X-chromosome-located microRNAs in immunity: Might they explain male/female differences?

The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females

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In this paper, we hypothesize that X chromosome-associated mechanisms, which affect X-linked genes and are behind the immunological advantage of females, may also affect X-linked microRNAs. The human X chromosome contains 10% of all microRNAs detected so far in the human genome. Although the role of most of them has not yet been described, several X chromosome-located microRNAs have important functions in immunity and cancer. We therefore provide a detailed map of all described microRNAs located on human and mouse X chromosomes, and highlight the ones involved in immune functions and oncogenesis. The unique mode of inheritance of the X chromosome is ultimately the cause of the immune disadvantage of males and the enhanced survival of females following immunological challenges. How these aspects influence X-linked microRNAs will be a challenge for researchers in the coming years, not only from an evolutionary point of view, but also from the perspective of disease etiology.

Keywords:
cancer; genomic context; immunity; miRNAs; X chromosome

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Abbreviations:
EDA-ID, ectodermal dysplasia with immunodeficiency; **eNOS,** endothelial nitric oxide synthase; **IPEX,** immunodysregulation-polyendocrinopathy and enteropathy-X-linked syndrome; **KO,** knockout; **LPS,** lipopolysaccharide; **miRISC,** miRNA-induced silencing complex; **miRNA,** microRNA; **miRNP,** miRNA ribonucleoprotein complexes; **MRE,** miRNA-recognition element; **NO,** nitric oxide; **PID,** primary immunodeficiency; **ROS,** reactive oxygen species; **TLR,** Toll-like receptor; **UTR,** untranslated region; **WAS,** Wiskott-Aldrich syndrome; **X-CGD,** X-linked chronic granulomatous disease; **XCI,** X chromosome inactivation; **X-EDA-ID,** X-linked anhidrotic ectodermal dysplasia with immunodeficiency; **XLA,** X-linked agammaglobulinemia; **XLP,** X-linked lymphoproliferative disease; **X-SCID,** X-linked severe combined immunodeficiency.

Introduction

In humans, as in other mammalian species, females not only live longer than males, but also show an improved survival outcome from shock episodes caused by sepsis, injury or trauma-hemorrhage [1–3]. For example, a retrospective study combining four major sepsis studies has shown a male preponderance for morbidity and mortality [4]. Similarly, Schröder et al. [5] reported a clear effect of gender on mortality in surgical patients with severe sepsis, with a significantly higher survival rate in women (74%) when compared to men (31%) after the onset of sepsis. Wichmann et al. [6] also found a significantly lower incidence of severe sepsis/septic shock in female intensive care patients in nearly all age groups studied. It is also reported that males experience more frequent and severe infections caused by bacteria or viruses from infancy to adulthood [7, 8]. However, it should be mentioned that the same mechanisms that endow females with a survival advantage to pathogenic insults also increase their susceptibility to develop autoimmune disorders later in life [9, 10]. This subject has been recently reviewed by us [11], and so it will not be explored further here. Although gender-specific immune responses may be partially explained by hormonal regulation [12, 13], males are *hemizygous* (Box 1) for all X chromosome-linked genes, which directly

Box 1

Glossary

CpG islands: Regions of at least 200 bp, rich in cytosine and guanines, linked by a phosphodiester bond. They are typically located within or near promoter regions, and have CpG contents 60% higher than expected. Methylation of CpG sites within the promoter of a gene leads to inhibition of its expression.

Female mosaicism: During early embryonic development, one of the two parental X chromosomes inherited by females is randomly inactivated in each individual cell. This state is then clonally transmitted to the daughter cells. Due to this mechanism, mammalian females have two genetically distinct types of cells and are, therefore, mosaics.

Hemizygous: Refers to individuals which have only one member of a chromosome pair, instead of the usual two. Thus, males are hemizygous for the X chromosome.

Lipopolysaccharide (LPS) (or endotoxin): Is an important structural component of the outer leaflet of the outer membrane of Gram-negative bacteria. It is a very toxic agent, and when administered to mice it is able to reproduce some of the clinical features of sepsis.

miRNA paralog clusters: These are evolutionary related clusters that arose by duplication within the genome. Usually, paralogs acquire new functions that can be related to the original cluster function.

Polycistronic precursor: A polycistronic mRNA precursor contains the genetic information of several genes, which are translated into different proteins that usually have related functions.

Primary immunodeficiencies (PIDs): Are genetic disorders usually diagnosed in early infancy and that occur when part of the immune system is missing or does not work correctly. X-linked PIDs include: X-linked agammaglobulinemia (XLA), Wiskott-Aldrich syndrome (WAS), X-linked neutropenia and myelodysplasia, X-linked chronic granulomatous disease (X-CGD), Properdin deficiency, immunodysregulation-polyendocrinopathy and enteropathy-X-linked syndrome (IPEX), X-linked severe combined immunodeficiency (X-SCID), X-linked lymphoproliferative disease (XLP), X-linked hyper-IgM syndrome, Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) and glucose-6-phosphate dehydrogenase deficiency.

Pseudoautosomal regions: Regions of sequence homology between sex chromosomes. These regions are located at both ends of the X and Y chromosomes, and have been retained in order to ensure proper segregation during male meiosis.

Reproductive isolation: Is a collection of mechanisms that prevents that crossing between members of two different species produces fertile offspring. These mechanisms are important for maintaining the integrity and conservation of species through time and ultimately result in speciation.

Skewed X chromosome inactivation: When the mechanism of XCI does not occur at random as expected. Females with skewed XCI have one of the parental X chromosomes preferentially inactivated in nearly all cells.

Speciation: The evolutionary process by which new species are formed.

X chromosome inactivation (XCI): Also called silencing. This is the mechanism by which one of the X chromosomes is randomly inactivated during early embryogenesis in all female cells in order to equal the X chromosome number of male cells.

X chromosome silencing escape: When genes or parts of the inactive X chromosome escape inactivation, due to inefficient methylation or due to reactivation mechanisms. When this occurs, females have two functional copies of the silencing-escaping genes in the cells where they are expressed.

Z chromosome: The ZW-sex determination system of birds is the opposite of the mammalian XY system. Female birds have two different kinds of sex chromosomes (ZW) and males have two of the same kind (ZZ).

exposes them to recessive mutations occurring on this chromosome. Additionally, the mechanism of *X-chromosome inactivation* (XCI) (Box 1), which is expected to equilibrate the gene expression of females to that of males, is often incomplete and some genes may be expressed by both X chromosomes in female cells [14]. Not only is this phenomenon of *silencing escape* (Box 1) frequent [14], but also, females with extreme *skewed* XCI (Box 1) may never be affected by the disease for which they are carriers [15]. Numerous X-located genes have a direct or indirect role in immunity (reviewed in ref. [11, 16]), and some of them are responsible for X-linked *primary immunodeficiencies* (PIDs) (Box 1) (reviewed in ref. [11, 17]). Naturally-occurring mutations on the X chromosome may be responsible for sex-specific immune responses, and possibly for the immunological advantage of females [18, 19]. In addition, the X chromosome also contains a number of microRNAs (miRNAs) involved in immunity, but it is not known whether they contribute to gender-specific immune responses. MiRNAs are important negative regulators of protein synthesis, by base-pairing to target mRNAs. These small double-stranded non-coding RNAs regulate gene expression by translational repression and/or messenger RNA degradation. The small mature miRNAs (~22 nucleotides in length) are processed from full-length (~2 kb in length) primary transcripts, the pri-miRNA, by the miRNA-mediated RNA interference pathway, which involves a series of RNase enzymes and accessory proteins [20–23]. The actions of miRNAs are mediated by the miRNA-induced silencing complex (miRISC) or miRNA ribonucleoprotein complexes (miRNP). The former complex, which includes the miRNA and a member of the Argonaute family of proteins, blocks translation and/or reduces mRNA stability by imperfect binding to the miRNA-recognition elements (MREs) within the 3′ untranslated region (UTR) of target genes [24]. Target-binding specificity is usually mediated by the "seed" region located between residues 2-8 at the 5′ of the miRNA. However, inhibitory-efficiency can be influenced by other factors such as cooperation between multiple MREs, spacing between MREs, proximity to the

stop codon, position within the 3′ UTR, AU composition, and target mRNA secondary structure [25]. The same miRNA can target several genes to degradation, and the same gene can be regulated by numerous miRNAs. In addition, miRNAs may be expressed in specific cell types and organs, and are essential in maintaining cell fate during embryogenesis.

Here, we hypothesize that X-linked miRNAs may contribute to the immunological advantage of females. We also provide an overview of the most important features associated with the X chromosome and a detailed map of all miRNAs located on human and mouse X chromosomes. We also highlight the ones which have a known role in both immune functions and cancer, and speculate that silencing escape and X-inactivation skewing, which are known mechanisms affecting X-linked genes, possibly influence X-linked miRNAs to the same extent.

The X chromosome: An eXceptional chromosome

Mosaicism of female cells confers them with a survival advantage

During the course of evolution, the emergence of a sex-determining mechanism was the main reason behind the separation of X and Y into a distinct pair of chromosomes [26]. This led to loss of recombination between them, except for the *pseudoautosomal regions* (Box 1), and to the progressive degeneration of the Y chromosome. Nowadays, the number of genes common between the two is less than 1% [27]. In contrast to the Y chromosome, the X chromosome is a *bona fide* chromosome that has retained a significant number of genes involved in spermatogenesis [28], cognitive functions [29], and immunity [11, 16]. Because females have two X chromosomes, and males have only one, the mechanism of XCI has evolved to compensate for the unequal gene expression between sexes. This mechanism results in *female mosaicism* (Box 1), which allows females to cope with recessive mutations that occur on the X chromosome. However, males have only one gene copy of X-linked genes and do not have rescuing mosaic cells. Therefore, in general, females are not affected by X-linked diseases, whereas males are always affected, and this is illustrated by the existence of numerous male-specific X-linked PIDs [30], such as Bruton (X-linked) agammaglobulinemia (XLA), X-SCID, WAS, X-linked anhidrotic ectodermal dysplasia with immunodeficiency (X-EDA-ID), X-CGD, incontinentia pigmenti, among others (reviewed in ref. [11, 17]). This extraordinary genetic advantage has endowed females with a cellular backup and with genetic diversity, which are advantageous when facing immune challenges [7, 8, 19, 30].

How might mutations in X-linked miRNAs affect sex-specific responses?

When a new recessive mutation arises on the X chromosome there are different possible scenarios (Fig. 1). If random XCI does not occur as expected, the immune advantage of females may become more evident. Extreme skewing of XCI favoring silencing of a detrimental mutation will eliminate nearly all cells containing the disadvantageous mutation in female carriers (Fig. 1C). This happens for example in female carriers of XLA, X-SCID, WAS, and incontinentia pigmenti [15]. Usually, these females do not show any of the symptoms associated with the disorder, or they are very mild. However, the opposite can also occur. Skewing may favor the inactivation of the normal allele, and female carriers will manifest various symptoms associated with the disease (Fig. 1D). This has been observed for example in female carriers for X-CGD [31]. In addition, up to 15% of the genes located on the X chromosome escape permanent inactivation and 10% show variable patterns of inactivation among females [14]. This means that females may have nearly twice the amount of given gene products when compared to males [14] (Fig. 1F). We speculate that if a gene escaping silencing is important for a particular signaling pathway, females will possibly have an enhanced response to pathogens. For example, several members of the Toll-like receptor (TLR) pathway are X-located (Box 2), and some of them have been shown to escape XCI, such as *BTK*, *IRAK1*, and *IKKγ/NEMO* [14], which could lead to higher NF-κB-dependent gene transcription in some females. Females are also mosaics for putative mutations affecting miRNAs and/or miRNA regulatory sequences on the X chromosome. However, so far, such mutations have not been reported in human populations. It is also not known whether X-linked miRNAs escape inactivation. But this is very possible. If 15% of the genes on the X chromosome escape permanent silencing, and 10% may also potentially do so [14], it is likely that miRNAs escape methylation, particularly if they are located within genes reported to escape inactivation. Furthermore, they are certainly subject to skewing. If one of the parental X chromosomes is preferentially inactivated, due to the presence of a detrimental mutation affecting proper cellular function, X-linked miRNAs are also subject to skewing. Altered miRNA expression and epigenetic aberrations are associated with disease development. Around 50% of the miRNAs are associated with *CpG islands* (Box 1), meaning that they are subject to DNA methylation [32]. For instance, DNA hyper- and hypomethylation have been associated with decreased or increased miRNAs expression and the development of carcinomas [33, 34]. Thus, any disturbances in miRNA expression can result in deregulation of gene networks, and ultimately lead to oncogenesis and immune dysfunctions. By bringing together all these facts, we theorize that disturbances in the normal inactivation pattern of miRNAs on the X chromosome, by means of silencing escape or inactivation skewing, may affect miRNAs-driven gene regulation and result in gender-specific responses.

microRNAs on the X chromosome: The importance of the genomic context

The X chromosome is enriched in miRNAs: A mammalian peculiarity?

According to the MicroCosm database (from EMBL) there are nearly 800 miRNAs described so far in the human

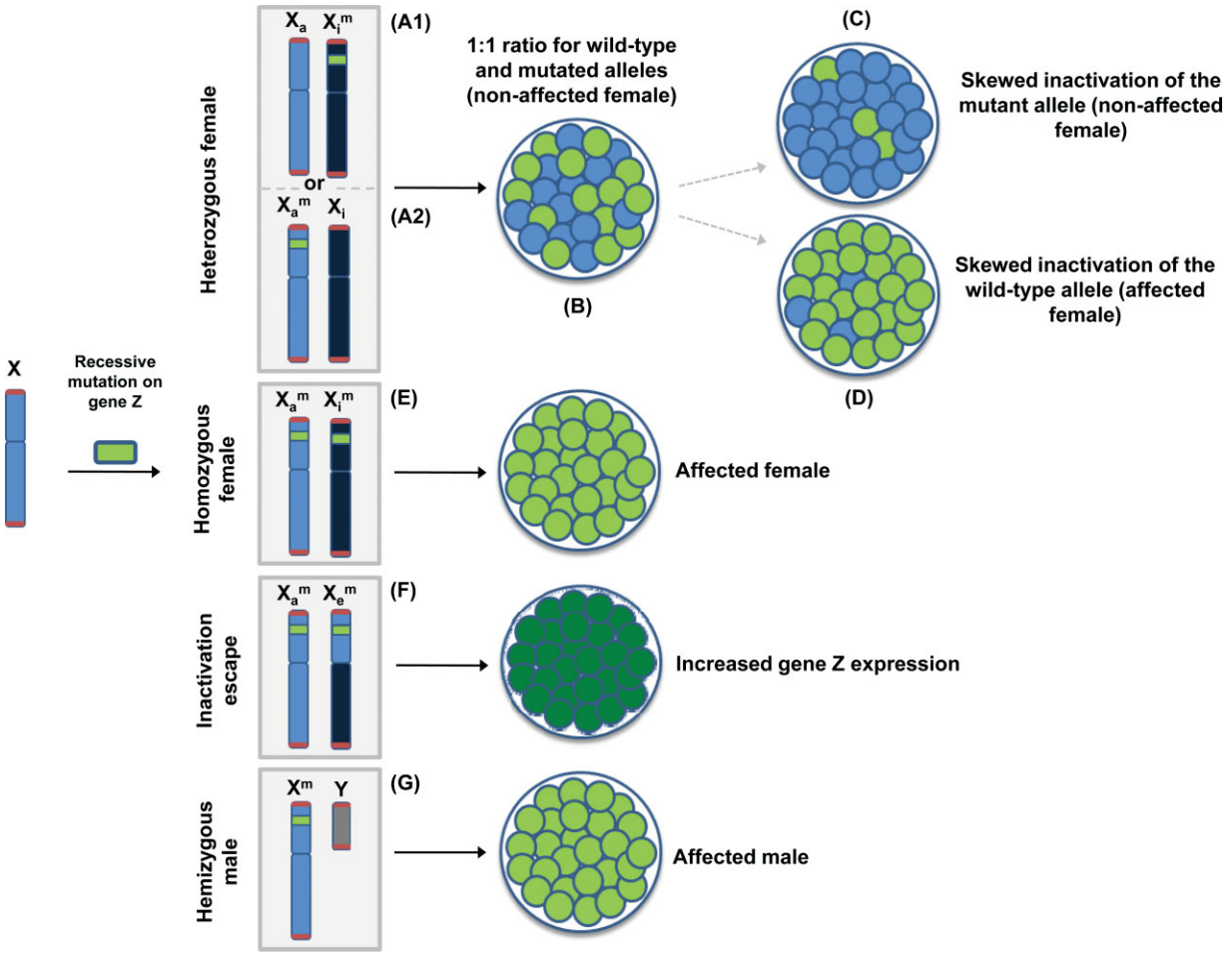

Figure 1. The possible outcomes of a newly-arisen recessive mutation on an X-linked gene. **A1**, **A2:** A heterozygous female for a new recessive mutation occurring on gene Z (depicted by a green rectangle) will have one of the parental X chromosomes inactivated in half of her cells and the other chromosome inactivated in the other half. **B:** Therefore, her organs will be mosaics, and the mutated allele will be present in only half of her cells. **C:** If the nature of this mutation is deleterious, females may have a skewed pattern of inactivation favoring the expression of the normal (or wild-type) allele in nearly all cells. **D:** If the expression of the mutated allele is favored, the wild-type allele will be inactivated in almost all cells. **E:** A homozygous female will carry the mutation in all her cells and will be affected by it. **F:** If a gene escapes inactivation (independently of being mutated or not), female cells may express the gene twice as much as males (represented by dark green circles). **G:** A hemizygous male will always be affected by a mutation present on the X chromosome. X_a: activated X chromosome; X_a^m: activated mutation-carrying X chromosome; X_i: inactivated X chromosome; X_i^m: inactivated mutation-carrying X chromosome; X_e^m: inactivation-escaping X chromosome carrying the mutation; X^m: X chromosome carrying the mutation; Y: Y chromosome. Small green and blue circles represent single cells carrying the mutated and the wild-type allele, respectively. Big circles represent any organ of the individual.

and mouse genomes, and bioinformatics predictions indicate that mammalian miRNAs regulate 30 to 50% of all protein-coding genes, and take part in the regulation of almost all cellular processes [35, 36]. According to a recent study, in several mammalian species, including humans and mouse, the X chromosome has a higher density of miRNAs when compared to autosomes and remarkably, the Y chromosome has no miRNAs [37]. Possible explanations for the absence of miRNAs on the Y chromosome are not known, but the authors speculate that the existence of possible X-specific properties or functions in mammals is the reason behind the higher concentration of miRNAs on the X chromosome [37]. Although these properties and functions have not been specified, they may be directly correlated with the mammalian XY sex-determination system, because in the chicken, whose sex chromosomes have an independent origin from that of mammals, the density of miRNAs on the *Z chromosome* (Box 1) is lower than on the autosomes [37]. A study made in *Caenorhabditis elegans* also showed that there is an enrichment of genes regulated by Dicer on the X chromosome, and that most of the misregulated gene targets in Dicer-deficient animals were implicated in innate immunity [38].

Only 57% of the miRNAs present on the mouse X chromosome are common to the human X chromosome

According to the Ensembl database, the human X chromosome has about 76 miRNAs and the mouse 65, and only 37 are common between the two species

Box 2

X-chromosome-linked members of the Toll-like receptor (TLR) pathway

Specific components of bacteria, viruses, fungi, and parasites bind to different TLRs, thereby triggering the host innate immune system. Genes encoding for several components of the TLR pathway are located on the X chromosome and are essential for NF-κB signaling. Among these components are *BTK*, *IRAK1*, *IKKγ*, *TSC22D3/GILZ*, and *CYBB/NOX2*. The central and fundamental role of these X-linked genes in TLR signaling is evident. Mutations affecting BTK, IRAK1, and IKKγ can have a significant impact on NF-κB signaling. For example, BTK was first identified to be involved in XLA, a male-specific X-linked PID, characterized by recurrent bacterial infections [30]. A variant haplotype of *IRAK1* was found to be associated with increased nuclear levels of NF-κB in LPS-stimulated neutrophils from septic patients and therefore correlated with a more severe outcome from sepsis [80]. Moreover, IRAK1 was recently shown to be involved in a mouse model of human inflammatory bowel disease in a sex-dependent manner [81]. *IKKγ* mutations are responsible for two important X-linked PIDs: incontinentia pigmenti, a severe immunodeficiency that usually affects only females, as males die in utero [30], and X-EDA-ID, where male patients are very susceptible to infections [82]. GILZ is induced by glucocorticoids, one of the most potent immunosuppressive molecules. GILZ decreases the sensitivity of macrophages to LPS and tumor necrosis factor and binds to NF-κB, thereby inhibiting its transcriptional activities [83, 84]. Furthermore, GILZ hampers the activity of AP-1 by inhibiting its binding to its cognate DNA [85]. So far, no haplotype variants have been described in the human population, and so it is difficult to know to what extent GILZ may be responsible for gender-specific immunological responses. Nevertheless, GILZ has been shown to be protective in a mouse model of induced colitis [86] and rheumatoid arthritis [87]. Reactive oxygen species (ROS) are essential for microbial killing and are produced in cells, such as phagocytes, in response to invading pathogens. Mutations occurring in *CYBB/NOX2*, a protein that catalyzes ROS formation, are responsible for X-CGD, an X-linked PID. Characteristically, neutrophils, eosinophils, monocytes, and macrophages of X-CGD-bearing male patients fail to generate oxygen radicals and show reduced killing of intracellular microbes [17]. Also, importantly, two members of the TLR family of receptors are located on the X-chromosome: *TLR7* and *TLR8*. The former has been shown to induce higher production of interferon alpha in females upon stimulation of cells with a synthetic TLR ligand [88]. In contrast, a naturally-occurring polymorphism in the coding sequence of TLR8 has been associated with protection against HIV-1 and *Mycobacterium tuberculosis* in human patients [89, 90].

(Fig. 2). Several miRNA clusters seem to be lineage specific, such as the miR-513∼514 cluster on the long arm of the human X chromosome. This cluster, found only in primates, is preferentially expressed in the testes, and has likely contributed to *speciation* (Box 1) [39]. Similarly, according to the miRNA database, several miRNAs seem to be specific to the rodent lineage (e.g. miR-743 to miR-871 in Fig. 2), and some of them have so far only been found in *Mus musculus* (e.g. miR-470 to miR-465a in Fig. 2). The gene content of the X chromosome is expected to be highly conserved among mammalian species. According to Ohno's Law, this is necessary to ensure equal expression of X-linked genes in females and males by means of dosage compensation [26]. Therefore, this mechanism created an evolutionary barrier to the exchange of genes between the X chromosome and the autosomes. For that reason, it is surprising to find so many exclusive miRNAs in the human and mouse X chromosomes. Although some nomenclature issues may be responsible for this discrepancy, we speculate that species-specific miRNAs may be on the origin of speciation and may have contributed to *reproductive isolation* (Box 1). MiRNAs are believed to be under strong selective pressure and, therefore, to be highly conserved among species due to their importance in development and disease regulation [40]. However, species seem to have their own specific miRNAs (as observed in Fig. 2). This suggests that these miRNAs have species-specific roles. In line with this, it has been argued that evolution of novel miRNA families contributed to the appearance of lineage-specific characteristics [41]. According to Heimberg et al. [41] the dramatic expansion of miRNAs lies behind the origin of vertebrate complexity. This suggests that the evolution of species-specific miRNAs may be at the root of vertebrate lineage divergence, by regulating gene expression in a species-specific manner. However, it should be stressed that many of the miRNAs predicted by computation-based approaches have not been experimentally confirmed, and so it is possible that some of them are false positives [42].

How might escape from silencing affect X-chromosome-located miRNAs?

miRNAs can be located in the intergenic genome (i.e. between genes), or within gene intronic regions. Intergenic miRNAs have independent transcription units, including promoters, transcript sequences, and terminators [33]. However, intragenic miRNAs may be found within introns of protein-coding genes in the same orientation and so they are believed to share regulatory motifs with their host genes [43, 44]. Nonetheless, some of the intronic miRNAs are antisense to their host gene, and may therefore possess their own independent transcription units inside the host intronic regions [42]. A number of X-located miRNAs are intronic in known protein-coding genes (Fig. 2) and so it is possible that they share the same transcription units with these host genes, and are co-regulated with

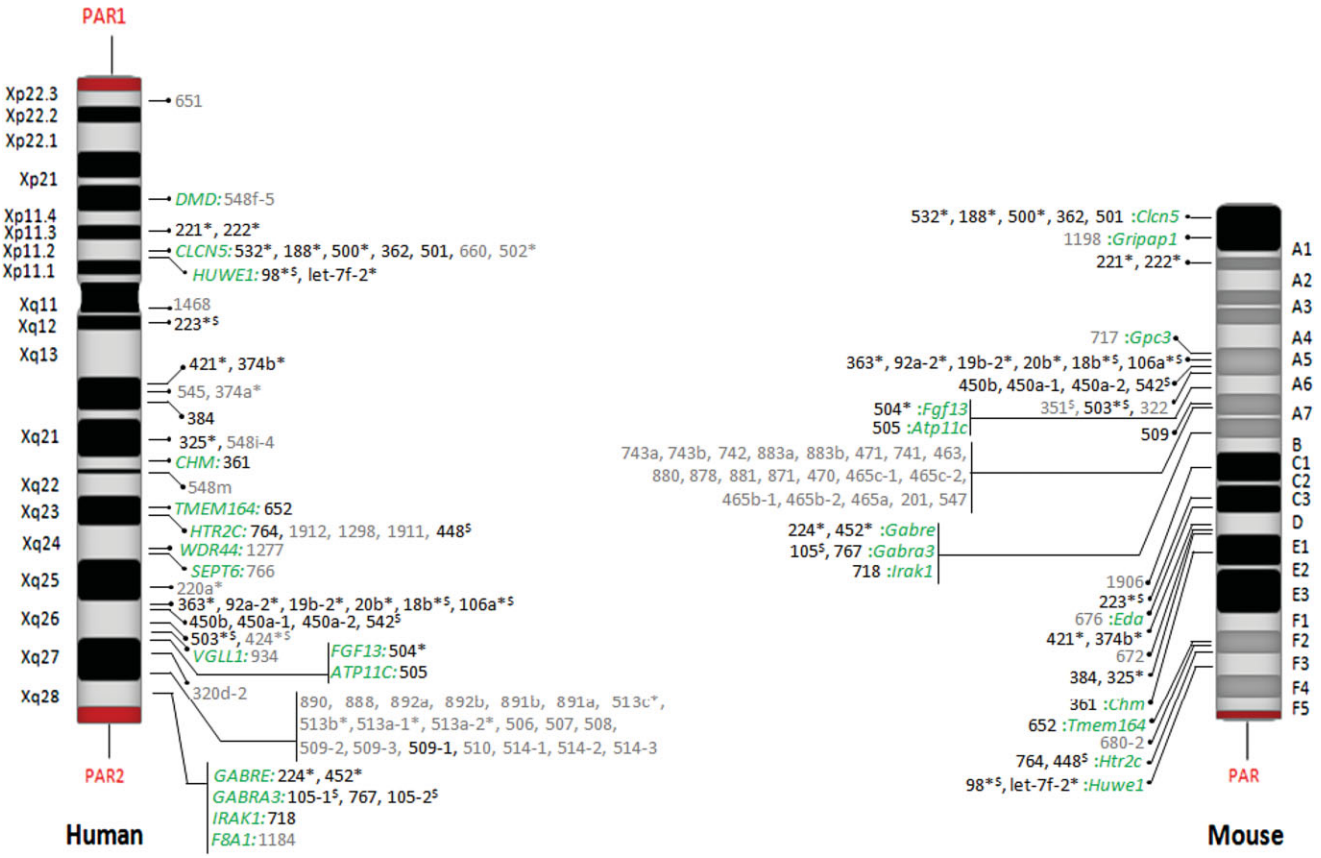

Figure 2. Map of microRNAs located on human and mouse X chromosomes. In black are depicted the miRNAs common to both species. In light gray are depicted the species-specific miRNAs. Some miRNAs are intragenic, and gene names are depicted in green before the miRNAs that they contain. (*) Indicates miRNAs which have a confirmed or putative role in cancer; ($) indicates miRNAs which have a confirmed or putative role in immunity (see Table 1 for more details). Human and mouse mapping positions are based on the Ensembl database. Information about genomic context was based on the miRBase database. ATP11C: ATPase, class VI, type 11C; CHM: choroideremia; CLCN5: chloride channel 5; DMD: dystrophin; Eda: ectodysplasin A; F8A1: coagulation factor VIII-associated 1; FGF13: fibroblast growth factor 13; GABRA3: gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3; GABRE: gamma-aminobutyric acid (GABA) A receptor, subunit epsilon; Gipap1: GRIP1 associated protein 1; Gpc3: glypican 3; HTR2C: 5-hydroxytryptamine (serotonin) receptor 2C; HUWE1: HECT, UBA and WWE domain containing 1; IRAK1: interleukin-1 receptor-associated kinase 1; SEPT6: septin 6; TMEM164: transmembrane protein 164; VGLL1: vestigial like 1; WDR44: WD repeat domain 44.

them. Moreover, some of them are located within genes that have been shown to escape XCI, such as *DMD*, *CHM*, *ATP11C*, or *IRAK1* (Fig. 2) [14]. Therefore, we hypothesize that these miRNAs also escape silencing. In reality, the inactivation status of human X-linked miRNAs is not known, and it would be interesting to perform a systematic study, such as that by Carrel and colleagues [14]. After evaluating the inactivation status of 624 X-located genes, they have found that silencing escape was much more frequent and variable than previously thought [14]. We believe that female mosaicism, silencing escape or skewed patterns of inactivation of X-linked miRNAs involved in immunity could lead to unbalanced miRNA expression between sexes, and to sex-specific immune responses. Considering that miRNAs have multiple targets across the genome, this could result in a cascade-like effect and lead to greater gender differences in terms of gene regulation than previously assumed.

X-linked miRNAs in immunity and cancer: What consequences might we expect?

The role of miRNAs in cancer has been extensively explored, and many miRNAs have been shown to behave as oncogenes or tumor suppressors in several types of cancer and are therefore referred to as "oncomiRs" [45, 46]. However, the same miRNA can have several targets and, for instance, miRNAs with well established roles in lymphoma and leukemia progression [47, 48] have also been found to be important for immune functions (reviewed in ref. [40, 49–51]). The role of chronic inflammation in carcinogenesis is well known, and the upregulation of miRNAs during chronic inflammation is thought to be a predisposing factor for the development of tumors [49, 52]. The general importance of miRNAs in shaping the immune system has been clearly illustrated by the deletion of Dicer. This is a key enzyme in miRNAs processing, and when deleted leads to reduced T cell numbers and to a complete arrest in B

cell maturation [53–55]. Thus, the role of miRNAs in disease onset and progression is unequivocal, and their broad mode of action is certainly one of the most important regulatory mechanisms of protein expression within the mammalian genome.

Might X-linked miRNAs be responsible for the high incidence of cancer in males?

So far, about 10% of all human miRNAs are known to be located on the X chromosome. Although the role of most X-linked miRNAs is not known, several of them participate in cancer onset and progression, and regulate the immune system at different levels. A list of X-located miRNAs with putative or confirmed roles in immunity and/or cancer can be seen in Table 1; this list is based on genetic association studies, expression profiling and functional studies, meaning that mechanistic information is still lacking for some miRNAs. It should be stressed that the gender effect in incidences of cancer is well-established and consistent worldwide [56–58]. Gender susceptibility to cancer at different ages is a phenomenon rarely addressed in epidemiological studies, yet the risks for males to develop cancer can be between two- and five-fold greater than in females [59]. Indeed, it is known that gender confers one of the greatest risks for contracting hematological malignancies such as leukemia and lymphomas [59]. However, male preponderance is also observed in other types of cancer such as Kaposi sarcoma, lip, larynx, mesothelioma, hypopharynx, urinary bladder, esophagus, tonsil, oropharynx, and other urinary organs [60]. Despite the fact that cancer rates tend to be higher among males than females, this is rarely the subject of investigation and the gender of samples used in miRNAs studies is hardly ever mentioned. In the same manner that males are hemizygous for mutations occurring on X-linked genes, they are also hemizygous for mutations on X-located miRNAs and their regulatory regions, and so we speculate that this may have an impact on cancer onset and development in males and to contribute to gender-specific susceptibility to cancer. Among X-linked miRNAs, the miR-221∼222 cluster is certainly one of the most extensively studied. Its deregulation is a hallmark of several types of cancer (Table 1), probably because one of its targets is the cell cycle regulatory protein $p27^{Kip1}$/CDKN1B, a tumor suppressor that is frequently inactivated in human cancers [61]. This cluster has also been shown to have anti-angiogenic properties, by inhibiting tube formation, migration and wound healing of endothelial cells in vitro [62]. miRNA-221∼222 can indirectly reduce the expression of endothelial nitric oxide synthase (eNOS). Nitric oxide (NO) is a key regulator of endothelial cell growth, migration, vascular remodeling and angiogenesis, and NO impairment is a feature of different cardiomyopathies, suggesting the involvement of this cluster in vascular and cardiac diseases (reviewed in ref. [63]).

Several X-located miRNAs have fundamental roles in immunity

Amongst the X-linked miRNAs involved in immune regulation, miR-223 is probably the most studied so far. MiR-223, which is also involved in cancer pathology (Table 1), is expressed in the myeloid lineage in the bone marrow, and is a regulator of neutrophil differentiation from myeloid precursors [64, 65]. However, the nature of this regulation is contradictory: according to Fazi et al., miR-223 is a positive regulator of granulocytic differentiation by targeting NFI-A [64], whereas Johnnidis et al. [65] suggest that miR-223 regulates both the generation and function of granulocytic cells by restricting their production and dampening their activation by targeting MEF2C and IGFR (Fig. 3). MiR-223 knockout (KO) studies support this last hypothesis. MiR-223 KO mice have increased numbers of granulocyte progenitors in the bone marrow and hypermature neutrophils in circulation, indicating that miR-223 negatively regulates granulocyte maturation [49, 65]. Moreover, these animals display an enhanced oxidative burst upon *Candida albicans* infection and develop inflammatory lung pathology after *LPS* (Box 1) challenge, showing that miR-223 is a negative modulator of the inflammatory response [49]. In parallel, miRNA-profiling of mice lungs exposed to aerosolized LPS showed a time-dependent increase of miR-223, confirming once more its importance in inflammation [66]. More recently, miR-223 was shown to be significantly reduced in septic patients, and the authors proposed that serum levels of this miRNA, together with miR-146a, should be used as biomarkers for sepsis [67]. Although the gender of the patients included in this study is known, a comparison between sexes was not done, and so we cannot discuss the possible differences between miR-223 expression in males and females, and whether this correlated with differences in mortality, if any.

Four additional X-located miRNAs are involved in hematopoietic lineage differentiation: miR-106a, miR-424, miR-542, and miR-503. Together with miR-17-5p and miR-20a, miR-106a was shown to negatively control monocytopoiesis by targeting AML-1, an inducer of monocyte differentiation and maturation, and subsequent downregulation of M-CSFR (Fig. 3) [68]. Moreover, miR-106a was also shown to downregulate IL-10, an important anti-inflammatory cytokine, in several transfected cell lines [69]. Interestingly, miR-106a is part of a cluster (miR-106a∼363) which has two *paralog clusters* (Box 1): miR-17∼92 and miR-106b∼25 located on human chromosomes 13 and 7, respectively. These clusters arose by a complex history of duplication and deletion events in early vertebrate evolution [70]. The miR-17∼92 cluster is well known for its importance in immune regulation because it is required for B and T lymphocyte maturation. Moreover, this cluster was shown to be involved in B cell lymphomas, and transgenic expression of miR-17∼92 resulted in breakdown of T cell tolerance and development of autoimmunity (reviewed in ref. [49]). The miR-106∼363 cluster, which includes miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2, and miR-363 (Fig. 2), was also shown to have oncogenic potential, as it was found to be overexpressed in T cell leukemias [71]. Therefore, we speculate that this cluster might have an important role in both innate and adaptive immunity by negatively regulating monocyte differentiation, as previously shown [68], but also by controlling lymphocyte development. MiR-424 synergizes with PU.1 (a myeloid

Table 1. miRNAs located on human and mouse X chromosomes involved in immunity ($) and/or cancer (*)

miRBase ID	Role in immunity	Role in cancer	Target(s)
mir-221*	–	Promotes cancer cell proliferation; anti-angiogenic; involved in glioblastoma, cutaneous melanoma, prostate cancer, breast cancer, lung cancer, liver cancer, thyroid cancer, bladder cancer, HCC, PTC, GISTs, PDAC, AML, ALL, FCL and neuroblastoma	CDKN1B; KIT; ERα; PTEN; TIMP3; BMF; HOXB5; CDKN1C; BIM
mir-222*	–	Promotes cancer cell proliferation; anti-angiogenic; involved in glioblastoma, cutaneous melanoma, prostate cancer, breast cancer, lung cancer, thyroid cancer, papillary carcinoma, urothelial carcinoma, colorectal adenocarcinoma, parathyroid carcinoma, HCC, GISTs, OTSCC, PDAC, AML and FCL; decreases susceptibility of tumor cells to cytolysis	CDKN1B; KIT; ERα; PTEN; TIMP3; MMP1; SOD2; BIM; ICAM-1; CDKN1C
mir-532*	–	Cutaneous melanoma	RUNX3
mir-188*	–	B cell-CLL	–
mir-500*	–	HCC	–
hsa-mir-502*	–	Breast cancer	SET8
mir-98* $	Together with let-7i regulates TLR-mediated epithelial innate immune response; reduction of IL-1β-mediated production of TNF in osteoarthritic tissue	Involved in HNSCC, AML, papillary carcinoma, breast cancer, lung adenocarcinoma, HCC, esophageal adenocarcinoma, pancreatic adenocarcinoma, ovarian and prostate cancers and hormone-refractory carcinoma	CIS; FUS1/TUSC2; RAS; MYC; HMGA2
let-7f-2*	–	Involved in renal cell carcinoma, AML, papillary carcinoma, breast cancer, lung adenocarcinoma, HCC, esophageal adenocarcinoma, pancreatic adenocarcinoma, ovarian and prostate cancers and hormone-refractory carcinoma; pro-angiogenic	RAS; MYC; HMGA2
mir-223* $	Upregulated during TLR4 signaling; regulator of granulocytic differentiation; regulation of LPS-induced IFNγ in splenic lymphocytes; regulation of erythropoiesis; involved in RA and HIV-1 latency; sepsis marker	Involved in HCC, bladder cancer, lung cancer, gastric cancer, ovarian cancer, adenocarcinoma of the esophagus, colorectal adenocarcinoma, PDAC, ATL, FCL, CLL, AML and ALL	MEF2C; NFI-A; E2F1; IGFR; LMO2; STMN1; RHOB
mir-421*	–	Involved in gastric cancer and neuroblastoma	CBX7; RBMXL1; ATM
mir-374*	–	Involved in lung cancer, colon cancer, AML and HCC	–
mir-325*	–	Squamous cell carcinoma of the tongue, Hodgkins lymphoma, AML	–
mir-448$	Induced by IFNβ; inhibition of HCV replication	–	–
hsa-mir-220a*	–	B cell-CLL, Hodgkin lymphoma, papillary carcinoma, lung adenocarcinoma	–
mir-363*	–	T cell leukemia; increased expression in Waldenström macroglobulinemia	–
mir-92a-2*	–	T cell leukemia	–
mir-19b-2*	–	T cell leukemia	–
mir-20b*	–	Involved in T cell leukemia and gastric cancer; improves survival of tumor cells; regulation of VEGF expression in breast cancer cells	HIF-1α; STAT3
mir-18b* $	Multiple sclerosis	Involved in T cell leukemia, gastric cancer; colon cancer and ERα-negative breast cancer	ERα

mir-106a*[$]	Control of monocytopoiesis; regulation of IL-10 (anti-inflammatory cytokine)	Involved in lung adenocarcinoma, gastric adenocarcinoma, colorectal adenocarcinoma, T cell leukemia, malignant mesothelioma, astrocytoma and neuroblastoma	AML-1; IL-10; RB; E2F1
mir-542[$]	Monocytic differentiation	–	–
mmu-mir-351[$]	Induced by IFNβ; inhibition of HCV replication	–	–
mir-503*[$]	Monocytic differentiation	Involved in parathyroid carcinoma, retinoblastoma, prostate cancer, AML and ACC	–
hsa-mir-424*[$]	Monocytic differentiation	Induced by estrogens in breast cancer cells; involved in lung carcinoma, intrahepatic cholangiocarcinoma, colon cancer, renal cell carcinoma, pancreatic adenocarcinoma, ovarian carcinoma, AML and ALL	NFI-A
mir-504*	–	GISTs	–
hsa-mir-513*	–	Hormone -refractory carcinoma	–
mir-224*	–	Involved in HCC, PDAC, papillary carcinoma, breast cancer, lung cancer, thyroid cancer, colon cancer, prostate cancer and FCL; regulation of cell migration and invasion	API-5; CD40; PAK4
mir-452*	–	Urothelial carcinoma	–
mir-105[$]	Modulation of TLR2 in human gingival keratinocytes	–	TLR2

ACC, adrenocortical carcinoma; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; AML-1, acute myeloid leukemia-1; API-5, apoptosis inhibitor-5; ATL, adult T cell leukemia; ATM, Ataxia-telangiectasia mutated; BIM, BCL-2 interacting protein; BMF, BCL-2 modifying factor; CBX7, chromobox homolog 7; CD40, CD40 antigen; CDKN1B, cyclin-dependent kinase inhibitor 1B; CDKN1C, cyclin-dependent kinase inhibitor 1C; CIS, cytokine-inducible Src homology 2-containing protein; KIT, stem cell factor receptor; CLL, chronic lymphocytic leukemia; E2F1, E2F transcription factor 1; ER, estrogen receptor; FCL, follicular lymphoma; FUS1/TUSC2, tumor suppressor candidate 2; GISTs, gastrointestinal stromal tumors; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIF-1α, hypoxia-inducible factor; HIV-1, human immunodeficiency virus type 1; HMGA2, high mobility group A2; HNSCC, head and neck squamous cell carcinoma; hsa, *Homo sapiens*; HOXB5, homeobox B5; ICAM-1, intercellular adhesion molecule 1; IFN, interferon; IGFR, insulin-like growth factor receptor; IL, interleukin; LMO2, LIM-only protein 2; LPS, lipopolysaccharide; MEF2C, myeloid ELF-1-like factor 2c; MMP1, matrix metalloproteinase 1; mmu, *Mus musculus*; MYC, c-Myc proto-oncogene; NFI-A, nuclear factor I/A; OTSCC, oral tongue squamous cell carcinoma; PAK4, p-21 activated kinase 4; PDAC, pancreatic ductal adenocarcinoma; PTC, papillary thyroid carcinoma; PTEN, phosphatase and tensin homolog; RA, rheumatoid arthritis; RAS, protein subfamily of small GTPases; RB, retinoblastoma; RBMXL1, RNA-binding motif protein X-linked-like 1; RHOB, RAS homolog gene family member B; RUNX3, Runt-related transcription factor; SET8, SET domain-containing protein 8; SOD2, superoxide dismutase 2; STAT3, signal transducer and activator of transcription 3; STMN1, stathmin 1; TIMP3, tissue inhibitor of metalloproteinase 3; TLR, Toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

specific transcription factor) to positively regulate monocyte to macrophage differentiation by targeting NFI-A (Fig. 3) [72]. The pre-miR-424 was found to be transcribed together with pre-miR-503 and pre-miR-542 as one transcript; therefore, it is likely that they regulate the same processes, such as monocytopoiesis [73]. Indeed, a second study confirmed that miR-424 and miR-503 are derived from a *polycistronic precursor* (Box 1) and those, together with miR-222 (also X-located) and miR-155, are pro-differentiation miRNAs of the monocytic lineage [74]. Finally, the authors also showed that miR-424 and miR-503 target a series of cell-cycle regulators, and downregulate miR-9, an anti-differentiation miRNA in human acute monocytic leukemia cells, leading to their differentiation [74]. Despite the important role of these miRNAs in early differentiation of myeloid cells, which can have a great impact in innate immune responses, their effect in gender-based immunity has never been studied. We believe that dysregulation of these and other miRNAs expression on the X chromosome, by means of irregularities in the process of DNA methylation such as may occur during silencing escape, may be partly responsible for differences in immune responses between genders. Moreover, naturally-occurring mutations in these miRNAs or their regulatory sequences may also contribute to gender differences in immune responses, but again such mutations have never been reported.

Summary of key open questions

The X chromosome contains several important miRNAs with an extensive role in cell lineage determination, immune regulation and oncogenesis but, to date, most of the X-linked miRNAs have no described functions. Moreover, the contribution of these miRNAs to the gender-specific predisposition for cancer or immune diseases has never been explored. These gaps in knowledge raise several questions, and provide new ground for future research, both from evolutionary and pathological perspectives: (i) Why is the Y chromosome devoid of miRNAs? It is known that the mammalian X

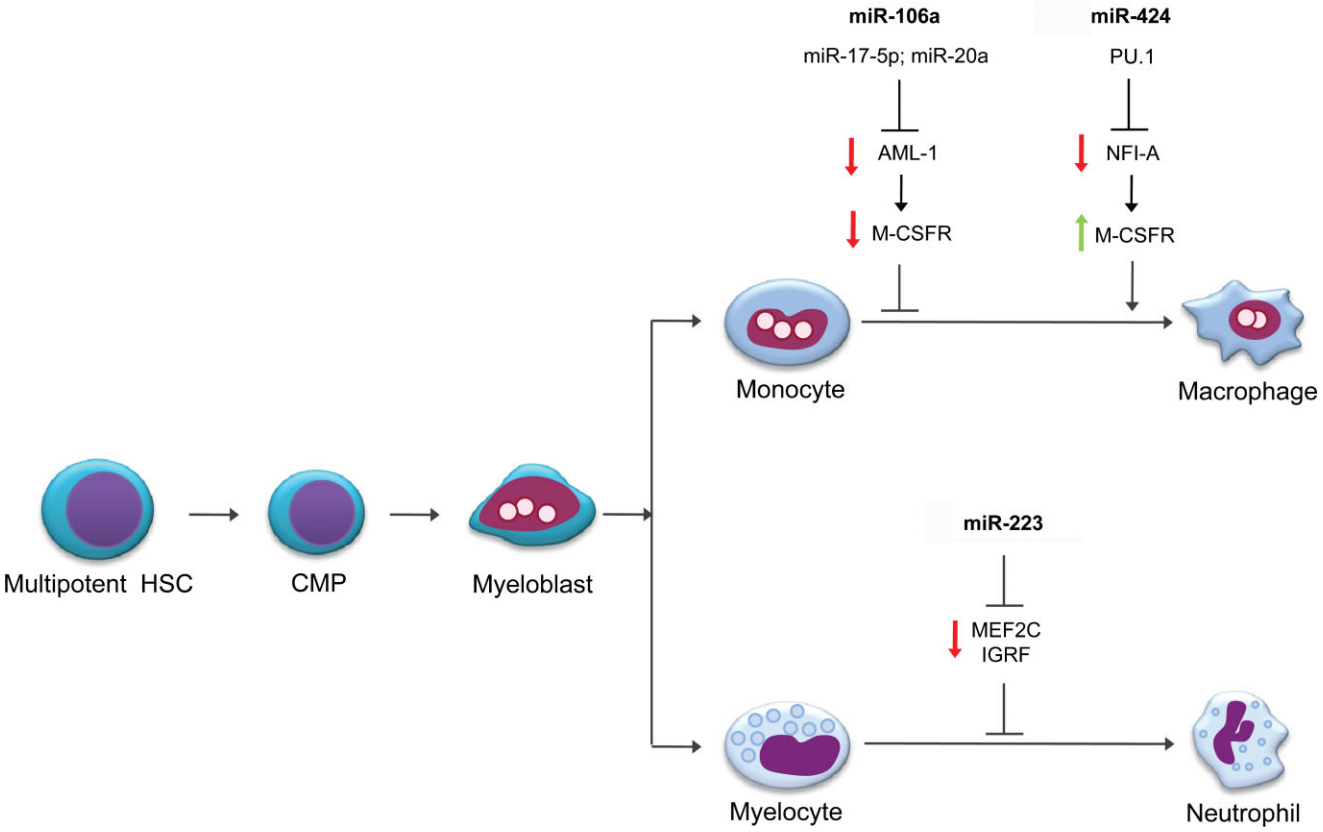

Figure 3. X-linked miRNAs involved in the differentiation of myeloid cells. MiR-106a and miR-424 control the differentiation of monocytes into macrophages: miR-106a, together with miR-17-5p and miR-20a, blocks monocytopoiesis by targeting AML-1 and subsequent M-CSFR downregulation; miR-424, together with PU.1, induces monocytopoiesis by targeting NFI-A, resulting in M-CSFR upregulation. MiR-223 negatively regulates neutrophil differentiation by targeting MEF2C and IGFR. AML-1: acute myeloid leukaemia-1; CMP: common myeloid progenitor; HSC: hematopoietic stem cell; IGFR: insulin-growth factor receptor; M-CSFR: macrophage-colony stimulating factor receptor; MEF2C: myeloid ELF-1-like factor 2c; miR: miRNA; NFI-A: nuclear factor I/A; PU.1: a myeloid transcription factor.

chromosome is enriched for miRNAs expressed in the testis [37, 39], so why aren't they Y-located? (ii) Why is the mammalian X chromosome richer in miRNAs than the autosomes? Do mammalian X-linked miRNAs have sex-specific functions that we are not aware of? (iii) The number of X-linked miRNAs common to human and mouse is limited. Why is this so and is this pattern extended to the rest of the genome? What are the consequences for cancer and immune research if several species-exclusive miRNAs have important roles in disease? (iv) The importance of the X chromosome in shaping immune functions and autoimmunity has been extensively recognized [7, 10, 16, 18, 19, 30, 75]. Are X-linked miRNAs also responsible for the immunological advantage of females and/or their predisposition to autoimmune diseases? (v) Intronic miRNAs that are encoded in the same orientation as their host gene are thought to share the same regulatory motifs with it, including the promoter region [76]. Intronic expansion or deletion may result in dysregulation of miRNA processing from the introns and lead to genetic diseases. This has been suggested for example for myotonic dystrophy [77] and fragile X mental retardation [78]. Do other X-linked diseases have the same etiology? In this context, one should be careful when planning gene KO strategies, to be sure that no intronic miRNAs have been affected, which could lead to misinterpretation of results. All these questions open the door for future research, which will ultimately contribute to our understanding of protein regulation by miRNAs and the importance of this process to define development and disease.

Conclusions and prospects

The role of miRNAs as immunomodulators is an emerging research field, where much has still to be done [40, 49–51]. Fine-tuning of protein expression is essential for maintaining homeostasis and for correct development and cellular function, and it is clear by recent advances in miRNA research, that minimal perturbations in miRNA-mediated regulation can have serious consequences [76, 79]. Thus, we believe that miRNAs on the X chromosome, because they are present in a particular genomic context, may influence sex specific responses. The myriad of functions and targets of miRNAs has obvious implications in the development of effective therapeutic strategies for cancer and immunity, and is likely to represent a challenge for researchers of both fields in the coming years.

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