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Review

EWSR1, a multifunctional protein, regulates cellular function and aging via genetic and epigenetic pathways☆

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ABSTRACT

Ewing's sarcoma (EWS) is a bone cancer arising predominantly in young children. *EWSR1* (*Ewing Sarcoma breakpoint region 1/EWS RNA binding protein 1*) gene is ubiquitously expressed in most cell types, indicating it has diverse roles in various cellular processes and organ development. Recently, several studies have shown that missense mutations of *EWSR1* genes are known to be associated with central nervous system disorders such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Otherwise, EWSR1 plays epigenetic roles in gene expression, RNA processing, and cellular signal transduction. Interestingly, EWSR1 controls micro RNA (miRNA) levels via Drosha, leading to autophagy dysfunction and impaired dermal development. *Ewsr1* deficiency also leads to premature senescence of blood cells and gamete cells with a high rate of apoptosis due to the abnormal meiosis. Despite these roles of EWSR1 in various cellular functions, the exact mechanisms are not yet understood. In this context, the current review overviews a large body of evidence and discusses on what *EWSR1* genetic mutations are associated with brain diseases and on how EWSR1 modulates cellular function via the epigenetic pathway. This will provide a better understanding of bona fide roles of EWSR1 in aging and its association with brain disorders.

1. Introduction

Ewing's sarcoma (EWS) is a bone cancer arising mostly in children. In 1921, EWS was originally described as “diffuse endothelioma of bone” by Dr. James R. Ewing, an American pathologist [1]. In 1992, Ewing sarcoma breakpoint region 1 (*EWSR1*)/*EWS* (herein termed *EWSR1*) gene was identified at chromosomal breakpoint *t*(11;22)(q24;q12) region from EWS and neuroectodermal tumors as a translocation-generated fusion gene between *EWSR1* and *FLI1* (*Friend leukemia integration 1*) [2]. The *EWSR1* gene encodes a RNA/DNA binding protein and involves in various cellular processes. EWSR1 is well-known as a multifunctional protein, which regulates transcription and RNA splicing, indicating that it is involved in diverse cellular processes. N-terminal domains of EWSR1 interacts with the basal transcription factor TFIID and RNA Polymerase II. Moreover, EWSR1 protein is able to modulate gene transcription via interaction with CREB-binding protein (CBP), suggesting that EWSR1 plays a role in basal transcription process [3,4]. Previous studies discovered that EWSR1 regulates the transcriptional activity of HNF4, OCT4 and BRN3A [5–7]. In addition, EWS also serves a role in posttranscriptional mRNA splicing by cooperating with multiple splicing factors [8]. As such, growing body of evidence indicates that EWSR1 concerts various cellular pathways by itself or via multiple interactions with other molecules in a gene-context dependent manner.

EWSR1 belongs to a TET (also known as FET) family of proteins, which includes Fused in Sarcoma/Translocated in Liposarcoma (FUS/TLS, herein referred as FUS) and TATA-box binding protein Associated Factor 15 (TAF15). EWSR1, FUS and TAF15 are related in both structure and function. TET members are DNA/RNA-binding proteins (RBPs) that mainly contain an N-terminal serine-tyrosine-glycine-glutamine

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(SYGQ)-rich domain that acts as a transcriptional activation domain, a central RNA recognition domain (RRM) and a C-terminal zinc finger domain involved in RNA and DNA binding, respectively, and multiple Arg-Gly-Gly (RRG)-rich regions (RRGs) in the C-terminal that affect RNA binding [9–12]. The amino acid sequences of these proteins share high homology (~70%), and are evolutionarily conserved from fish to human [10]. TET proteins are expressed in most cell and tissue types, and predominantly reside in the nucleus [13]. In addition, they also have functional similarities. TET members contribute diverse roles in physiological cellular functions, and are involved in the regulation of RNA metabolism. Importantly, recent studies have shown that mutations of TET family genes are closely linked to certain neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) (or termed as frontotemporal lobar degeneration [FTLD]), and essential tremor.

Epigenetic mechanisms unveil ambiguous and mysterious features and phenotypes derived from gene activity that cannot be explained by conventional wisdom of genetics beyond DNA mutations. In this paradigm, epigenetic changes include reversible modifications in DNA (methylation) and post-translational modifications in histone protein (acetylation, methylation, phosphorylation, and etc.) that eventually affect gene expression. Involvement of noncoding RNAs at post-transcriptional regulation of mRNA level is also categorized as a component of epigenetic pathway. It has been shown that epigenetic modifications and signatures contribute to the pathogenesis of many human disorders. Despite the strong association between EWSR1 mutations in cancers and neurodegenerative disorders, its epigenetic roles are not well known. Accordingly, if EWSR1 functions through the epigenetic pathway, it can provide novel insights to identify unseen biological markers and therapeutic targets to treat EWSR1-related disorders. Taken together, this review paper outlines and discusses the genetic and epigenetic roles of EWSR1 in cellular function, aging, and brain disorders.

2. Genetic aspects: *EWSR1* and *TET* family gene mutations are linked to neurodegenerative disorders

Mutations in *TET* family genes encoding FUS, EWSR1, and TAF15 have recently been demonstrated to be associated with several neurodegenerative disorders including ALS, FTD/FTLD, and essential tremor that causes involuntary and rhythmic shaking (Fig. 1) [14–17]. ALS is a neurodegenerative disease that is typically described by the degeneration of lower and upper motor neurons, which leads to a progressive and fatal muscle paralysis (amyotrophic) [18–20]. FTD is caused by the degeneration of neurons in the frontal and temporal lobes, and clinically characterized by progressive disorders of behaviors and personality, as well as language skills. Notably, FTD is ranked as the second most common dementia, just after Alzheimer's disease [21]. The findings of *TET* mutations in ALS and FTD pathology have made a shift in understanding the pathological mechanisms, from RBP aggregation to problem of RNA metabolism [22]. Here, we focus on mutations of *EWSR1* and other members of *TET* family underlying ALS and FTD pathogenesis.

2.1. FUS

FUS mutations are identified as a common genetic cause for ALS, accounting for around 4% of familial ALS (fALS) cases, while not frequently seen in sporadic ALS (sALS) [23–28]. Over 50 mutations in the *FUS* gene have been identified in ALS, and about two-thirds of mutations are missense changes clustering in exons 13–15, encoding the last RRG-rich domain and a PY nuclear localization signal (Fig. 1). The majority of the C-terminal mutant FUS proteins show cytoplasmic redistribution due to the defects of transportin-mediated nuclear import, resulting in a loss of nuclear function and an aggregation of cytoplasmic FUS proteins. The remaining mutations are located in exons 3, 5, and 6, encoding SYGQ-rich domain and the first RGG domain [16,25–27]. It has been documented that mutations in *FUS* account for less than 1% of sALS [28], whereas 35 different pathogenic *FUS* mutations have been reported in fALS in only 2 years [27,29–31]. The FUS-immunoreactive cytoplasmic inclusions were observed in postmortem analysis of fALS cases [26,30]. In ALS patients, a hallmark feature of FUS-associated neuropathies is the aggregated cytoplasmic FUS proteins. To analyze pathological alterations within ALS related to *FUS* mutations, Higelin et al. used the models of human induced pluripotent stem cells (hiPSCs) and hiPSC derived motor neurons, and observed the FUS mislocalization in motor neurons that express mutant *FUS* [32], which is correlated with the findings in ALS patients. Nonetheless, it remains unsolved how the mutations in *FUS* cause the development of ALS. FTD neuropathology in most cases is identified by abnormal cellular aggregations of tau protein (FTD-tau) or transactive response DNA-binding protein with molecular weight of 43 kDa (TDP-43) (FTD-TDP). However, *FUS* mutations were also reported in FTD pathology, and even new subgroup of FTD has been categorized based on FUS pathology with the presence of abnormal cytoplasmic FUS-positive inclusions [33,34].

2.2. EWSR1

In contrast to FUS, a few *EWSR1* mutations have been found in sALS patients. Couthouis et al. performed complete sequencing of the last four exons encoding the last RGG- and PY-NLS domains in the C-terminus, which are equivalent to regions in *FUS* and *TDP43* contributing to ALS pathology. Two missense mutations, G511A and P552L, were identified in 2 sALS cases out of 817 samples and none in 1082 control individuals, suggesting their potential cause for ALS pathogenicity (Fig. 1). G511A and P552L are located in the last RGG domain of EWSR1. Intriguingly, both ALS-linked mutant EWSR1 proteins result in a formation of cytoplasmic EWSR1 inclusion in primary motor neurons cultured from mouse spinal cord and rat embryos, whereas wild-type (WT) EWSR1 primarily reside in the nucleus [35]. It has been well known that main pathological hallmarks of TET-associated pathology in ALS include the mislocalization of TET proteins to the cytoplasm. Interestingly, ALS-associated mutant EWSR1 proteins show increased proclivity to form aggregated proteins than WT EWSR1, which suggests the possibility of these mutations causing accelerated aggregation in the affected motor neurons. Furthermore, overexpression of mutant *EWSR1* in *Drosophila* causes neurodegeneration in the nervous system. Both in vitro and in vivo data support that mutant EWSR1 is able to confer neurodegeneration in similar manner to FUS, TAF15 and TDP-43 [35]. On the other hand, Ticozzi et al. screened the coding regions of *EWSR1* in fALS patients and healthy individuals; however, no coding variants were found in *EWSR1* [13]. Therefore, whether mutations in *EWSR1* contribute significantly to the pathogenesis of fALS or not awaits further investigation. To date, there is very little data on *EWSR1* mutations in FTD pathology. EWSR1 cytoplasmic inclusions were observed in FTD cases with FUS pathology, suggesting its potential involvement in pathogenic mechanisms of FTD [28]. However, it remains to be further investigated to determine whether EWSR1 represents as a cause of FTD pathogenesis.

2.3. TAF15

TAF15 gene mutations are observed in both sALS and fALS patients (Fig. 1) [13,36,37]. Using a yeast functional screening, missense variants in *TAF15* gene (G391E, R408C and G473E) were found in sALS cases, while absent in a large number of healthy individuals. The ALS-associated mutant TAF15 proteins also accelerate TAF15 aggregation in primary spinal cord neurons. Notably, mutant TAF15 proteins confer neurodegeneration in *Drosophila* with more severe phenotypes than WT TAF15. In sALS cases, mutant TAF15 was mislocalized to the cytoplasm of motor neurons in the spinal cord [37]. Mutations of *TAF15* (*A31T* and *R395Q*) were also discovered in fALS cases that belong to three

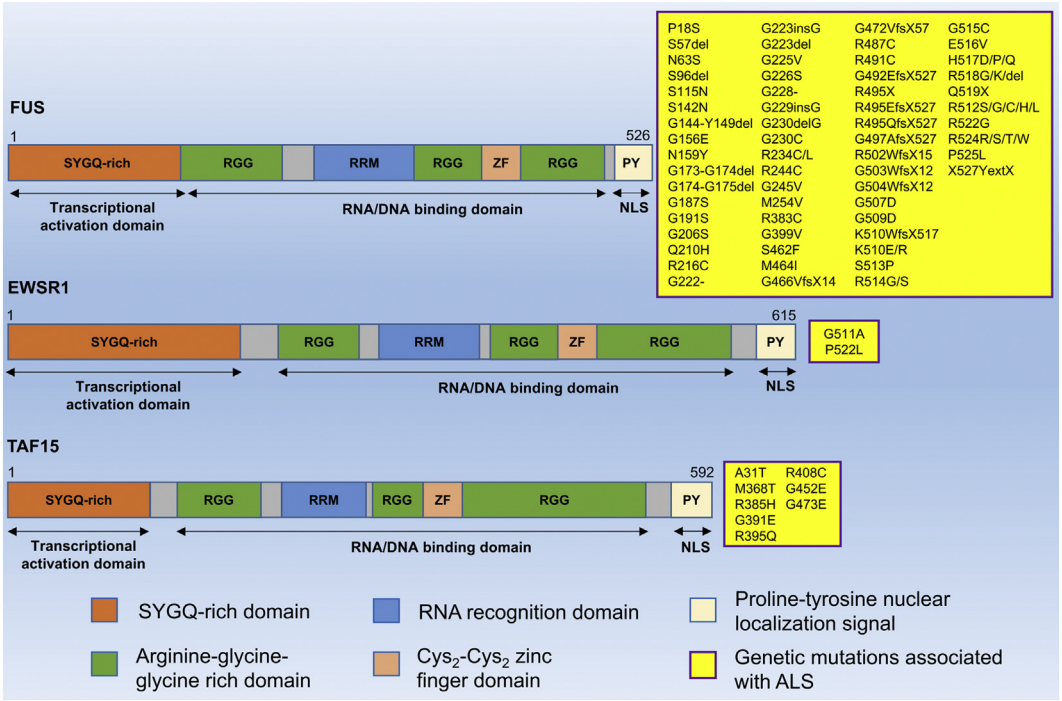

Fig. 1. Scheme representing the domain structure and ALS-associated mutations of TET family (FUS, EWSR1, and TAF15). TET members have similar domain structure, mainly contain an N-terminal serine-tyrosine-glycine-glutamine (SYGQ)-rich domain that acts as a transcriptional activating domain, a RNA recognition domain (RRM) and a zinc finger domain involved in RNA and DNA binding, respectively, multiple Arg-Gly-Gly (RRG)-rich regions (RRGs) in C-terminal that affect RNA binding, and prolie-tyrosine (PY) region functioning as nuclear localization signal (NLS). Yellow boxes present many kinds of missense-mutations that are found in *FUS*, *EWSR1*, and *TAF15* gene, respectively.

unrelated pedigrees, while not in over 1100 healthy cases [13]. These studies suggest that further investigations are needed to identify whether *TAF15* genetic mutations are the cause of ALS pathogenesis. TAF15 has been implied as a potential candidate for understanding FTD pathology. A recent study has shown that TAF15 is found in cytoplasmic aggregations in the neurons of FTD-FUS patients, suggesting that TAF15 is likely involved in the pathological processes of FTD-FUS [36].

3. *Ewsr1* knock out (KO) mouse model reveals bona fide functions of EWSR1

In order to determine the physiological roles of WT (wild type) EWSR1, Dr. Lee's group established a conventional *Ewsr1* KO mouse [38]. Since then, the *Ewsr1* KO (−/−) mouse model has successfully been utilized to decipher many important in vivo roles of EWSR1 (Fig. 1).

3.1. EWSR1 deficiency shows defects in multiple organs and cell types

Ewsr1-deficient mice are born according to the Mendelian ratio [38]. However, all of *Ewsr1* homozygous KO (−/−) mice are born smaller than their littermates and a few surviving *Ewsr1* KO (−/−) mice in 129SvEv/Black Swiss mixed background (10% survival rate) remain smaller in the body size and weight in adulthood. Congenic *Ewsr1* KO (−/−) mice in C57BL6/J or 129SvEv background exhibit complete postnatal lethality within 24 h of birth, but the cause of death remains unresolved.

Surviving129SvEv/Black Swiss background *Ewsr1* KO (−/−) mice show severe lymphopenia and a cell-autonomous pre-B cell development defect compared to littermate control mice [38]. The flow cytometric analysis indicates that the number of pre-B cells is significantly reduced compared to WT mice, while the number of pro (Progenitor) B cells appeared to be normal. *Ewsr1* KO (−/−) mice display reduced cellularity of thymi and spleens.

All *Ewsr1* KO (−/−) mice are sterile and have relatively small testis and ovary compared to littermate WT mice [38]. Further analysis showed impaired development of spermatogonium to spermatid and complete lack of mature sperms in 12-week-old *Ewsr1* KO (−/−) mice. Furthermore, *Ewsr1* KO (−/−) spermatocytes showed a reduced meiotic recombination and XY asynapsis, resulting in massive apoptosis of spermatocytes. In 12-week-old female *Ewsr1* KO (−/−) mice, most ovaries were devoid of maturing ovarian follicles, and no corpora lutea was present.

3.2. EWSR1 and adipogenesis

Park et al. discovered that EWSR1 is indispensable for brown fat lineage determination [39]. Brown adipose tissues (BATs) from *Ewsr1* KO embryos are developmentally arrested. A mechanistic study shows that *Ewsr1* deficiency halts brown preadipocyte differentiation due to loss of Bmp7, a critical brown adipogenic factor during BAT development. Upon adipogenic stimulation, EWSR1 interacts with Y-box binding protein 1 (YBX1) to activate Bmp7 transcription. Accordingly, loss of *Ewsr1* or *Ybx1* function results in the reduction of Bmp7 expression and brown adipogenesis. Considering the facts that EWSR1 is a transcriptional co-factor in the brown fat adipocyte fate determination and BAT is potentially applicable for reducing obesity, the EWSR1-YBX1-BMP7 pathway may be a new therapeutic target to control obesity.

3.3. EWSR1 and mitochondrial function

Interestingly, EWSR1 participates in mitochondria function and cellular energy homeostasis by modulating the stability of PGC-1α (Peroxisome proliferator-activated receptor γ Coactivator) protein, a key regulator of mitochondria biogenesis, [40]. Park et al. identified that *Ewsr1* deficiency leads to a rapid degradation of PGC-1α by increasing ubiquitination and proteolysis of PGC-1α via proteasome

pathway [40]. The reduction of PGC-1α, in turn, markedly reduces mitochondria abundance and activity in brown adipocytes and skeletal muscles of *Ewsr1* KO mice. Rescue of EWSR1 activity in *Ewsr1*-deficient cells complements PGC-1α activity and restores mitochondrial abundance. How is PGC-1α protein vulnerable to ubiquitination and degradation under *Ewsr1* deficiency condition? FBXW7 (F-box/WD40 domain protein 7), an E3 ubiquitin ligase, is involved in PGC-1α ubiquitination in *Ewsr1*-deficient cells. Knockdown of *Fbxw7* in *Ewsr1*-deficient cells restores PGC-1α protein level and mitochondria abundance. The decrease of mitochondrial biogenesis coincides with the significant reduction of respiration and fatty acid β-oxidation genes in the liver of *Ewsr1* KO mice.

3.4. The global effect of EWSR1 deficiency on cellular senescence and aging

Ewsr1-null fibroblasts exhibit cellular senescence while *Ewsr1*-deficient mice show an aging-like phenotype [38]. The immunocytochemistry analysis using senescence-associated β-galactosidase staining verified that the cellular senescence is significantly elevated in *Ewsr1 homozygous* ($^{-/-}$) mouse embryonic fibroblasts (MEFs) compared to *Ewsr1* WT ($^{+/+}$) MEFs. In addition, Li et al. further determined that gamma-irradiation (7 Gy) exacerbates the death of *Ewsr1* mutant mice compared to *Ewsr1* WT mice. Upon irradiation, *Ewsr1* mutant mice lived up to 60 days while littermate control mice (*Ewsr1*$^{+/+}$ and *Ewsr1*$^{+/-}$) survived beyond 120 days [38].

How is EWSR1 deficiency linked to cellular aging? It is well known that cellular senescence is triggered by the attrition of telomere due to the shortening of the telomere length beyond a critical threshold [41,42]. However, mouse cells possess long telomeres. Accordingly, it seems unlikely that the early onset of senescence in *Ewsr1* mutant cells is due to telomere attrition. The other molecular pathways via p53 and RB may be involved in the cellular senescence of *Ewsr1* mutant cells. However, no significant changes in p53, p21, and p19^{ARF} levels were found in *Ewsr1*$^{-/-}$ MEFs. On the other hand, the phosphorylated Rb (pRb) level was markedly reduced in *Ewsr1*$^{+/+}$ MEFs. Considering that the reduced pRb levels are more likely to result in proliferation rather than cellular senescence, the cellular senescence of *Ewsr1*$^{+/+}$ MEFs might be independent of the RB pathway. Interestingly, after more than 10 passages, an elevated level of p16^{INK4A}, a marker of cellular senescence, was observed in *Ewsr1*$^{-/-}$ MEFs compared to *Ewsr1*$^{+/+}$ MEFs [43].

EWSR1 deficiency also contributes to hematopoietic stem cell senescence [44]. Cho et al. reported that EWSR1 is essential for stem cell quiescence by observing that *Ewsr1* KO mice show the early onset of senescence in hematopoietic stem progenitor cells [44]. *Ewsr1*-deficient hematopoietic stem cells exhibit a phenotypic change such as an increase in β-galactosidase activity and a molecular change such as a robust increase of p16^{INK4a} compared to *Ewsr1* WT mice. These findings indicate that EWSR1 plays a significant role in maintaining the hematopoietic stem cell lineage and its deficiency may trigger aging processes in a cell type-specific manner. At the moment, it is not known whether free radicals are up or down regulated under EWSR1 deficiency condition. In addition, parameters of free radical injury are not determined in EWSR1 deficiency cells and animal models. On the other hand, it is curious about whether modulation of free radicals can ameliorate or rescue EWSR1 deficiency-associated cellular dysfunction and aging. To address these questions, the future study remains to be investigated.

3.5. EWSR1 deficiency leads to neuronal atrophy and abnormal motor function

Recently, we have reported that *Ewsr1* KO mice show neuroanatomical changes followed by motor dysfunction as one of behavioral phenotypes (Fig. 2) [45]. Overall, the neuronal sizes in the cortex, striatum and hippocampus were significantly reduced in homozygous *Ewsr1* KO (−/−) mice than that of *Ewsr1* WT mice. Furthermore, it was found that both *tyrosine hydroxylase* (*Th*) mRNA and TH protein, a dopamine synthesizing enzyme, are significantly reduced in *Ewsr1* KO mice compared to *Ewsr1* WT mice. The immunoreactivity of TH and Protein Phosphatase 1 Regulatory Inhibitor Subunit 1B (PPP1R1B)/dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) was markedly decreased in the striatum and substantia nigra of *Ewsr1* KO (−/−) mice. PPP1R1B/DARPP-32 is an important molecule that mediates signal transduction in the medium spiny neurons of the striatum [46]. This finding indicates that *Ewsr1* deficiency down-regulates TH level and, in turn, reduces PPP1R1B/DARPP-32 phosphorylation. Concurrent with the molecular changes of TH and PPP1R1B/DARPP-32 level, *Ewsr1* KO (−/−) mice exhibit a significant increase of forelimb and hindlimb clasping movements compared to *Ewsr1* WT mice. Taken togehter, these results suggest that *Ewsr1* deficiency deregulates dopaminergic signaling pathways by reducing TH and PPP1R1B/DARPP-32 activity and subsequently leads to motor dysfunction. However, future studies are necessary to elucidate a precise mechanism on how EWSR1 regulates the motor function and other behaviors using multiple molecular and behavioral analyses.

4. EWSR1 deficiency alters microRNA processing via Drosha

As a part of epigenetic components, microRNAs (miRNAs) play significant roles on regulating gene expression. miRNAs are transcribed by RNA polymerase II from the genome. miRNA is a short non-coding RNA consisting of about 22 nucleotides and plays a role as a post-transcriptional regulator in gene expression. miRNAs bind to complementary target sequence of messenger RNA (mRNA), and degrade target mRNAs or inhibit their translation into proteins. Long primary transcript miRNAs (called pri-miRNAs) are processed in the nucleus by DROSHA, a member of the ribonuclease III family (RNase III), and converted into precursor miRNAs (pre-miRNAs) [47]. In turn, the pre-miRNA is being exported to the cytoplasm via XPO5/EXPORTIN-5 and is further processed by DICER1, a double-stranded RNA-specific endoribonuclease [48]. Although many studies have characterized the role of miRNAs under normal and disease conditions, no studies have shown whether EWSR1 is involved in the regulation of miRNA levels.

Our group found for the first time that EWSR1 indirectly regulates the expression of microRNAs (miRNAs) via an induction of DROSHA (Fig. 3) [43]. *Ewsr1* deficiency elevates expression of Drosha and, in turn, increases miR-29b and miR-18b levels. Interestingly, both miR-29b and miR-18b directly target collagen IV alpha 1 (*Col4a1*) and connective tissue growth factor (*CTGF*) mRNAs and reduce their mRNA levels by negatively regulating the post-transcriptional pathway in *Ewsr1* KO mouse MEFs. Consequently, the increased expression of *Drosha*, miR-29b, and miR-18b and the reduction of Col4a1 and CTGF lead to impaired epidermal and dermal development, resulting in the abnormal skin development and aging in the *Ewsr1* KO mice. In contrast, loss of Drosha function restores Col4a1 and CTGF protein levels by normalizing miR-29b, and miR-18b expression in *Ewsr1* KO mouse MEFs. Collectively, our previous study indicates that EWSR1 presents the epigenetic effector function in the post-transcriptional regulation of Col4a1 and CTGF via the Drosha-miRNA-dependent pathway. This evidence proves a novel epigenetic role of EWSR1 in miRNA biogenesis and dermal morphogenesis.

5. EWSR1 regulates autophagy via an epigenetic modulation of UVRAG

Autophagy is a well-known intracellular self-digestive process that disassembles dysfunctional macro-molecular components to maintain cellular homeostasis [49,50]. In response to stress, autophagy often plays a key role by removing damaged organelles and recycling nutrients and energy within the cell [51–53]. Moreover, it has been reported that excessive activation or inactivation of autophagy is

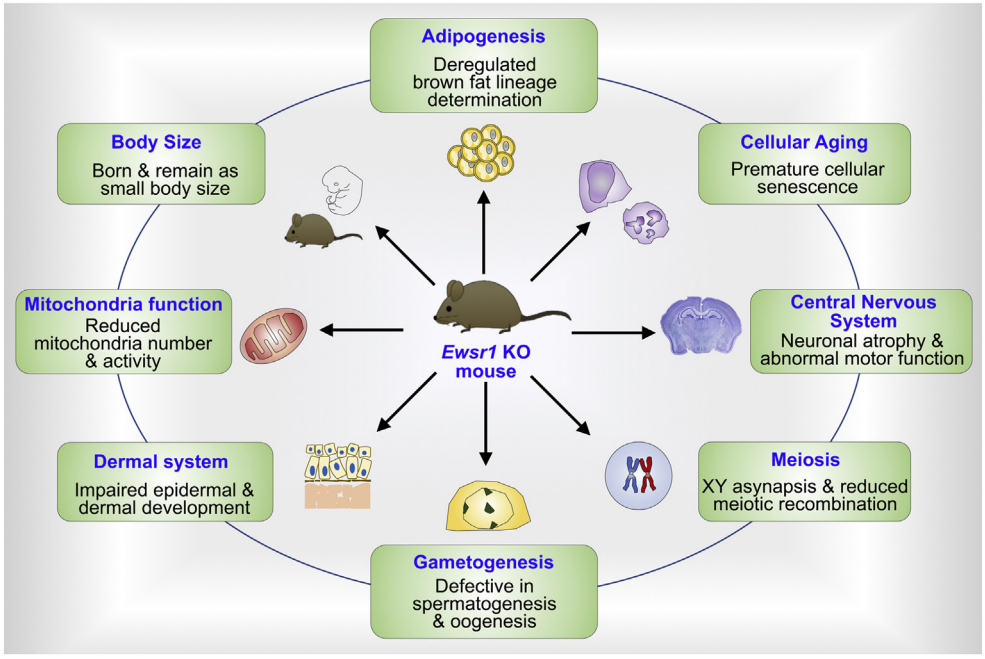

Fig. 2. EWSR1 is a multifunctional protein and its deficiency impairs tissue-specific functions. *Ewsr1* knockout mice exhibit smaller body size, deregulated brown fat lineage determination, premature cellular senescence, neuronal atrophy and abnormal motor function, XY asynapsis and reduced meiotic recombination, defective in spermato- and oogenesis, impaired epidermal and dermal development, and reduced mitochondria number and activity.

associated with various diseases, including neurodegenerative disorders and cancer [51,52,54,55].

miRNAs are known to regulate the autophagy-related genes and their activities. Also, miRNAs modulate autophagy at different stages such as autophagic induction, vesicle nucleation, vesicle elongation and completion, by targeting autophagy complexes via different miRNAs [56–58]. Recently, although there has been plenty of evidence that miRNAs modulate autophagy, their target genes and precise roles in the autophagy pathways have not been fully defined yet. In this context, our group previously investigated whether EWSR1 plays a role in the autophagy pathway or not using *Ewsr1 null* (−/−) MEFs. Two novel findings were identified as follows: First, *Ewsr1* deficiency up regulates microprocessor complexes and miR125a and miR351. Interestingly, EWSR1 indirectly regulates UVRAG expression at the post-transcriptional level via miR125a and miR351 [59]. Second, UVRAG dysfunction subsequently leads to an aberrant deregulation of autophagy pathway. Decreased expression levels of *Uvrag* mRNA and protein are correlated with the altered autophagy pathway in *Ewsr1* KO (−/−) mice.

UVRAG is a mammalian ortholog of yeast Vps38 and a promoter of autophagy [51,60,61]. It forms distinct complexes with BECN1 (mammalian ortholog of yeast Vps30/Atg6) and the class III phosphatidylinositol 3-kinase (whose catalytic subunit [PIK3C3] is the mammalian ortholog of yeast Vps34) and contributes to both autophagosome formation and maturation [60,62]. UVRAG suppresses cancer cell growth by promoting autophagy, its deficiency leads to decrease in autophagy and uncontrolled cell proliferation [60]. Based on the previous finding that DROSHA level is elevated by *Ewsr1* deficiency, it is hypothesized that DROSHA-miRNA dependent pathway may be involved in UVRAG expression [43]. Our group found that *Uvrag* mRNA is inversely correlated with elevated DROSHA levels in the cytoplasm of *Ewsr1 null* (−/−) MEFs [59]. In addition, miRNA microarray analysis verified that mir125a and miR351 are significantly increased in *Ewsr1*$^{-/-}$ MEFs. Indeed, miR125a and miR351 directly target and degrade *Uvrag* mRNA. Moreover, *Ewsr1* KO mice show that the levels of miR125a and miR351 are significantly increased, whereas the levels of UVRAG and LC3-II (autophagy marker) are significantly reduced compared with

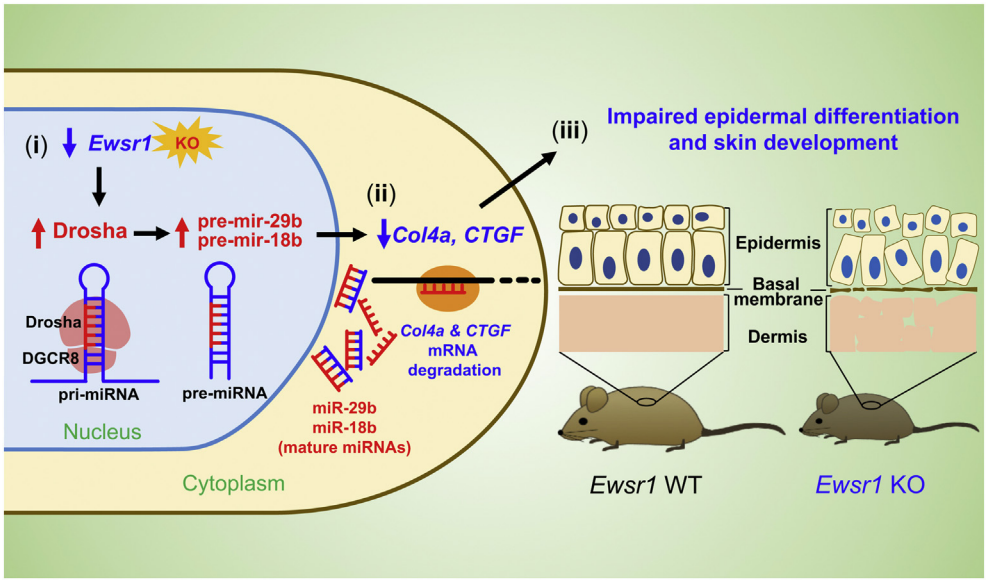

Fig. 3. EWSR1 deficiency influences epithelial cell senescence through an epigenetic modulation of miRNA processing via Drosha. (i) EWSR1 deficiency increases *Drosha* expression and certain miRNAs (miR-29b and miR-18b) in the nucleus of epithelial cells. (ii) In turn, miR-29b and miR-18b degrades *Col4a1* and *CTGF* mRNAs in the cytoplasm of epithelial cells. (iii) Consequently, reduction of Col4a1 and CTGF leads to impaired epithelial cell senescence and dermal development.

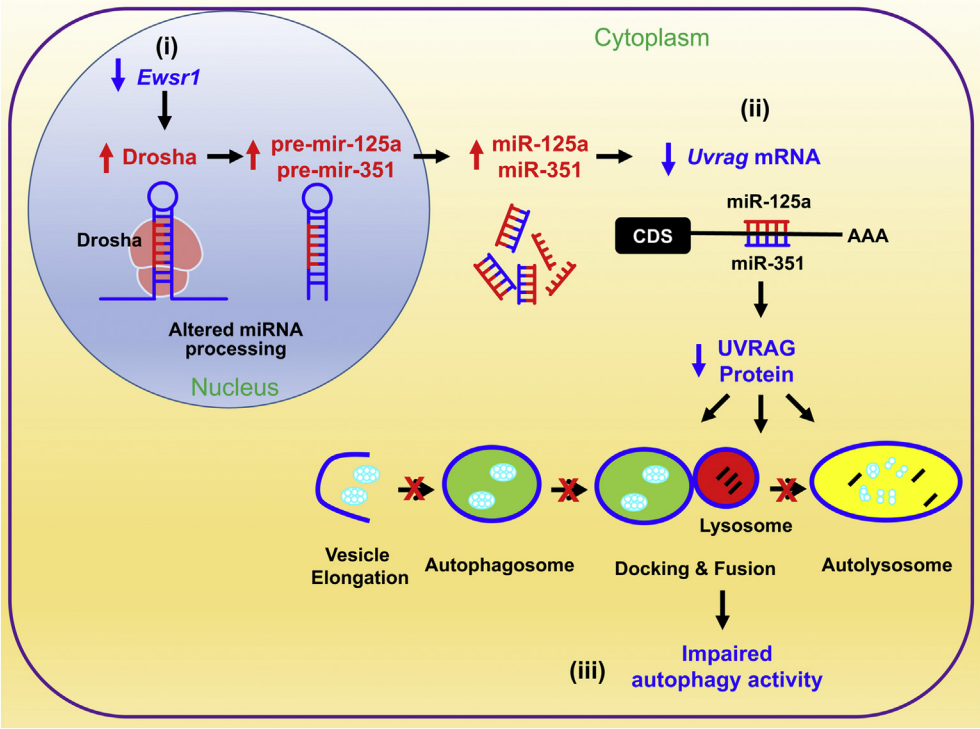

Fig. 4. EWSR1 plays an epigenetic role in the regulation of autophagy via a post-transcriptional modulation of UVRAG level. (i) EWSR1 deficiency increases the abnormal processing of pri-miRNAs to pre-mir125a and pre-mir351 by Drosha in the nucleus compartment. (ii) Next, elevated mature miRNA products (miR125a and miR351) degrade *Uvrag* mRNA in the cytoplasmic compartment. (iii) As a result, impaired UVRAG-dependent autolysosomal pathway leads to autophagy and cellular dysfunction. This figure is adopted from *Autophagy* (2015) 11 (5):796–811.

littermate control mice. Together, the previous study suggests that EWSR1 indirectly regulates autophagy via an epigenetic modulation of UVRAG level (Fig. 4). Thus, *EWSR1*-mediated regulation of UVRAG and autophagy may be a potential therapeutic target for restoration of the cellular function.

6. The role of EWSR1 in regulating stem cells

The *EWSR1* gene plays a crucial role in the regulation of stem cells and the development of a number of different tumors. During early development, Oct-4 (octamer-binding transcription factor 4), also known as POU5F1 (POU domain, class 5, transcription factor 1), that encodes a key regulator of stem cell pluripotency, is expressed to maintain the totipotent status of embryonic stem and germ cells. EWSR1 is fused to POU5F1 in hidradenoma of the skin and mucoepidermoid carcinoma of salivary glands [63]. POU5F1-mediated transactivation is stimulated by EWSR1 protein in mouse and human embryonic stem cells [6]. In 85–90% of cases, Ewing's sarcoma (ES) is characterized by the expression of the EWSR1-FLI1 chimeric protein resulting from the chromosomal translocation, which links the transcription regulating domain of EWSR1 to the ETS DNA-binding domain of FLI1 [64]. This EWSR1-FLI-1 fusion oncoprotein is responsible for the transcriptional deregulation of target genes, such as the CD99 membrane receptor. Expression of CD99 contributes to the ES oncogenesis by modulating the growth and differentiation of tumor cells [65]. Also, EWSR1 is chimerically fused to DDIT3 (DNA Damage Inducible Transcript 3) by the myxoid liposarcoma-specific chromosomal translocation. Suzuki K et al. investigated the molecular mechanisms underlying EWSR1-DDIT3 fusion protein-mediated phenotypic selection of putative target multipotent mesenchymal cells during myxoid liposarcoma development [66]. A better understanding of this mechanism is pivotal to elucidate the direct lineage reprogramming process in oncogenic sarcoma transformation mediated by EWSR1-fusion proteins [66].

7. Therapeutic approaches to target EWSR1

EWSR1, EWSR1-fusion protein, EWSR1-interacting molecules, and its downstream pathways can be ideal therapeutic targets to treat ES or EWSR1-related disorders. Interestingly, transcriptional activation of protein kinase PKC-ß (PRKCB) is directly regulated by the chimeric EWSR1-FLI1 protein in EWS. PRKCB loss induces apoptosis in vitro and prevents tumor growth in vivo. PRKCB possesses an enzymatic activity that can be directly targeted by small compounds. Accordingly, in the perspective of therapeutic strategy, blocking PRKCB activity in EWS is a new promising approach [67]. Poly (ADP-ribose) polymerase-1 (PARP) protein plays a role in the regulation of the cell cycle, apoptosis, and etc. It has shown that EWSR1-ETS fusion protein could be sensitive to PARP inhibitors such as Olaparib, Veliparib, and Iniparib [68]. Trabectedin is an antitumoral agent that modulates EWSR1-FLI1 transcriptional functions, causing DNA damage. The combination of a PARP inhibitor and Trabectedin highly inhibits proliferation and induces apoptosis in EWS cells [68]. Sanker et al. described that the Nucleosome Remodeling Deacetylase (NuRD) complex directly binds to EWS-FLI1 oncoprotein and regulates transcriptional activity of EWS-FLI1 target genes [68]. It has been widely known that the EWS-FLI1 plays a driver of proliferation and transformation in ES. Daniel et al. investigated that the combination effects of the histone deacetylases inhibitor suberoylanilide hydroxamic acid (SAHA) and Lysine-specific demethylase1 inhibitor (HCI-2509) on different biological functions in ES. The combination of SAHA and HCI-2509 inhibits the essential driver of this sarcoma and tumor growth and is proposed as a novel treatment strategy for ES patients [69].

Otherwise, ES cells express high level of histone lysine specific demethylase 1 (LSD1) expression. In this context, it is proposed that LSD1 inhibition may block the function of EWS-ETS proteins [70]. Furthermore, checkpoint kinase 1 (CHK1), a modulator of cell survival under the condition of impaired DNA replication, is a candidate of therapeutic targets in ES [71]. Small-molecule CHK1 inhibitor combined with gemcitabine shows elevated toxicity both in vitro and in vivo models of ES.

8. Conclusion

EWSR1 participates in various functions which are crucial for the regulation of tissue development and cellular homeostasis. We overviewed that i) genetic mutations of EWSR1 are associated with neurodegeneration, ii) EWSR1 deficiency leads to epigenetic alteration such as miRNA processing, and iii) EWSR1, as if "Jack of all trades, master of none", plays diverse molecular functions and its deficiency affects many cellular functions including autophagy and mitochondrial activity. Loss of EWSR1 function also contributes to the hypersensitivity of ionizing radiation and premature cellular senescence and aging. Even though several groups have studied in depth about EWSR1, there are still many areas that have not yet been explored. For example, conditional KO or knock-in EWSR1 animal models are necessary to determine the EWSR1's multifunctional or undescribed roles in an organ-specific or a cell type-specific manner. For example, to study desmoplastic small round cell tumor characterized by *EWSR1-WT1* translocation, Vanoli et al. developed a strategy using the combination of CRISPR-Cas9 genome editing and homology-directed repair to select human mesenchymal stem cells containing the *EWSR1-WT1* translocation with fusion transcript expression under the control of the *EWSR1* promoter and conditionally using Cre recombinase. A similar strategy was recently applied to generate conditionally inducible *EWSR1-WT1* and *EWSR1-FLI1* fusion genes in a human cell line (HEK293). This approach provides multiple advantages and expected to be a model for studying the tumors driven by chromosomal translocations [72,73]. Another group also developed a mouse model harboring conditional expression of *EWS-FLI1* fusion transcripts under the control of Prx1-Cre, which is expressed in the primitive mesenchymal cells in the limb bud of embryo. The *EWS-FLI1;Prx1-Cre* mice showed developmental defects of the limbs without tumors. Thus, it was clearly demonstrated that EWS-FLI1 is not able to initiate sarcoma formation by itself. Nonetheless, conditional deletion of *p53* in *EWS-FLI1; p53$^{flox/flox}$;Prx1-Cre* triple transgenic mice produced a poorly differentiated sarcoma. These data imply that sarcomagenesis can be induced via the cooperation of EWS-FLI1 and inactivation of the p53 tumor suppressor pathway [74].

In addition, studies using multiple behavioral and molecular analyses are required to better understand the mechanisms by which EWSR1 regulates brain and motor neuron functions. It is with great anticipation that future studies will further scrutinize and unravel new functions of EWSR1, which could be utilized to fuel new research areas in cancer, neurodegeneration and perhaps open a new field of research.

Transparency document

The Transparency document associated with this article can be found, in online version.

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