Review

Ongoing hurdles in harnessing XIST to treat human disease: Review Article

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Ongoing Hurdles in Harnessing XIST to Treat Human Disease: Review Article

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Abstract: XIST RNA is one of the most important long non-coding RNAs and plays a significant role in X-chromosome inactivation during early mammalian development. This epigenetic process ensures dosage compensation between males (XY) and females (XX) by silencing one of the two X chromosomes in females. Beyond its classical function, XIST is emerging as a biomolecule of interest due to its involvement in diverse pathological conditions and genetic diseases and many promising therapies are emerging in this context. Here, we delve into the therapeutic potential of targeting XIST RNA in treatment of genetic disorders.

Keywords: XIST; LncRNA; Down; Syndrome; cancer; autoimmune; genetic; chromosomes; epigenetic; stem cell; therapeutic; silencing; ceRNA; regulation; trisomic; trigger; differentiation

1. Introduction

The mammalian X chromosome is a well-known carrier for many critical genes for human development and functionality. Because females have two copies of X chromosome while males have only one copy, several epigenetic mechanisms have developed to equalize gene expression and maintain balance of the normal human physiology. The most important one was through X-chromosome inactivation (XCI), a process that transcriptionally silences one X chromosome in each cell. The major key role in this process is done by X-linked X-inactive-specific transcript (Xist), a long non-coding RNA that triggers silencing of genes located on active X-chromosome, which is then transformed into a heterochromatic, transcriptionally silent state. It is considered a classic example of IncRNAs which act as regulatory molecules in the context of cellular development and disease.

2. XIST and Normal Biological Function: X Inactivation

X-inactive–specific transcript (*Xist*) was first discovered in the early 1990s [1] and is considered one of the first long non-coding RNA to be discovered by that time [2]. It is a basic component of the *Xic* – X-chromosome inactivation center [3] – along with two other RNA genes (*Jpx* and *Ftx*) and two protein genes (*Tsx* and *Cnbp2*) [4]. XIST is a 17–22 kb RNA transcribed from the X-inactivation center (XIC) of the inactive X chromosome [5]. It lacks coding potential but contains conserved repeat regions ("A" to "F") critical for its function [6, 7]. Repeat A, located at the 5' end, interacts with chromatin modifiers to initiate silencing, while other domains contribute to XIST's stability and localization [8]. The regulatory roles of Xist RNA are extensive, including epigenetic changes, gene expression, cellular identity, and sex chromosomal inactivation. In addition to being expressed in almost all females, XIST is expressed in very few developmental contexts in males including human preimplantation embryos, primordial germ cells, testicular germ cell tumors, and a subset of male cancers of diverse lineages [33].

Many studies in animal models have shown the critical role of RNA silencing in regulating different cellular functions, including the scaffolding for protein recruitment and regulating the function of the inactive X-chromosome (Xi) in 3D-space [9]. Moreover, many studies have shown

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that over 30 RNA-binding proteins directly interact with Xist [10]. Between these, SAF-A protein was shown to mediate Xist anchoring to the nuclear matrix [11].

XIST achieves XCI by recruiting chromatin-modifying complexes such as PRC2 (Polycomb Repressive Complex 2) and other histone modifiers, leading to the establishment of heterochromatin [12, 13]. XIST's interaction with nuclear scaffold proteins ensures its localization on the inactive X chromosome, forming a dense Barr body [14]. The long noncoding transcript *XIST* (19 kb in humans, and 17 kb in mice) has been suggested to play vital roles in random XCI. [15, 16]. Random XCI has three phases: initiation, establishment, and maintenance [17] and has been extensively investigated in mice. After the genetic transcription of XIST, XIST RNA-containing complexes gradually expand, allowing it to spread across Xi. Meanwhile, these complexes change chromatin architecture and thus compact the chromosome, leading to progressive gene silencing along the Xi.

During normal biological development, XIST activation leads to X inactivation (also termed lyonization) in females [23]. Females harbor two X chromosome copies, while males have an X and a Y copy. One of the two X chromosomes in females undergoes random XIST activation, with the long non-coding RNA progressively winding and spreading the XIST complex around the chromatin structures in each cell to achieve silencing during early embryonic development. Silencing of one of the two copies in females ensures normalization of the X-linked gene dosages between sexes. This type of silencing is maintained indefinitely in the cells.

3. XIST and Disease Pathogenesis: Autoimmune Disorders, Malignancy, and General Health

Long non-coding RNAs (lncRNAs) such as XIST, have emerged as critical regulators of different diseases. Dysregulation of XIST levels can cause an imbalance in gene expression through epigenetic modifications, thereby contributing to autoimmune disorders, cancer and heart disease.

Historically, autoimmune disorders such as systemic lupus erythematosus (SLE) are known to afflict females more so than males, although the underlying reason for this observation was not known. Recent studies have shown a higher prevalence of autoantibodies against XIST complexes in females, thereby providing a potential explanation [34]. With X inactivation in females, the increased expression of XIST naturally silences the extra X chromosome but also leads to greater likelihood of an auto-immune reaction in females, recognizing XIST and its associated complexes as foreign, and thereby leading to autoantibodies against these molecules. Blood samples from individuals diagnosed with autoimmune conditions were found to have antibodies to many of the Xist complexes, with specific antibodies associated with specific autoimmune diseases. A second study similarly reported that XIST ribonucleoprotein (RNP) complex, comprising numerous autoantigenic components, is an important driver of sex-biased autoimmunity and that patients with autoimmune disorders displayed significant autoantibodies to multiple components of XIST RNP [39], further supporting the role of the long non-coding RNA in the development of autoimmune disorders.

XIST has been implicated in a growing list of cancers, where it may act as both an oncogene promoting tumor progression or suppressor in preventing cancer [52]. Aberrant XIST expression influences cell proliferation, invasion, and metastases by regulating gene expression, as well as chemotherapy resistance [37]. In breast cancer, XIST is aberrantly expressed in cancerous cells with high XIST expression levels being associated with treatment resistance and poor patient outcomes [41,53,54]. The high expression levels are thought to promote cancer-like stem cells, which are more resistant to chemotherapies. Low expression of XIST correlates with greater sensitivity to chemotherapies and prolonged survival [55]. Conversely, other studies have reported loss of XIST expression in female breast, cervical and ovarian tumors [39,40,41]. In ovarian cancer, decreased XIST promotes cancer cell stemness and promotes resistance to chemotherapy treatments like carboplatin [49]. XIST also regulates DNA replication, chromosome segregation, and cell cycle checkpoints. In blood-borne leukemias, XIST suppresses oncogene levels, which are typically associated with genomic instability and contribute to tumor progression and worsened prognosis [18,19]. As hematopoietic cells mature and accumulate, loss of normal mechanisms suppressing XIST function can promote dysregulation of a wide range of X-linked genes, including tumor-associated gene de-suppression, thereby driving tumorigenesis. Related studies have also revealed that the loss

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of Xist in mouse hematopoietic cells leads to the reactivation of the inactivated X chromosome leading to a genome-wide change in gene expression, thus inducing aggressive and fatal leukemia [36]. Conversely, XIST upregulation can alter specific micro –RNA signaling pathways and thereby promote liver metastasis [47], inhibit apoptosis and increase cell growth and invasion, and resistance to chemotherapy in oral, gastric and colorectal cancerous cells [44]. Such studies suggest that XIST can act as a "molecular sponge" to inhibit microRNAs (miRNAs) that normally suppress oncogenes, allowing cancer-related genes to be expressed freely. Similar molecular processes have been implicated in non-small cell lung cancers and glioblastoma multiforme [46,40]. Taken in this context, stabilization of XIST expression may be paramount in maintaining normal cellular processes while dysregulation with either increased or decreased levels of expression promotes tumorigenesis.

The requirement for XIST homeostasis extends to maintenance of normal biological functions such as cardiovascular and central nervous system health [38,47]. XIST regulation of various micro-RNAs can influence low density lipoprotein function in vascular endothelial cells and subsequent atherosclerosis [64]. Additionally, vascular endothelial growth factor signaling through micro-RNA regulation modulates endothelial function [57]. Cardiac hypertrophy due to cardiac failure has also been linked to XIST, micro-RNA changes and toll-like receptor 2 [59]. Fibrosis that develops after cardiomyocyte death is also associated with XIST, which again regulates various micro-RNA expression levels responsible for fibrosis-related protein functions [48]. Finally, XIST is upregulated in cardiomyocytes under anoxic conditions and myocardial infarction, leading to increased immediate early gene expression through micro-RNA changes. Silencing of the long non-coding RNA promoted cell viability and suppressed cell death through this same pathway [56]. Thus, atherosclerosis, hypertrophy, and cardiac fibrosis, as well as myocardial infarction, are all conditions linked to dysregulation of XIST RNA expression. In an analogous manner, XIST has been proposed to influence neurodegeneration in Parkinson's disease by regulating miRNA function through the BDNF/TrkB pathway, and consequent neuroinflammation. Similar XIST-dependent processes likely exist across various organ systems and would be important in maintenance of cellular function.

4. Therapeutic Applications with XIST

Given the association of XIST and human disease, various therapeutic strategies targeting the long non-coding RNA and its associated complexes have been proposed. Conventional approaches using RNA-based therapies such as small interfering RNA (siRNA) and antisense oligonucleotides (ASOs) can be used to modulate XIST expression or downregulate pathways linked to XIST dysregulation [20, 26, 27]. CRISPR-dCas9 systems coupled with XIST-targeting guide RNAs to modify gene expression profiles reflect another means of control over this system [28]. Development of epigenetic small molecule inhibitors which target XIST- associated complexes, leading to reduced activity, are also underway [21]. Enhancing XIST function using RNA stabilizers [24] and developing small molecules targeting chromatin remodelers show promise as well. Finally, use of XIST to silence an entire non-sex-linked chromosome has been reported in treatment of aneuploidy such as Down syndrome [34, 37, 60]. A complicating variable in all such approaches is the ability to fine tune XIST expression and cell specific localization, as both over- and under-expression, as well as cell specific targeting, of this long non-coding RNA can disrupt epigenetic modifiers in different organ systems. Lack of temporal and spatial specificity could alter gene expression and function to either improve or worsen normal and disease states.

5. Down Syndrome and XIST

Down syndrome (DS) is the most common genetic disorder and occurs in one out of 800 live births. DS individuals live fairly normal life spans but are hindered primarily by neurological issues including mental retardation, seizures, and early onset Alzheimer's disease (AD) [61]. DS arises from both genetic and epigenetic effects that result from the triplication of the approximate 500 genes (with 163 protein coding genes identified), six micro RNAs, and many ORFs and potential functional mRNAs that reside on chromosome 21 (HSA21). Taken in this context, the trisomy in DS would be expected to disrupt innumerable cellular processes and pathways required for CNS development and maintenance [62].

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Overcoming the challenges in treating a polygenic disorder such as DS may require an unconventional approach. Rather than pharmacological targeting of specific pathways- none of which have proven efficacious, optimal treatment of DS requires inhibition of the entire HSA21. During early embryonic development, the X-inactivation specific transcript (*XIST)* gene on HSAX acts as a trigger for X chromosome-wide inactivation. *XIST* encodes for a non-coding RNA, which when upregulated accumulates within the chromosome territory of the future inactive X chromosome. It recruits polycomb group complexes thereby modifying the chromatin and silencing gene expression. Recent transgene experiments integrating *XIST* into autosomes lead to silencing of the autosome and suggest that this gene could recapitulate the chromosome-wide inactivation of any autosome in the same manner as HSAX silencing [6, 64, 65]. In fact, insertion of *XIST* into the *DYRK1A* locus on HSA21 in DS pluripotent stem cells silences the trisomic copy and rescues cell proliferation defects [60].

Several limiting factors restrict the use of XIST gene therapy for treatment of DS. **First**, the requirement for genomic recombination- insertion of XIST into the trisomic copy limits the number of cells that can be efficiently induced to incorporate the desired construct. ***Second***, *specificity is required that only one of three HSA21 copies in DS can be targeted to incorporate XIST*. **Third**, the efficiency of delivery of both XIST and other regulatory components to the cells for integration will be needed. **Fourth**, the ability to regulate XIST expression may be critical to achieve clinical efficacy.

6. Genetic Recombination and XIST

The CRISPR/Cas system derives from a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages [66,67,68]. CRISPR itself comprises a family of DNA sequences in bacteria, that encode small segments of DNA from viruses that have previously been exposed to the bacterium. These DNA segments are used by the bacterium to detect and destroy DNA from similar viruses during subsequent attacks [69]. In a palindromic repeat, the sequence of nucleotides is the same in both directions. Each repetition is followed by short segments of spacer DNA from previous exposures to foreign DNA (e.g., a virus or plasmid) [70]. Small clusters of Cas (CRISPR-associated system) genes are located next to CRISPR sequences. These observations form the basis of the CRISPR/Cas9 system in eukaryotic cells that allow for genome editing. By delivering the Cas9 nuclease complexed with a synthetic guide RNA (gRNA) into a cell, the cell's genome can be cut at a desired location, allowing existing genes to be removed and/or new ones added [71,72]. The Cas9-gRNA complex corresponds with the CAS III CRISPR-RNA complex.

While genome modification through the CRISPR-Cas9 allows for insertion or deletion of genetic material, the efficiency by which these processes occur is dependent on the mechanism of repair of the DSBs. In NHEJ, several nucleotides are frequently lost from the ends of the DSBs, and lead to frameshift mutations and subsequent knockout of the targeted alleles at high efficiency [64]. In HDR, genetic material integrates into the genome by homologous recombination, thereby allowing for knockin of genetic segments, albeit at low efficiency [65]. A significant technological hurdle arises in the use of CRISPR-Cas9 for knocking of genetic material.

Several approaches have been directed toward increasing the efficiency of HDR for CRISPR-Cas9, each limited by special caveats [73]. First, the suppression of NHEJ molecules to promote the HDR pathway has been reported to improve the efficiency of HDR by 4 to 8 folds. The effects were seen with inhibition of NHEJ molecules (KU70, KU80, or DNA ligase IV), application of ligase IV inhibitor SCR70, or co-expression of adenovirus 4 E1B55K and E4orf6 proteins (to promote ligase IV degradation) [74], albeit the applicability and reproducibility in other cell systems has not been substantiated over time [75]. Second, timed delivery of Cas9-guide RNA ribonucleoprotein complexes by cell synchronization with nocodazole in HEK293T, human primary neonatal fibroblast and human embryonic stem cells increased rates of HDR by up to 38% compared to unsynchronized cells [76]. Perturbation of the DNA and toxicity from microtubule polymerizing agents poses potential issues with this methodology. Third, modification of the HDR donor by double cleavage increased HDR efficiency to 30% when combined with cell synchronization (CCND1 or nocodazole) [77] with this approach again limited by toxicity. Fourth, optimization of the CRISPR/Cas9 system (electroporation, CRISPR/Cas9 dosage, homologous arm lengths, and dosing of various

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synchronizing agents) have been shown to reach HDR efficiency of 29.6% at specific "safe harbor" sites such as the Rosa26 locus, which is relatively conserved across species [75]. Customization of this type of approach for different systems would prove effort intensive. Fifth, microhomology-mediated end-joining enables efficient integration of exogenous donor DNA but also is limited by ease of use [78,79]. Summarily, while these various approaches show low to moderate CRISPR/Cas9 knocking efficiency, they also carry significant inherent limitations.

More recent work using modifications of CRISPR/Cas9 with other approaches to increase DNA insertion efficiency. While the insertion efficiency of double stranded DNS (dsDNA) through CRISPR/Cas9 remains poor [81,82], the insertion of single-stranded oligodeoxynucleotides (ssODN) at Cas9 cleavage sites through an HDR pathway is much higher. Recent work suggests that increasing the length of the 3′ or 5′ overhangs for the recipient genomic DNA after CRISPR/Cas9 cleavage through endonucleases can mimic ssDNA and thereby increase the efficiency of integration for donor dsDNA with efficiency of 30-40% with large constructs up to 14 kB (U.S. Patent Application No.: 62/849,504). Alternatively, the PASTE (Programmable Addition via Site-specific Targeting Elements) provides a novel approach which includes a Cas9 enzyme that cuts at a specific genomic site, guided by a strand of RNA that binds to a chosen site. This allows specificity of targeting for insertion of a 46 DNA base pair landing site. This insertion does not require double-stranded breaks which may be detrimental to the cell and allows for use of integrases, that subsequent permit insertion of large DNA payloads with efficiency ranging from 5 to 60% [80]. Use of such approaches would allow for efficient insertion of XIST.

7. Selective Chromosomal Targeting with XIST

Even with increased efficiency of XIST genomic integration, a second technical issue must be addressed for practical implementation of this approach in DS. XIST targeting would necessarily involve integration into only one of the three HSA21 chromosomes. This issue has never been addressed, in part because the efficiency of genomic integration is so low that the likelihood of having two or more HSA21 copies within the same cell incorporated into the genome would be very low. However, the origin of nondisjunction in DS leading to the trisomic HSA21 predominantly (80-95%) occur during meiosis I within cells of maternal origin [83,84,85,86]. Non-disjunction leads to failure of homologous chromosome to separate during anaphase such that one of the gametes will have an extra HSA21 chromosome while the other will be missing an HSA21 chromosome. Importantly, each of the HSA21 chromosomes from the mother will be distinct and this uniqueness will allow for specificity of targeting. Each of the three HSA21 chromosomes is distinct and will therefore harbor different SNPs. A SNP-based PAM targeting approach through CRISPR allows for targeting of one of the three HSA21 copies.

8. Delivery systems and regulation of XIST

Several other issues remain in the development of XIST directed silencing. Both over-expression and under-expression of XIST can cause disruption of micro-RNA dependent control over gene expression. In this sense, a tunable form of XIST will be required. Use of inducible promoters may be the best way currently with which to address this problem. Additionally, efficient and tissue-specific delivery of XIST-targeted therapies remains a hurdle. At present, electroporation and lipofectamine provide a means of introducing XIST into cells, but each has its limitations. Electroporation is limited to the area that can be covered and remains traumatic due to the electrical current. Lipofectamine can cover a larger region, but this too may be limited topographically. Other advances in nanoparticle technology, magnetic particles and viral vectors will likely need to be explored to address these issues [21].

9. Conclusions

We reviewed the XIST RNA's unique role in epigenetic regulation which makes it a promising therapeutic target for a variety of diseases and genetic conditions. While challenges remain ahead, advancements in RNA biology, delivery systems, and genome-editing technologies are rapidly

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expanding the scope of XIST-based interventions. Continued research will lead the way for novel treatments, raising the therapeutic potential of this very important lncRNA.

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Abbreviations: XIST: X inactive-specific transcript, XCI: X-chromosome inactivation, RNP: Ribonucleoprotein, lncRNA: Long non-coding RNAs (lncRNAs), ceRNA: Competing endogenous RNA, CCSC: chronic compressive spinal cord injury, iPSCs: induced pluripotent stem cells.

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