors were demonstrated to have EMT characteristics such as low E-cadherin and high vimentin and ZEB1 levels. Furthermore, miRNA array analysis identified miR-200c and miR-141 to be downregulated in malignant tumors as compared to low-grade IPMN tumors. This underpins the importance of the miR-200c-ZEB1 pathway in the progression of IPMN and presents it not only as a prognostic biomarker but also as a potential target for IPMN therapy [113].

In addition to EMT and metastasis, miR-200c expression has potential to predict therapy resistance as well. Ovarian and breast cancer patients showing incomplete response to paclitaxel treatment expressed low levels of miR-200c along with significant increase in beta-tubulin and ZEB1 expression when compared with patients showing complete response to paclitaxel [109, 118]. Furthermore, in NSCLC patients, high expression of miR-200c has been found to be correlated with bigger tumor size and worse overall survival suggesting an oncogenic activity of miR-200c [114, 115]. In terms of tumor subtype classification, oncocytoma and chromophobe structures in renal tumors can be distinguished according to miR-200c and miR-139-5p expression patterns [121]. Moreover, miR-200c together with differential expression of other miRNAs is a candidate to distinguish hepatocellular carcinoma (HCC) from intrahepatic cholangiocarcinoma [112].

Besides its potential to be used as a biomarker in tissues, miR-200c level in serum as circulating miRNA has also been proven to be a promising prognostic and diagnostic factor for several diseases including various cancer types. For instance, increased levels of circulating miR-200 family members in serum have been shown to be associated with serous epithelial ovarian cancer (SEOC) [131] (Table 1). High level of miR-200c in sera of ovarian [134] and esophageal cancer patients [135] is also correlated with poor prognosis. Furthermore, presence of miR-200c in patients' sera as circulating miRNA has been validated as a diagnostic marker for gastric cancer [127]. miR-200c has been shown as a significant predictor of tumor stage in colorectal cancer as high level of this miRNA in serum is able to significantly discriminate stage IV from stage I–III colorectal cancer patients. In addition, these high serum levels are positively correlated with lymph node metastasis, distant metastasis, poor prognosis, and tumor recurrence in colorectal cancer [125]. Similarly, in prostate cancer, together with four other circulating miRNAs, elevated levels of miR-200c have been shown to be higher in sera taken from patients with metastatic castration-resistant prostate cancer as compared to healthy controls [132]. Dysregulated level of miR-200c in serum has also been observed in non-cancer diseases such as Crohn's disease [126] and Kawasaki disease [128] which further proves its fate as a diagnostic biomarker.

Circulating tumor cells (CTCs), on their own, are shown to be indicators of cancer metastasis, but are

Table 1 miR-200c as a biomarker

Biomarker	Disease	Status	Reference
Intracellular marker			
Tissues	*Breast cancer*	Paclitaxel treatment response	[109]
	Colorectal cancer	Metastasis	[110]
	ICC	Diagnosis, subtype Classification, EMT	[111, 112]
	IPMN	Malignancy, EMT	[113]
	Non-small cell lung cancer	EMT, metastasis, Gemcitabine drug response	[114–117]
	Ovarian cancer	Paclitaxel treatment response	[118]
	Epithelial ovarian cancer	Patient survival	[119]
	Renal cell carcinoma	Diagnosis, prognosis, metastasis, subtype classification	[120–122]
	Bladder cancer	Prognosis	[123]
CTCs	*Breast cancer*	Metastasis	[124]
Extracellular marker			
Serum	*Breast cancer*	Metastasis (via CTC)	[50]
	Colorectal cancer	Prognosis, metastasis	[125]
	Crohn's disease	Diagnosis	[126]
	Gastric cancer	Diagnosis, prognosis	[127]
	Kawasaki disease	Diagnosis	[128]
	Lung cancer	Prognosis	[129]
	Ovarian cancer	Diagnosis (via exosomes)	[130]
	SEOC	Diagnosis	[131]
	Prostate cancer	Metastasis	[132]
Urine	*Chronic kidney diseases*	Diagnosis (via exosomes)	[133]

Diseases and their status related to miR-200c expression are listed with the location where significant miR-200c expression has been detected to be used as a biomarker

ICC intrahepatic cholangiocarcinoma, *IPMN* intraductal papillary mucinous neoplasm, *CTC* circulating tumor cells, *SEOC* serous epithelial ovarian cancer

difficult to detect [136]. Deep miRNA-array analysis of circulating miRNAs in the sera of metastatic breast cancer patients and healthy controls has demonstrated that miR-200c levels were significantly higher in CTC-positive patients compared to CTC-negative patients and healthy controls suggesting a role for circulating miRNAs in predicting CTC status and associated metastatic disease [50].

miRNAs are not only circulating in extracellular fluids, but they are also secreted from tissues to their extracellular environment in exosomes. For instance, levels of miR-200c and seven other miRNAs in tumor-secreted exosomes were found to be similar to those in ovarian tumor cells confirming diagnostic potential of profiling circulating tumor exosomes [130]. Furthermore, exosomal secretion of miR-200c in urine has been observed in various chronic kidney diseases [133] and in systemic diseases such as lupus [137]. As a whole, miR-200c has a huge potential to be used as a biomarker of diagnosis, disease progression, and predicting treatment response.

Future perspectives

The role of miR-200 family, especially miR-200c, in regulating cancer metastasis and acquisition of drug resistance, two major interlinked hallmarks of cancer, has been very well established. However, a new insight came from a recent study in breast cancer where miR-200 overexpression has been shown not to affect onset of lung metastasis but to increase post-metastatic chemosensitivity suggesting the potential of miR-200-based EMT-targeting strategy in enhancing chemosensitivity [82]. Further understanding of underlying mechanisms will help improve and re-shape future EMT-targeting strategies.

With the advances in the field of non-coding RNAs over the last decade with the help of technological advancements in deep sequencing, researchers have now started focusing on the interactions among various kinds of non-coding RNAs, e.g., long intergenic RNAs, circular RNAs and miRNAs, etc., to understand the developmental and disease-associated mechanisms. Recently, a long non-coding RNA named ATB

has been shown to be activated downstream of TGF-β, which competitively binds to miR-200 family in HCC cells, upregulates ZEB1/2 transcription factors, and induces EMT [138]. Therefore, future research on miR-200c function should also include lncRNAs, in addition to proteins, to have a better understanding of its mechanism of action.

Importantly, cell-cell interaction via exosome transport within the tumor microenvironment is of great importance in maintaining tumor state and developing drug resistance. In this line, pre-treatment of A549 cells with exosomes collected from cisplatin-treated counterparts decreased miR-200c expression along with altering the expression of different miRNAs [139]. Untangling the puzzles of the tumor microenvironment will further improve our knowledge about involvement of miR-200c and its trafficking in regulating tumor state.

Tumor suppressor miRNAs, like miR-200c, have potential to be used as therapeutic agents, but their delivery and stability in body fluids is of great concern. Major challenges include (1) poor penetration of miRNAs in tumor tissues, (2) miRNA degradation in body fluids, (3) miRNA-associated immunotoxicity and neurotoxicity, (4) inefficient gene silencing due to poor intracellular delivery of miRNAs, (5) off-target effects, and (6) unavailability of sufficient miRNA-processing enzymes. Multiple miRNA modification techniques have been described to better overcome these barriers (reviewed in [140]). For example, targeted delivery of aptamer-conjugated let-7g in a xenograft model of lung adenocarcinoma has shown promising results in reducing tumor growth [141]. In addition, combining miRNAs with other therapy agents is another option to better utilize miRNAs as therapeutic tool. For instance, miR-200c has been found to increase the radiosensitivity by directly regulating oxidative stress response genes PRDX2, GAPB/Nrf2, and SESN1 which led to the inhibition of DNA double-strand break repair, increased levels of reactive oxygen species, and upregulated p21 [142].

Recently, miR-34, a well-known tumor suppressor miRNA that is in a double-negative feedback loop with Snail, like miR-200c and ZEB1, has been entered in a phase-I clinical trial (NCT01829971) for the treatment of HCC [143]. Furthermore, regarding the control of multiple oncogenes in different tumor tissues, miR-200c can be considered as a promising targeted therapy molecule similar to miR-34a. Therefore, a broad range of tumor suppressor functions conducted by miR-200c reviewed here also need to be assessed in clinical trials for the benefit of patients using miR-200c-based therapeutics.

Concluding remarks

Regulation of gene expression by miRNAs is not to serve just as a switch—turning protein production on or off—but to fine-tune the overall gene expression output providing robustness to cell-specific programs. In this article, we reviewed miR-200c, a well-established, exceptional miRNA, which fine-tunes gene regulation in various diseases, especially in cancer. Notably, miR-200c simultaneously regulates major elements of cancer progression, i.e., proliferation, EMT, and drug resistance, in a context-dependent manner. Commonly known mesenchymal transcription factors ZEB1 and ZEB2 are among the direct targets of miR-200c; thus, it has established a strong link with EMT regulation. In addition, miR-200c is also correlated with and regulates chemoresistance, multidrug resistance, targeted therapy resistance, radiotolerance, and stemness through targeting and modulating the activities of key molecules involved in these processes. Overall, miR-200c functions at the crossroad of EMT and drug resistance reversal and has great potential to be used as therapy sensitizer and biomarker for several cancer tissues due to its presence in both intracellular as well as extracellular environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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