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Guilherme José Ruivo Pereira

Inativação do cromossoma X: implicações na patologia humana

X-Chromosome Inactivation: implications in human disease

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Dedicatória

Aos meus pais Marcelo e Isabel, que deram todo o apoio que puderam (e até demasiado) durante o curso, a nível de tempo e disponibilidade, nos momentos bons e nos menos bons, física e emocionalmente.

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X-Chromosome Inactivation: implications in human disease

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Abstract

X-Chromosome Inactivation (XCI) is a process involved in the pathogenesis of several diseases. In this mini review we discuss the known mechanisms associated with XCI, when and how it initiate, spreads and maintain, as well as the mechanisms that allow some genes to escape from it. We address skewed XCI, the condition that happen when this process is not fully randomized, and its consequences to the phenotype of some pathologies. We debate about the known pathologies implicated, including X unbalanced rearrangements, X-autosomal balanced translocations, Turner and Klinefelter syndromes and also for X-linked diseases and his consequences for males and females. Some pathologies were discussed more in detail such as intellectual disability with a recognize relation with XCI. Finally, possible future implications for genomic therapy and treatment of patients and list areas that need further research on this topic were addressed.

Introduction

In humans, female cells carry two X-chromosomes, whereas male cells have only one X chromosome plus a Y chromosome. Y chromosome contains less genetic material (approximately 70 genes) than the X chromosome (approximately 900-1500) (Disteche 2016). This contributes to a big imbalance between men and women regarding genes, imbalance that has two mechanisms for compensation: X chromosome upregulation or X chromosome inactivation (XCI) (Fang *et al*. 2019).

Focusing on XCI, the first hypothesis was formulated by Mary Lyon in 1961, and it consisted on "that mosaic phenotype is due to the inactivation of one or other X chromosome early in embryonic development" (Disteche and Berletch 2015). This reasoning was proven to be accurate, first based on mice, and later confirmed as a law in all mammals. The so-called Lyon Law is still the most important contribute to this topic, more than 50 years after its formulation.

It happens during early female embryonic development (Monk *et al.* 1987; Disteche and Berletch 2015) and it starts shortly after embryo implantation (Fang *et al*. 2019). The cells lineage maintain the initial chosen chromosome (either the paternal or the maternal X chromosome) throughout all the development till the adult age (Fang *et al*. 2019) while the other is silenced resulting in the so-called Barr body (Galupa and Heard 2018), a condensed heteropycnotic structure in the interphase nuclei of female cells (Disteche and Berletch 2015). This provides dosage compensation for eutherians between the sexes (Dixon-McDougall and Brown 2016).

Logically, XCI randomness creates a coexistence between both chromosomes activated of an approximate ratio of 1:1. This is extremely relevant to phenotypic changes, as mutations that could lead to serious complications in males are compensated by another chromosome in 50% of the female cells (Cantone and Fisher 2017). This process of randomness follows a normal distribution, what almost mandatorily creates examples of very unbalanced ratios. This process is called "skewing of XCI", and the extreme cases (over 90% of the cells with inactivation of the same chromosome) skewed X-chromosome inactivation (Cantone and Fisher 2017). Non-random inactivation also exists in special cases where a group of cells has a proliferative advantage (or disadvantage) based on X-chromosome mutations, what causes one to outgrow the other.

It is also important to refer that XCI is not a permanent state, as it can be reversed (Payer 2016). It is physiologic for example when it happens on haploid cells (like the spermatozoid – the paternal X is silenced till fertilization happens), but it can also be pathological (in some congenital or acquired diseases) (Disteche and Berletch 2015). Age-related X reactivation may also happen, and that this process could be related with neurological and oncological problems (Leppig *et al.* 2001).

Methods

To write this mini-review, two searches (first on 20/07/2020 and second on 23/02/2021) were conducted in PubMed, Scopus and Cochrane Library, including all articles written in English, Spanish and Portuguese. The searched was made using the following query: (X-chromosome inactivation [MeSH Major Topic]) OR (Lyon Hypotesis [MeSH Major Topic]). Inclusion criteria included studies made in humans and related with pathology while exclusion criteria included studies made in animals. Year of publication from the articles was not an exclusion criteria, but most recent studies were treated as more relevant.

XCI regulation and function

XCI comprises a series of highly organized events that result in the transcriptional silence of one of the two female X chromosomes. The process starts with the counting and choosing of the X-chromosome that will be silenced followed by three steps referred usually as: XCI initiation, XCI spreading and XCI maintenance.

The initiation of XCI is controlled by a regulatory locus named X-inactivation centre (Xic) that contains clustered genes and regulatory sequences involved in the inactivation process. It is defined as the region that is necessary and sufficient to trigger XCI when present in two copies (Galupa and Heard 2018). The proximal limit of the human X-inactivation center (XIC) has been localized between Xq13.1 and Xq13.2 chromosome region.

This region was found to have a gene that encodes noncoding RNA (IncRNA), named X-inactive specific transcript *(XIST*) that is transcribed only from the future inactive X chromosome (Xi). The process of silencing is still largely unknown but according to

literature *XIST* RNA may binding to concrete sites after starting in the XCI, beginning a cascade of epigenetic changes that results in the formation of the Barr body (Pinter *et al.* 2012; Payer 2016; Posynick and Brown 2019). Without a functional *XIST* gene, there is no X-chromosome inactivation, and its partial decrease of activity in one group of cells (due to a mutation in X-chromosome) causes an unbalance that can lead to non-random inactivation (Bicocchi *et al.* 2005).

The choice of the X chromosome that becomes silenced is also molecularly influenced: X controlling element (Xce) locus is fundamental to that process, even if there are no certainty about how it works. Even though, randomness is also an important component of the inactivation (Monkhorst *et al.* 2008). Epigenetics are also relevant, and even some degree of heritable gene silencing could influence this process (Disteche and Berletch 2015).

XCI spreading is a not well-known process but following the most recent studies, XIST spreads in cis across the inactive chromosome, recruiting PRC2 (polycomb repressive complex 2) and spreading it due to the formation of a silent nuclear compartment, with repressive chromatin modifications, which the most known may be H3K27me3 (Engreitz *et al.* 2013). XIST RNA recruits several proteins, namely SHARP, who help in this process, as SHARP recruits SMRT, a protein that activates the histone deacetylase HDAC3 for silencing. Several studies refer also the importance of A-repeat within the XIST gene, as it interacts both as a silencing component, and as a binding interactors (Disteche and Berletch 2015).

XCI spreading studying is extremely relevant as it may be possible in future, as technology improves, to correct trisomy 21 by insertion of a XIST transgene on chromosome 21, offering help to individuals with Down syndrome (or other trisomies). This is “the major first step towards chromosome therapy”, and it is certainly a matter that deserves more studies (Jiang *et al.* 2013).

The maintenance of gene silencing in X-inactivation is a complex phenomenon that involves the interaction of many different cellular mechanisms and pathways. Furthermore, recent studies showed that XCI is not as stable as previously thought. Reactivation of some Xi genes was observed in normal and diseases tissues, for example upon ageing, in autoimmune diseases and cancer (Bicocchi *et al.* 2005). The normal progression of this process is not the same in every cell lineage: it has been reported that

immune cells, for example, have a diverse process of XCI maintenance, what actually has been reported as a possible cause of the well-known imbalance in autoimmune diseases between men and women (Sierra and Anguera 2019).

One of the most intriguing questions regarding X inactivation is how (and why) some loci are able to escape gene silencing. In human, up to 25% of X-linked genes can escape from XCI - 15% of them constitutively and about 10% of them variably between individuals (Galupa and Heard 2018). This biallelic expression results in a higher dosage of these genes in females suggesting that these genes might have female-specific roles (Berletch *et al.* 2011).

Historically, we know that there are several different pathways that lead to XCI, and some of the genes involved in this process are the same in multiple species. The most popular explanation for why this process started comes from the fact that there was a need to have Y homology in the X-chromosome, for questions of balance. However, half or more than half of the escaping genes have no Y correspondence. This difference is probably explained simplistically by Darwinism – the cases that XCI escape gave reproductive advantage to the female probably created this difference (Posynick and Brown 2019).

We can also relate several elements that benefit or reduce this escape. Regions without silencing elements, like X added region (XAR), have more escaping genes, while regions that have less Y homology have subsequently more presence of heterochromatin and are more silenced.

These genes that escape X-inactivation are responsible for many of the different phenotypes that some diseases can present. As such, and due to the fact that we have drawn a clear correlation between DNA methylation and escape from XCI (Payer 2016; Halmai *et al.* 2020) , we can now project that understanding this can have a lot of potential to create innovative ways of treatment for people that suffer from this X-linked disorders (Halmai *et al.* 2020).

XCI - consequences for human diseases

The existence of skewed X-chromosome inactivation allows the development of multiple diseases, in non-common forms, mainly X-linked diseases in women.

X-chromosome numerical and structural abnormalities

The occurrence of XCI has many implications in both numerical and structural abnormalities of the X-chromosome.

XCI has a protective effect in human chromosome aneuploidies, such as Turner syndrome (X) and Klinefelter syndrome (XXY), because it balances the chromosomes causing a softer, milder disease in comparison with autosomal trisomies (the few tolerated have severe consequences) (Cantone and Fisher 2017). Turner syndrome individuals have low stature, as the gene *SHOX* partially escape inactivation in the Xi of women, giving them an average stature (Blaschke and Rappold 2006; Disteche and Berletch 2015).

In the case of X-autosome balanced translocations, there is an interesting order of events regarding XCI: the normal X chromosome tends to be inactivated (75%), giving usually a normal phenotype. However, two situations can happen: even an initiation point of XCI is imprinted just before the translocation, what actually silences also the translocated gene (what could lead to different pathologies), or the skewed XCI happens on the translocated chromosome (25%) of females, which leads to a functional X disomy and autosomal monosomy (Mattei *et al.* 1982). One example was reported in a girl carrier of a translocation between X and 11 chromosomes and that have a phenotype compatible with Coffin-Lowry syndrome (Yamoto *et al.* 2020). In unbalanced translocations, if the abnormal X is inactivated, the inactivation spreads to the autosomal region, and the phenotype is often abnormal. Other structural abnormalities such as X chromosome deletions, or extra X rings may be tolerated because of the preferential inactivation of the abnormal X chromosome, being random XCI more associated with an abnormal phenotype.

X- linked diseases

A correlation between Intellectual Disability (ID) and skewed XCI has been established (Plenge *et al*. 2002; Gieldon *et al.* 2017). In males (with only one copy of X-chromosome), ID in autism spectrum syndromes and other mental impairment diseases, are way more prevalent. However, skewed XCI can cause intermediate or even almost complete phenotypes of these diseases in women. It has been reported that deletions of *KDM5C* or *IQSEC2* cause ID, however, while in men they are normally severe, in women it depends on dosage sensitivity: women with skewed or extremely skewed X-chromosome inactivation have more clinical severe consequences. *KDM6A* gene (responsible for Kabuki syndrome) also have effects related with the dosage, and as such,

have great impact in women with skewed X-chromosome inactivation (Disteche and Berletch 2015). Other examples are ATR-X, Lowe oculocerebrorenal syndrome, Allan-Herdon-Dudley syndrome and autism spectrum diseases (Aarabi *et al.* 2019).

Going to systemic diseases, a good example is Fabry disease, a rare metabolic disease caused by the deficient activity of α-galactosidase A, which usually affects males in a severe way, while women range from asymptomatic (the majority) to severely affected, in cases relatable to the existence of skewed X-chromosome inactivation (Echevarria *et al.* 2016).

Other example could be the skin diseases: incontinentia pigmenti, focal dermal hypoplasia, Conradi–Hünermann–Happle syndrome, oral–facial–digital syndrome type 1 or MIDAS (microphthalmia, dermal aplasia and sclerocornea) syndrome are all lethal X-linked traits that not only occur in men, but also in women with skewed XCI. There are also other non-lethal pathologies associated, such as hypohidrotic ectodermal dysplasia of Christ–Siemens–Touraine, IFAP (ichthyosis follicularis–alopecia–photophobia).

Another interesting disease that could make the woman range from simply being carrier to have serious consequences to that woman is haemophilia. Skewed X-chromosome inactivation can create serious consequences, reducing the clotting factor in the blood to similar level to men that are affected by the disease (Shoukat *et al.* 2020).

There are also some case reports of situations where skewed or high skewed X-chromosome inactivation could lead to most normal phenotypes: the gene *SHOX*, deleted in Leri-Weill dyschondrosteosis, can be compensated by the other chromosome (Sun et al. 2019).

Another important point is that not only deletions cause disease: a failure of the XCI mechanisms can also cause it: hypometilation of the X-chromosome, or mutations in the XCI escape system, also cause disease. For example, reactivation of the silenced X-chromosome has been documented in breast or renal cancer (Disteche and Berletch 2015) and the hypothesis has been made that also in other cancers, but studies in the area are not ample.

It’s also relevant to refer a problem to be solved: usually all the studies were done with small cohorts, and there is a little amount of bibliography related to this topic. Moreover, every data should be taken carefully, and more studies with bigger cohorts should be

performed, to distinguish which diseases are and which aren't related with XCI, and what future treatments could be applied in that cases.

Concluding remarks

XCI understanding, namely how it starts, spreads, maintains, and how escape from it happens can help us finding genomic treatments that will certainly be fundamental on the many diseases that get affected by this mechanism, from ultra-rare to common pathologies such as Down Syndrome, Turner Syndrome or Klinefelter Syndrome. Also due to skewed X-chromosome inactivation, many women have different phenotypes of diseases that are X-linked. However, still a lot of work has to be done on this area, mainly with bigger cohorts, and with the exploration of the differences between cell groups, such as immune system cells, and also new methods of treatment, like some that are previously referred, such as transgene insertions.

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MAIN TEXT. This should be divided into sections with first-level headings (centred and in boldface) such as Introduction, Materials and methods, Results and Discussion. These may also be descriptive headings, such as may be appropriate, for example, for review articles, or theoretical papers. There may be subsections with short, descriptive headings. Major subsections within sections may be placed under second-level headings (flush left, in italics and boldface, free-standing); these may contain further subsections under third-level headings (flush left, in italics and boldface, and text runs on after a colon). Sections and subsections are not numbered. Footnotes are not allowed. Where appropriate, first person and active voice are preferred. Spelling should conform to the preferred spelling of the latest edition of the Concise Oxford Dictionary.

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Sambrook J., Fritsch E. F. and Maniatis T. 1989 *Molecular cloning: a laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Via S. 1994 The evolution of phenotypic plasticity: what do we really know? In *Ecological genetics* (ed. L. A. Real), pp. 35–57. Princeton University Press, Princeton.

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Scale for the Assessment of Narrative Review Articles

Justification of the article's important for the readership – 2

Page 2 – "X-Chromosome Inactivation (XCI) is a process involved in the pathogenesis of several diseases (…). We debate about the known pathologies implicated"

Pages 8/9 – "It's also relevant to refer a problem to be solved: usually all the studies were done with small cohorts, and there is a little amount of bibliography related to this topic. Moreover, every data should be taken carefully, and more studies with bigger cohorts should be performed, to distinguish which diseases are and which aren't related with XCI, and what future treatments could be applied in that cases."

Page 9 – "XCI understanding, namely how it starts, spreads, maintains, and how escape from it happens can help us finding genomic treatments that will certainly be fundamental on the many diseases that get affected by this mechanism, from ultra-rare to common pathologies such as Down Syndrome, Turner Syndrome or Klinefelter Syndrome"

Statement of concrete aims or formulate of questions – 2

Page 2 – "We address skewed XCI, the condition that happen when this process is not fully randomized, and its consequences to the phenotype of some pathologies. We debate about the known pathologies implicated (…) and his consequences for males and females."

Page 2 – "Finally, possible future implications for genomic therapy and treatment of patients and list areas that need further research on this topic were addressed."

Description of the literature search – 2

Page 4 – "To write this mini-review, two searches (first on 20/07/2020 and second on 23/02/2021) were conducted in PubMed, Scopus and Cochrane Library, including all articles written in English, Spanish and Portuguese. The searched was made using the following query: (X-chromosome inactivation [MeSH Major Topic]) OR (Lyon Hypotesis [MeSH Major Topic]). Inclusion criteria included studies made in humans and related with pathology while exclusion criteria included studies made in animals. Year of publication from the articles was not an exclusion criteria, but most recent studies were treated as more relevant."

Referencing – 2

Pages 4/5 – "The process of silencing is still largely unknown but according to literature *XIST* RNA may binding to concrete sites after starting in the XCI, beginning a cascade of epigenetic changes that results in the formation of the Barr body (Pinter *et al.* 2012; Payer 2016; Posynick and Brown 2019)."

Page 6 – "One of the most intriguing questions regarding X inactivation is how (and why) some loci are able to escape gene silencing. In human, up to 25% of X-linked genes can escape from XCI - 15% of them constitutively and about 10% of them variably between individuals (Galupa and Heard 2018). This biallelic expression results in a higher dosage of these genes in females suggesting that these genes might have female-specific roles (Berletch *et al.* 2011)"

Page 6 – "These genes that escape X-inactivation are responsible for many of the different phenotypes that some diseases can present. As such, and due to the fact that we have drawn a clear correlation between DNA methylation and escape from XCI (Payer 2016; Halmai et al. 2020) , we can now project that understanding this can have a lot of potential to create innovative ways of treatment for people that suffer from this X-linked disorders (Halmai et al. 2020)."

Scientific reasoning – 2

Page 7 – "A correlation between Intellectual Disability (ID) and skewed XCI has been established (Plenge *et al*. 2002; Gieldon *et al.* 2017)."

Appropriate presentation of data – 2

Non-applicable.

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