H19X-encoded miR-424(322)/-503 cluster: emerging roles in cell differentiation, proliferation, plasticity and metabolism

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Received: 15 September 2018 / Revised: 5 November 2018 / Accepted: 13 November 2018 / Published online: 24 November 2018
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Abstract

miR-424(322)/-503 are mammal-specific members of the extended miR-15/107 microRNA family. They form a co-expression network with the imprinted IncRNA H19 in tetrapods. miR-424(322)/-503 regulate fundamental cellular processes including cell cycle, epithelial-to-mesenchymal transition, hypoxia and other stress response. They control tissue differentiation (cardiomyocyte, skeletal muscle, monocyte) and remodeling (mammary gland involution), and paradoxically participate in tumor initiation and progression. Expression of miR-424(322)/-503 is governed by unique mechanisms involving sex hormones. Here, we summarize current literature and provide a primer for future endeavors.

Keywords H19X · miR-424 · miR-322 · miR-503 · Tumor suppressor gene · EMT · Hypoxia

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs 18–24 nucleotides (nt) in length that regulate posttranscriptional gene expression [1]. miRNA genes are transcribed by RNA polymerase II to produce pri-miRNA, which is cleaved by Drosha to give rise to hairpin-structured pre-miRNA, ~60–100 nt in length. Exportin transports pre-miRNA into the cytoplasm, where it is processed by Dicer to produce a ~22 nt double-stranded intermediate comprising the mature miRNA strand and its complementary strand. The mature miRNA is loaded into the RNA-induced silencing complex (RISC) where it binds to the 3′ UTR of target mRNAs by partial sequence complement in the “seed” region, causing degradation of the mRNA transcript or inhibition of its translation.

The miR-15/107 family of microRNAs shares the “AGC AGC” sequence within the “seed” region, starting at either the first or the second nucleotide from the 5′ end [2]. They are critical regulators of cell division, apoptosis, stress response and metabolism, and involved in cancer, cardiovascular and neurodegenerative disorders. miR-424 (ortholog of rodent miR-322) and miR-503 are mammal-specific members of the miR-15/107 family. They are encoded as one cluster by H19X, located in human Xq26.3 [3]. Their expression is more dynamic and tissue restrictive than other miR-15/107 family members. miR-424(322)/-503 regulate fundamental processes such as cell cycle, epithelial-to-mesenchymal transition and hypoxia, drive tissue differentiation and remodeling, and paradoxically participate in tumor initiation and progression. Here, we summarize a decade of literature and provide a primer for future investigation concerning miR-424(322)/-503.

miR-424(322)/-503 are unique members of the miR-15/107 miRNA family

The miR-15/107 family includes ten miRNAs based on the presence of “AGCAGC” in the “seed” region situated at positions 2–7 from the 5′ end of mature microRNAs [2].
A

hsa-miR-107
hsa-miR-103a-3p
hsa-miR-15a-5p
hsa-miR-15b-5p
hsa-miR-16-5p
hsa-miR-195-5p
hsa-miR-497-5p
hsa-miR-503-5p
hsa-miR-424-5p
hsa-miR-646

AGCAGCAUUGUACAGGCGUAUCA
AGCAGCAUUGUACAGGCGUAUGA
UAGCAGCACAUAAUGGUUUUGUG
UAGCAGCACAUCAUGGUUUACA
UAGCAGCAGUAUAAUUGGC
UAGCAGCAACACUGGUUUUG
UAGCAGCGGAAACAGUUCUGCAG
CAGCAGCAUUUCAUGUUUUGAA
AAGCAGCUGCCUCUGAGGC

B
However, there is no consensus regarding the criteria of miRNA family classification; distinct classifications were proposed for these ten miRNAs [4–9]. We have adopted the classification by Finnerty et al. [2], but this is likely to change with new functional data accumulated and new miRNAs identified (Fig. 1a).

miR-15/107 family members are only expressed in chordates, with several being mammal specific (miR-195, -497, -503, -424 and -646) [2]. Genes of miR-15/107 family members are genomically associated with protein-coding genes or lncRNAs. They also show conserved tandem organization: miR-15 with miR-16, miR-424(322) with miR-503, and miR-497 with miR-195. To explore the evolutionary relation of miR-424(322)/-503 to others, we have updated a phylogenetic tree originally built by Necsulea et al. [3], using “stem-loop” pre-miRNA sequences in Homo sapiens, Monodelphis domestica (mdo), Macaca mulatta (mml), Mus musculus (mmu), Ornithorhynchus anatinus (oan), Gallus gallus (gga) and Xenopus tropicalis (xtr). miR-103 and -107 are the least closely related, and omitted in the plot. miR-424(322) is most closely related to miR-15c in mdo, oan, gga and xtr, while miR-503 is most closely related to miR-16c in gga, oan and xtr.

Supporting a common phylogenetic origin, miR-15/107 family members have similar expression patterns and functions. miR-15a, -15b, -16, -322 and -503 are dynamically upregulated during serum starvation and contact inhibition, with miR-503 showing the highest fold change [10]. miR-15a, -15b, -16 and -497 are essential for the switch from expansion to differentiation in precursor B lymphocytes [11]. On the other side, loss of either miR-15a/-16-1 or miR-15b/-16-2 by genomic deletion causes B cell chronic lymphocytic leukemia [12–14]. miR-15/-16 family members also drive NK cell maturation, by targeting Myb [15]. miR-15/107 family members inhibit cell proliferation in many tissue types. They are broadly upregulated after birth and cause cardiomyocyte mitotic arrest in rodents [16, 17]. Additionally, miR-15/107 family members respond to cellular stresses such as hypoxia, ischemia, ultraviolet, environmental toxin, etc., and induce adaptive changes in angiogenesis and cellular metabolism [18–23]. As will be detailed in the rest of this essay, the function of miR-424(322)/-503 overlaps with other family members, but is unique under temporal and spatial regulations and works in distinct processes.

**Association with the imprinted paradigm lncRNA, H19**

Necsulea et al. surveyed eight organs in 11 tetrapod species for the expression profiles of ncRNAs [3]. They have identified approximately 400 lncRNA genes that are at least 300 million years old. These lncRNAs evolve rapidly in terms of sequence and expression levels, but conserve tissue specificity. One evolutionarily conserved co-expression network is predicted to regulate placenta development. This network comprises H19 and the lncRNA that encodes the miR-424(322)/-503 cluster, which was hence named H19X.

H19 is best known as the imprinted paradigm [24–29]; except under rare pathological conditions, the H19 gene is only expressed from the maternal allele, while the adjacent IGF2 gene is only expressed from the paternal allele. Imprinting provides an important mechanism of gene dose control, allowing expression from only one allele, while the other is epigenetically silenced. Many imprinted genes are involved in placenta development; H19 regulates placenta growth in late gestation, via its “spinoff” miR-675 [30]. Overexpression of H19 causes embryonic and perinatal lethality [31]. H19 is quickly downregulated in most tissues except skeletal muscles after birth [32, 33].

H19X resembles H19 in several ways. First, H19X may be imprinted (see following section). Second, miR-424 is downregulated by hypoxia in trophoblasts, and higher miR-424 levels are associated with fetal growth restriction, indicating a role in placenta growth regulation [34, 35]. Third, expression of H19X is striated muscle restricted during embryogenesis. In addition to these suggestive evidences, it will be important to know if H19 and H19X are mutually regulatory, and if they cooperate with or compensate for each other in genetic models. Addressing these questions may provide insights into the intricate mechanisms of gene dose control and coordination in embryonic development.

**Structure of the H19X locus**

The human H19X locus encodes seven non-coding RNAs, including a number of microRNAs (miR-424, 503, 542, 450-1, 450-2, and 450b) and a long non-coding RNA (miR503HG), spanning a region of ~7 kb pairs on Xq26.3 (Fig. 2). The microRNAs are highly conserved in mammals. The lncRNA has similar expression patterns as miR-424(322)/-503, agreeing with being the host gene of the miRNA cluster. The ENCODE project has identified clustered H3K27Ac and DNase I signals, while the GeneHancer project has identified a high-confidence cluster of regulatory elements in the upstream regulatory region and gene bodies of miR-424 [36], miR-503 and miR503HG, suggesting that the H19X locus is actively transcribed and intricately regulated.
In human and mouse genomes, H19X is situated between PLAC1 and HPRT on the 5' and 3' ends, respectively. PLAC1 is a placenta-specific protein whose expression is restricted in the trophoblast lineage. PLAC1 is paternally imprinted, and deficiency of it causes placentomegaly [37]. In marsupials, the downstream gene of H19X is RNA-on-the-silent X (Rsx), an Xist-like lncRNA that drives X-inactivation [38]. Collectively, the genomic location and indication in placenta growth suggest that H19X is probably imprinted.

The precursor sequence of the miR-424(322)/-503 cluster encodes both -5p and -3p mature miRNAs. According to expression levels, miR-322-5p, miR-424-5p and miR-503-5p are predominant over their -3p counterparts. The -3p miRNAs are not related to the miR-15/107 family. The -5p miRNAs are not related to the miR-15/107 family. Throughout this manuscript, miR-322, miR-424 and miR-503 refer to -5p mature miRNAs. There are few studies about the function of -3p miRNAs encoded by the miR-424(322)/-503 cluster; how these -3p miRNAs contribute to described phenotypes is completely unknown.

Roles in cell fate specification and differentiation

Cardiomyocyte differentiation

In the embryo proper, expression from the H19X locus first appears in Mesp1-marked mesendoderm cells. Mesp1 is a bHLH factor that is transiently expressed at the onset of gastrulation [39, 40], marking a bipotent cardiac and skeletal muscle precursor population [41]. By surveying the transcriptome of this precursor population, we found that miR-322 and -503 were highly enriched, together with other H19X ncRNAs [42]. A knockin LacZ reporter demonstrated that expression from H19X is restricted to the developing heart, somites and skeletal muscles (Fig. 3a). miR-322/-503 augment the cardiomyocyte differentiation program while inhibiting neuroectoderm cell fates. Mechanistically, miR-322/-503 target Celf1 (note: only functionally and biochemically validated direct targets are included in this review), an RNA-binding protein that regulates RNA alternative splicing and decay and has a tight neuroectoderm association in embryos [42] (Fig. 3b). Mesendoderm formation and cardiac differentiation represent the first epithelial-to-mesenchymal transition (EMT) in life; as discussed later, regulating EMT is one of the chief mechanisms of miR-424(322)/-503 in carrying out their functions.

Skeletal muscle differentiation

Agreeing with specific expression in skeletal muscle precursor cells, miR-322(424)/-503 promote skeletal muscle differentiation [43]. Skeletal muscle differentiation starts with a G1 phase cell cycle arrest through inhibition of CDK2 [44, 45], miR-322(424)/-503 target Cdc25A, the phosphatase that removes inhibitory phosphorylation on CDK2 and causes cell cycle progression [43]. This effect of miR-322(424)/503 is implicated in inducing cell cycle quiescence during muscle differentiation.

Most miR-15/107 family members are broadly expressed [2]. As to whether some members have relative tissue specificity, such as whether miR-103/-107 are highest in the brain, and whether miR-15/-16 are higher in hematopoietic cells, discrepant results exist [46–50]. Striated muscle restriction appears to be unique for miR-322/-503. It is one place
where H19 and H19X cross path. H19 is broadly expressed in embryos, but restricted in postnatal skeletal muscles \([33, 43]\), whereas H19X shows early restriction which is lost after birth \([42]\). Whether their functions are related or even coordinated remain to be determined.

**Monocyte differentiation**

The transcription factor PU.1 synergizes with miR-424 in driving transcriptional commitment in the differentiation from promyelocytic blasts to monocyte/macrophage lineages.
[51]. PU.1 transactivates miR-424; miR-424 then targets NFI-A, whose downregulation is required for the commitment of two myeloid-specific pathways (granulocyte and monocyte/macrophage). miR-424, therefore, has a high hierarchical position in monocyte/macrophage lineage differentiation. Other mechanisms of miR-424 in inducing monocyte differentiation have also been described. miR-155, miR-222, miR-424 and miR-503 are induced by phorbol myristate acetate which promotes terminal differentiation of acute myeloid leukemia cells blocked in a progenitor cell state; miR-155 arrests cells in G2 phase, miR-424/-503 arrest cells in G1 and miR-222 induces apoptosis [52]. Interestingly, miR-424/503 directly targets the anti-differentiative miR-9 [52]. In chronic myeloid leukemia, miR-424 suppresses proliferation by directly targeting the oncogenic BCR-ABL tyrosine kinase fusion gene [53].

One of the best characterized functions of miR-15/-16 is in hematopoiesis and leukemia [54, 55]. miR-15a/-16-1 are lost by 13q deletions in chronic lymphocytic leukemia, which leads to activation of miR-15/-16 target, BCL2 [56, 57]. There appears a trend where miR-15/-16 regulate lymphoid cell differentiation, whereas miR-424(322)/-503 contribute to myeloid cell differentiation [11, 51, 52]. These miRNAs are predicted to target overlapping sets of mRNAs; therefore, they are likely controlled by different lineage commitment transcription factors to carry out similar functions in the lymphoid and myeloid compartments, respectively.

Roles in regulating proliferation and apoptosis, and as a tumor suppressor

Molecular targets in cell division and apoptosis

miRNAs have been long known to modulate cell cycle. Members of the miR-15/107 family induce G1 arrest by targeting primary cell cycle regulators including CDK1, CDK2, CDK6, cyclin D1, cyclin D3 and cyclin E1 [5, 6, 58–60]. Together with other miR-15 family members, miR-424(322)/-503 are upregulated in G1-arrested cells, serum starvation, contact inhibition and cellular senescence, and the change in miR-322/-503 levels is a multitude higher than others [10, 61]. Moreover, miR-15/-16 regulate cell death by targeting BCL2, which is an important mechanism in chronic lymphocytic leukemia [56, 57]. Like miR-15/-16, miR-424(322)/-503 are pro-apoptotic. Regulatory functions in cell division and apoptosis underlay the involvement of miR-424(322)/-503 in many biological processes, such as differentiation, organ homeostasis and carcinogenesis. To this front, Llobet-Navas et al. exemplarily dissected how miR-424(322)/-503 regulate mammary gland involution and work as a breast cancer tumor suppressor gene [62, 63].

Function in mammary gland involution and as a tumor suppressor

The mammary gland continuously undergoes tissue remodeling [64]. During pregnancy, secretory alveoli develop and form a dense lactiferous epithelial tree. During weaning, the alveoli and secretory duct structure collapses, known as involution. Llobet-Navas et al. described a cascade of molecular events in the involuting process [62]. Weaning activates the TGF-β signaling pathway, which upregulates the expression of the primary transcript of miR-424(322)/-503. Once processed, the mature miRNAs target important genes involved in cell division (Cdc25A) and survival or death decisions (Bcl-2 and Igf1r), resulting in significant reduction in the activity of the AKT and ERK1/2 pathways. These are conducive to inhibited growth and increased apoptosis of mammary epithelial cells. Ablation of the miR-424(322)/-503 gene leads to compromised regression of the secretory acini of the mammary gland. Further, ablation of miR-424(322)/-503 promotes breast tumorigenesis after pregnancy in animal models [63]. miR-424(322)/-503 is frequently lost in a subset of aggressive primary breast tumors which are chemoresistant, due to increased activity of BCL-2 and IGFIr. Other miR-15/107 family members are not among the most changed during mammary gland involution, supporting that regulating mammary epithelial cell growth is a unique function of miR-424(322)/-503.

A large corpus of evidence supports that miR-424(322)/-503 work as a tumor suppressor whose deletion or downregulation contributes to tumor initiation or aggressive behavior. We summarize the findings in Table 1, with apologies to colleagues whose works are not included due to space. We choose to highlight a few where multiple lines of evidences are available, with emphasis given to gynecological cancers. Several groups independently uncovered that miR-424 is downregulated in cervical cancers; lower miR-424 is correlated with poor prognostic clinicopathological parameters [67, 68, 82–85]. Xu et al. identified that miR-424 targets CHK1, which is inversely correlated to miR-424 levels [68]. Ectopic miR-424 enhanced apoptosis and blocked G1/S transition, and suppressed cell migration and invasion in cervical cancer cell lines. Intriguingly, this mechanism is indicated in human papillomavirus infection, a causative event of cervical and other anogenital cancers [83]. HPV E6 and E7 proteins suppress the levels of miR-424, while CHK1 is augmented. As a downstream effector kinase in the ATR DNA repair pathway, increased CHK1 contributes to viral genome amplification.

Lower miR-424 levels are also associated with epithelial ovarian cancers [78, 86]. Ectopic miR-424-5p arrests ovarian cancer cells in G0/G1 phase, via directly inhibiting cyclin E1 [86]. In ovarian clear cell carcinoma, a subtype of epithelial ovarian cancer associated with poor prognosis and
chemoresistance, miR-424 targets doublecortin-like kinase 1 (DCLK1) which is associated with cancer stem cells in multiple cancers [78]. Moreover, miR-424(322) modulates the PD-L1/PD1 and CD80/CTLA4 immune checkpoint [77]. PD1 and CTLA4 are T cell-expressed immunomodulatory receptors, whereas PD-L1 (to PD1) and CD80 (to CTLA4) are binding partners present on tumor cells and macrophages, and dendritic cells, respectively. PD-L1/PD1 and CD80/CTLA4 interactions result in reduced CD8+ cytotoxic T-lymphocyte proliferation and survival, and ultimately immune tolerance [87]. In chemoresistant epithelial ovarian cancers, miR-424(322) directly targets PD-L1 and CD80. Lower miR-424(322) and higher PD-L1 correlate to chemoresistant phenotypes [77]. Restoration of miR-424(322) hence represents a new opportunity in increasing chemosensitivity in ovarian cancers.

### Regulation by hormones

The expression of miR-424(322)/-503 is significantly altered during several hormone-controlled processes. Endometriosis is a benign gynecological disease among women in reproductive age, characterized by the presence of endometrial glands and stroma in locations other than the uterine cavity. Several groups reported the down-regulation of miR-424 or miR-503 in endometrial tissues vs. controls, or in ectopic vs. eutopic endometrial tissues [88–90]. There is an inverse correlation between miR-424 and the levels of VEGF-A [89]. Higher VEGF-A levels may be responsible for elevated angiogenic activity in endometriotic lesions. Ovary granulosa cells support the growth and maturation of follicles, and they undergo constant morphological and functional changes. miR-424/-503 are highly expressed in granulosa cells, with varying expression levels during the menstrual cycle [91–93]. miR-424/-503 regulate proliferation of granulosa cells, but how they affect follicle growth and maturation is unknown [91, 94].

Two groups have reported that expression of miR-424(322)/-503 is responsive to estrogen in MCF-7 breast epithelial cells. One group captured the temporal profiles of miRNAs and identified both miR-424 and miR-503 as among the most upregulated by E2 [95]. The other group captured the temporal profiles of mRNAs following E2 exposure and reversely predicted miRNAs that may be regulated. miR-424 was among the top findings and experimentally vetted [96]. Though these studies support the close association of miR-424 and -503 with estrogen, it is unknown if an estrogen receptor element is present on the upstream regulatory region and essential for H19X transcription. Efforts in this area will provide new insights into hormone-related disease mechanisms. Further, as miR-424(322)/-503 emerge as a critical regulator of muscle differentiation, growth

### Table 1 Tumor suppressor roles of miR-424(322)/503

| miR/Cluster | Cancer site | Pathology | Target | Refs. |
|-------------|-------------|-----------|--------|-------|
| miR-424-5p  | Breast      | Basal-like| DCLK1  | [65]  |
| miR-322/503 | Breast      | NA        | CDC25A, CCNE1, BCL-2, IGFR1 | [62, 63] |
| miR-503     | Breast      | NA        | PYK2   | [66]  |
| miR-424-5p  | Cervix      | Adenocarcinoma | KDM5B | [67]  |
| miR-424     | Cervix      | Cervical epithelial carcinoma | CHK1 | [68]  |
| miR-503     | Esophagus   | ESCC      | CCND1  | [69]  |
| miR-503     | Esophagus   | ESCC      | HOXC13 | [70]  |
| miR-503     | Esophagus   | ESCC      | PRKACA | [71]  |
| miR-424     | HSC         | CML       | BCR-ABL| [53]  |
| miR-424-5P  | Liver       | HCC       | ICAT   | [72]  |
| miR-503     | Liver       | HCC       | FGF2, VEGF-A | [73] |
| miR-503     | Liver       | HCC       | EIF4E  | [74]  |
| miR-503     | Liver       | HCC       | Cyclin D3, E2F3 | [75] |
| miR-503     | Lung        | NSCLC     | PI3K p85, IKK-β | [76] |
| miR-424(322)| Ovary       | Epithelial ovarian carcinoma | PD-L1, CD80 | [77] |
| miR-424     | Ovary       | OCCC      | DCLK1  | [78]  |
| miR-503     | Prostate    | NA        | ZNF217 | [79]  |
| miR-424     | Ovary       | Endometrial carcinoma | E2F7 | [80]  |
| miR-503     | Uterus      | Endometrial carcinoma | CCND1 | [81]  |

ESCC esophageal squamous cell carcinoma, CML chronic myelogenous leukemia, HCC hepatocellular carcinoma, NSCLC non-small cell lung cancer, OCCC ovarian clear cell carcinoma, EEC endometrioid endometrial cancer
and metabolism [42, 43, 97], their hormone link may help explain sex differences in muscle physiology and diseases.

**Roles in epithelial-to-mesenchymal transition and tumor progression**

**Relation to the TGF-β pathway and epithelial-to-mesenchymal transition (EMT)**

The transforming growth factor beta family comprises structurally related proteins that regulate cell proliferation, differentiation, apoptosis and other functions [98–100]. TGF-β proteins bind to TGF-β type II receptors, which phosphorylate the type I receptor on its serine residues, and the latter becomes activated. Next, type I receptors phosphorylate R-SMADs (SMAD2 and SMAD3 for TGF-βs), increasing their affinity to coSMAD (SMAD4). Finally, the RSMAD/coSMAD complex translocates to the nucleus where it functions to drive gene transcription. The TGF-β pathway is regulated at multiple levels; R-SMADs are regulated by inhibitory SMADs (I-SMADs) and proteasome-mediated degradation. There are two I-SMADs: SMAD6 competes with R-SMADs for SMAD4 binding, whereas SMAD7 competes with R-SMADs for binding to type I receptors. Both I-SMADs are downstream targets of TGF-β signaling, providing a negative feedback mechanism. E3 ubiquitin ligases SMURF1 and SMURF2 regulate the levels of R-SMADs via proteasome-mediated degradation.

Mounting evidences support that TGF-β signaling is one of the main drivers of miR-424(322)/-503 biogenesis [62, 101–104]. In mammary epithelial cells, activated TGF-β signaling leads to increased transcription of miR-424(322)/-503, through an upstream SMAD-binding site [62]. Gu et al. found that SMAD4 is required for miR-503 transactivation, through the same SMAD-binding site, during smooth muscle cell differentiation in mesenchymal stem cells [102]. Additionally, TGF-β upregulates miR-424 in glioblastoma, cardiac fibrosis, myofibroblast differentiation from lung epithelial cells and formation of cancer-associated fibroblasts (CAFs) [101, 103, 104].

In some processes, miR-424(322)/-503 provide a feedforward mechanism to amplify TGF-β signaling (Fig. 4). In distraction osteogenesis, a clinical strategy to promote bone formation, miR-503 is one of the most upregulated miRNAs. miR-503 targets SMURF1, and positively modulates TGF-β signaling which is active during the early stage of distraction osteogenesis [105]. During TGF-β-induced EMT in human lung epithelial cells and TGF-β-regulated intestinal epithelial homeostasis, miR-424(322)/-503 target SMURF2 and enhance TGF-β signaling [103, 106]. The inhibitory SMAD7 is also a target of miR-424, which relieves the negative effects of SMAD7 on R-SMADs and contributes to smooth muscle cell differentiation in mesenchymal stem cells [102].

miR-424(322)/-503 help carry out classic function of TGF-β, such as growth inhibition and EMT, by serving as an effector and feedforward regulator. Confoundingly, miR-424 may also negatively regulate TGF-β signaling by targeting TGFBR3 [107, 108]. Thus, miR-424(322)/-503 target selection may be specific for individual processes, but more likely, miR-424(322)/-503 may target a few TGF-β pathway components simultaneously; the net outcome is a balance among all the interactions. This agrees with the notion that miRNAs regulate a network of genes by fine-tuning their expression levels, and hence coordinating a biological process.

miR-424(322)/-503-mediated EMT contributes to tumor progression in some cancers. Drasin et al. described stage-dependent roles of miR-424 in breast cancer [107]: downregulated miR-424 leads to tumor initiation, while subsequent upregulation facilitates metastasis. Twist and Snai1, classic transcription factors of mesenchymal programming, drive miR-424 expression. Elevated miR-424 induces EMT and cancer stemness-associated genes, by selectively targeting TGFBR3. In metastases, miR-424 is downregulated, which facilitates MET [107]. Completion of the EMT–MET axis allows metastatic tumor outgrowth at the new site. The importance of miR-424(322)/-503 in regulating EMT and cellular plasticity has also been demonstrated in colorectal cancer, prostate cancer and tongue squamous cell carcinoma [108–110].

**Other mechanisms contributing to tumor progression**

Suppressor of cytokine signaling (SOCS) factors are negative regulators of the JAK/STAT pathway. Lowered SOCS expression leads to higher JAK/STAT activity, which is associated with many cancers [111]. In oral squamous cell carcinoma, SOCS2 is downregulated and inversely correlated with the level of miR-424-5p. There exists a signal-amplifying loop in which IL-8 drives activity of STAT5, STAT5 drives miR-424 expression. Elevated miR-424 induces EMT and cellular plasticity which facilitates MET [107]. Completion of the EMT–MET axis allows metastatic tumor outgrowth at the new site. The importance of miR-424(322)/-503 in regulating EMT and cellular plasticity has also been demonstrated in colorectal cancer, prostate cancer and tongue squamous cell carcinoma [108–110].

Dallavalle et al. reported another mechanism of miR-424 in activating STAT proteins. In prostate tumors, miR-424 is upregulated due to lower expression of ESE3/EHF, which binds to the promoter of miR-424 and represses its transcription. E3 ubiquitin ligase COP1, an miR-424 target, is downregulated. Consequently, several oncogenic transcription factors including STAT3 evade proteasome-mediated degradation and become activated [113].
miR-424-5p facilitates gastric cancer cell proliferation and invasion by targeting LATS1, a core component of the Hippo pathway [114]. Circular RNA_LARP4 neutralizes the activity of miR-424-5p by serving as a molecular sponge. This is a rare demonstration of a posttranscriptional mechanism in regulating miRNA accessibility.

In summary, the role of miR-424(322)/-503 in cancer is highly contextual. While a tumor suppressive role has been established in breast cancers, oncogenic functions are suggested in glioblastoma and melanoma [63, 101, 115, 116]. The elegant works in breast cancer demonstrate the dynamic roles of miR-424(322)/-503 through the initiation and progression of the disease, exemplifying the complexity of gene regulation exerted by miRNAs. Potential redundant or cooperative roles of other miR-15/107 family members pose additional challenges in understanding the role of miR-424(322)/-503 in cancer. Despite the confounding issues, miR-424(322)/-503 has emerged as critical regulators of a variety of cancer hallmarks. Additional genetic evidences, as well as better appreciation of the dynamic interactions of miR-424(322)/-503 with its target network may bring about new therapeutic opportunities.

**Roles in stress response**

Expression of miR-15/107 family members is responsive to a variety of cellular stresses, including UV damage, environmental toxin, hypoxia and ischemic injury [18–23]. Among miR-15/107 family members, miR-424(322)/-503 show the
highest responsiveness under stress. [10]. Here we summarize the underlying mechanisms that miR-424(322)/-503 use to alleviate damages and help adaptation during cellular stresses.

**Hypoxia and ischemia**

Reduced availability of oxygen can occur in physiological conditions such as wound healing and physical exertion as well as pathological situations such as stroke and myocardial ischemia. A complex adaptation system centered around hypoxia-inducible factor (HIF) is responsible for restoring oxygen and nutrient homeostasis [117] (Fig. 5a). Under normoxia, HIF-1α is maintained at low levels due to active proteasome-mediated degradation. The degradation process starts with hydroxylation on two proline residues, P402 and P564. Hydroxylated HIF-1α is recognized by von Hippel–Lindau (VHL) protein, which brings hydroxylated HIF-1α to the VCBCR (VHL, elongin C, elongin B, cullin 2, and RBX1) E3 ubiquitin ligase complex. Poly-ubiquitination and proteasome-mediated degradation ensue. In hypoxia, HIF-1α becomes stabilized and forms a heterodimer with HIF-1β. The dimer migrates into the nucleus where it binds to hypoxia response elements and transactivates genes regulating metabolism, angiogenesis and erythropoiesis.

miR-424 and miR-210 are the most upregulated miRNAs in hypoxic vascular endothelial cells which sit at the frontline of responding to hypoxia. Ectopic miR-424 stabilizes both HIF-1α and HIF-2α, through targeting cullin 2 (CUL2), the scaffold protein for the E3 ubiquitin ligase complex [118] (Fig. 5b). Accordingly, miR-424 overexpression promotes in vitro angiogenesis and neovascularization in mice. Induction of miR-424 in hypoxia is through a C/EBPα-RUNX-1/PU.1 cascade, in which C/EBPα in cooperation with RUNX-1 transactivates PU.1 expression, and PU.1 transactivates miR-424 transcription through a PU.1-binding site [118]. Hypoxia induces miR-424 in a myocardial infarction mouse model as well as a hind limb ischemia mouse model, demonstrating the response is widespread in multiple tissue types [118].

Hypoxia induces miR-424 in cancer cells as well. Zhang et al. reported that in melanoma and colon cancer cell lines, hypoxia transactivates miR-424 expression via a hypoxia response element present on the promoter of miR-424. Increased miR-424 renders resistance to apoptosis-inducing drugs doxorubicin and etoposide [119].

Cerebral ischemia induces an acute increase in miR-424 levels in the peri-infarct cortex in a middle cerebral artery occlusion/reperfusion mouse model. Ectopic miR-424 reduces neuronal cell apoptosis and infarct volume, accompanied by increased activity of MnSOD [120]. In neuronal culture, H2O2 upregulates miR-424 expression, similar to ischemia or hypoxia exposure. Thus, it appears that the dynamically reactive miR-424 provides an acute means of reducing oxidative stress [120].

**Endoplasmic reticulum stress**

Deregulation of normal endoplasmic reticulum (ER) function leads to a conserved cellular response, unfolded protein...
response (UPR) [121]. It can trigger cell death if ER stress is prolonged. There are three ER transmembrane proteins serving as sensors of unfolded protein accumulation in the ER lumen: PERK1, IRE1, and ATF6. They represent three branches of signaling pathways in restoring homeostasis. Thapsigargin and tunicamycin, drugs causing accumulation of unfolded protein in the ER lumen, downregulate miR-424(322)/-503 in a PERK1-dependent fashion. miR-424 modulates the activity of two branches of UPR: it directly binds to the 3′-UTR of ATF6 transcripts and inhibits translation, whereas it regulates the activity of “regulated IRE1 dependent decay” (RIDD) on the IRE1 branch. Together, PERK-induced downregulation of miR-424(322)/-503 optimizes the activity of IRE1 and ATF6 during ER stress, hence serving as a node to coordinate UPR [122].

Roles in metabolism

Consistent with a role in adapting cells to changed environment, miR-424 has been shown to regulate major metabolic switches. TGF-β or PDGF-induced CAF formation is accompanied by metabolic switch from oxidative phosphorylation to aerobic glycolysis [104]; miR-424 plays a critical role in the process. TGF-β upregulates the expression of miR-424, and miR-424 directly targets isocitrate dehydrogenase 3a, an enzyme catalyzing the conversion from isocitrate to α-ketoglutarate (α-KG) in the tricarboxylic acid (TCA) cycle. Upregulated miR-424 causes a drop in α-KG, which contributes to the stability of HIF-1α. Proteasome-mediated HIF-1α degradation requires HIF-1α hydroxylation on two proline residues, P402 and P564; the activity of the responsible enzyme, proline hydroxylase, requires oxygen and α-KG. Structural analogs of α-KG, such as succinate and fumarate, inhibit proline hydroxylase activity [123–125]. Increased miR-424 effectively drops the ratio between α-KG and succinate/fumarate, and reduces HIF-1α degradation [104]. This is another mechanism that miR-424 uses to stabilize HIF-1α (Fig. 5). Stabilized HIF-1α transactivates genes involved in glycolysis.

Diabetic levels of glucose significantly drive down miR-424 expression in breast cancer cells [126]. Lowered miR-424 levels lead to higher expression of its target gene, CDC42. CDC42 induces the expression of transcription factor PRDM14, which is associated with poor prognosis in breast cancer patients [126]. Wang et al. reported decreased miR-150, miR-146a and miR-424 in peripheral blood mononuclear cells from type I diabetic patients, and the decrease is associated with ongoing autoimmuneity of pancreatic islet [127]. However, it is not clear how miR-424 is suppressed under hyperglycemic conditions, and how this change contributes to adaptive or pathological alterations. Research in this area is not yet sufficient to build a unitary framework, but miR-424(322)/-503 apparently influences metabolic pathways, which serve as bridges linking many processes discussed so far.

As biomarkers

Using circulating miRNAs as biomarkers has gained tremendous research interests [128, 129]. Many cell types, such as reticulocyte, dendritic cell, B cell, T cell, mast cell, epithelial cell, as well as tumor cell, release miRNAs. They are incorporated into exosomes/extracellular vesicles (EVs) and transferred to body fluids, such as plasma, urine and saliva. Exosomes/EVs carry and deliver mRNAs and miRNAs into recipient cells and exert profound physiological and pathological functions [130, 131]. Meantime, these circulating RNAs constitute a new category of non-invasive disease markers.

Circulating miR-15/-16 show correlation with several cancer types, including glioma, esophageal adenocarcinoma, cervical cancer and breast cancer [132–135]. They exhibit prognostic value in melanoma and acute heart failure [136, 137]. miR-424(322)/-503, especially miR-424, often constitute miRNA signatures with high predictive power for disease outcomes. Bye et al. assayed 179 miRs in the serum of 112 healthy participants who either suffered from fatal AMI within 10 years or remained healthy. They established a model for predicting future AMI consisting of miR-106a-5p, miR-424-5p, let-7g-5p, miR-144-3p and miR-660-5p, with 74.1% and 81.8% correct classification for men and women, respectively [138]. de Andrade et al. showed in 39 ALS patients/39 controls that miR-424 and miR-206 were higher in patient plasma and the baseline levels were associated with clinical deterioration [139]. Two groups independently demonstrated the prognostic value of miR-424 in non-small cell lung cancers. One model includes four miRNAs (miR-200c, miR-424, miR-29c and miR-124), whereas the other includes six miRNAs (miR-29a, miR-542-5p, miR-502-3p, miR-376a, miR-500a, miR-424), each holding prognostic value for overall survival [140, 141]. A 3-miRNA signature (miR-199a, miR-29c and miR-424) was found to distinguish breast cancer patients from controls [142]. We have summarized recent reports about miR-424(322)/-503 as circulating biomarkers in a variety of diseases (Table 2). Though there are many caveats regarding using circulating miRNA as biomarkers, it is clear that miR-424 is one of the best candidates that may be vetted in larger cohorts.

A major challenge is that the source of the circulating miRNAs is often unknown. It is critical to distinguish whether they are from a primary lesion such as cancer, or a secondary and reactive source, such as lymphocytes or muscles. Technical issues include the lack of a housekeeping circulating RNA control to normalize among individuals.
Conclusions and future perspectives

Over a decade of research has accumulated a large body of knowledge related to the miR-424(322)/-503 cluster. Individually or together, these miRNAs are involved in placenta, heart and skeletal muscle development during embryogenesis. They regulate core cellular processes including cell cycle control and EMT. They are the most dynamic in responding to a range of cellular stresses, including hypoxia and ischemia, and help restore homeostasis. Hormonal regulation over the biogenesis of these miRNAs and their involvement in physiological and pathological processes of female reproductive organs prompt an important question, are they one of the deciding factors of sex differences? The identification of miR-424(322)/-503 as biomarkers in many human diseases, especially the frequent detection in plasma samples, poses exciting clinical opportunities. However, this is still a new research subject; there remain many challenges awaiting exploration.

Many important hypotheses are not validated. The connection to H19 has not been supported by genetic evidence in animal models. Does miR-424(322)/-503 overexpression stall placental development, like in H19 transgenic mouse? Is the H19X locus paternally imprinted? Genetic ablation of H19 or miR-424(322)/-503 causes very mild systemic phenotypes: H19 KO animal has mild overgrowth [29], whereas miR-424(322)/-503 KO animal has mild white fat accumulation [62]. Will double-knockout animal display more pronounced phenotypes related to organism growth or homeostasis? Answering these questions would provide important insights into ncRNA regulatory mechanisms of growth control.

It remains difficult to rank the importance of molecular targets of miRNAs in a biological process. Most studies rely on computation programs or transcriptome survey to predict miRNA targets, and select one or two for additional investigation. Such strategies have intrinsic weaknesses: miRNAs are known to target many genes simultaneously, and mainly through affecting protein abundance, not mRNA abundance. With the advancement of technologies such as reverse phase protein array (RPPA), we may include protein arrays into the toolbox of miRNA target identification. This note is especially important for miR-424(322)/-503, as they are highly dynamic and their identified targets sometimes occupy opposite sides of signaling pathways.

Developing therapeutic interfering strategies require understanding of the redundancy and coordination between miR-424(322)/-503 and other miR-15/107 family miRNAs. It is a challenging task to inhibit the activities of miR-424(322)/-503, because other miR-15 family members may be parallel or compensatory. Thus, developing strategies that target all the miRNAs sharing the same seed sequence
would provide conclusive evidence for the function of miR-424(322)/-503 in important processes, and also form the base for therapy development.

Finally, building connections among the currently separated processes is critical. For instance, under cellular stress, cell proliferation, plasticity and metabolism may be well orchestrated, and miR-424(322)/-503 may coordinate the expression of a network of genes and help cells adapt and regain homeostasis. Systemic dissection of these processes, especially at protein and organism levels, would likely yield important insights that are currently unavailable.

Acknowledgements We thank Robert J. Schwartz, M. David Stewart and Xiaopeng Shen for helpful discussions. The work was supported by a startup fund from the University of Houston (to YL), American Heart Association Grants (11SDG5260033, 16GRNT27760164, and 18IPA34170360 to YL) and a Grant from the US Congressionally Directed Medical Research Program (PR162075 to YL).

Compliance with ethical standards

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

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