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Human X chromosome inactivation and reactivation: implications for cell reprogramming and disease

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SUMMARY

X chromosome inactivation (XCI) is an exemplar of epigenetic regulation that is set up as pluripotent cells differentiate. Once established, XCI is stably propagated, but can be reversed *in vivo* or by pluripotent reprogramming *in vitro*. Although reprogramming provides a useful model for inactive X (Xi) reactivation in mouse, the relative instability and heterogeneity of human ESCs and iPSCs, hampers comparable progress in human. Here we review studies aimed at reactivating the human Xi using different reprogramming strategies. We outline our recent results using mouse ESCs to reprogram female human fibroblasts by cell-cell fusion. We show that pluripotent reprogramming induces widespread and rapid chromatin remodelling in which the human Xi loses *XIST* and H3K27m3 enrichment and selected Xi genes become reactivated, ahead of mitotic division. Using RNA sequencing to map the extent of human Xi reactivation, and chromatin modifying drugs to potentiate reactivation, we outline how this approach could be used to better design strategies to re-express human X-linked loci. As cell fusion induces the expression of human pluripotency genes that represent both the 'primed' and 'naïve' states, this approach may also offer a fresh opportunity to segregate human pluripotent states with distinct Xi expression profiles, using single-cell-based approaches.

KEYWORDS: human X chromosome inactivation, epigenetic, cell reprogramming, gene reactivation

1. INTRODUCTION

Gene dosage compensation between XX females and XY males is achieved in mammals through transcriptional silencing of one of the two female X chromosomes. This process, called X chromosome inactivation (XCI), was first postulated in 1961 by Mary Lyon who observed a variegated pattern in the coat of heterozygous female mice with mutations in X-linked genes. Mary studied the inheritance of this trait across generations and understood that in heterozygous females the mutant X chromosome was expressed in some cells and the normal X chromosome in others. This lead her to hypothesise the random inactivation of one of the two X chromosomes [1]. Later studies confirmed that XCI is randomly established during early development, and it is inherited throughout the following somatic divisions [2].

The randomness of XCI together with its stability through mitotic inheritance results in mosaicism in female somatic tissues where cells expressing alternate X chromosomes (i.e. Xa^1Xi^2 and Xi^1Xa^2) coexist in an approximate 1:1 ratio. This has important implications for X-linked Mendelian diseases, including structural and numerical abnormalities. Deleterious mutations of X-linked genes that lead to death or disease in males can, in heterozygous females, be compensated by the expression of the wild-type allele in around 50% of the cells. Importantly, the percentage of cells expressing one or other X chromosome varies in different females and the degree of 'skewing' (towards cells bearing wild-type factors) leads to differences in the penetrance of X-linked diseases within the population and to variable phenotypic expression (reviewed in [3, 4]). Skewing of XCI can also be extreme so that in most cells the same X chromosome is silenced, thus mimicking a homozygous phenotype [5-9]. Structural abnormalities of one of the two X chromosomes provide an example in which the same X chromosome is inactivated in virtually all viable cells as a consequence of selection. In balanced X:autosome translocations, where silencing spreads into the autosomal segment, cells that inactivate derivative X would be generally selected against and eliminated during embryogenesis to avoid deficits in autosomal gene expression [10]. In the case of unbalanced X:autosome translocations, in which the cells maintain only one aberrant chromosome, this may be inactivated in order to reduce the genetic unbalance [11, 12]. Similarly, X chromosomes carrying deletions or duplications are often inactive, mitigating against the effects of gene dosage imbalance [12].

A protective effect of XCI is also apparent in human X chromosome aneuploidies, such as X0 monosomy (i.e. Turner syndrome) and XXY trisomy (i.e. Klinefelter syndrome). Patients with these numerical abnormalities have a much less severe phenotype than comparable autosomal abnormalities. Autosomal monosomies are embryonically lethal in man, with trisomies of chromosomes 15, 18 and 21 being tolerated, but associated with malformation. In contrast, the relatively mild impact of X aneuploidies probably reflects the fact that in humans all but one X chromosome are inactivated, and the phenotypic abnormalities observed in these individuals probably reflect the altered expression of X-linked genes that normally escape X inactivation [13]. In human, it was shown that about 12-20% of genes are expressed from the inactive X chromosome (Xi) [14]. These genes that are believed to 'escape' silencing vary among tissues and also among different female individuals thus contributing to phenotypic variability in X aneuploidies.

Recent studies showed that XCI is not as stable as it had been previously thought. Reactivation of some Xi genes was observed in normal and disease tissues, for example upon ageing [15-17], in autoimmune diseases [18] and in cancer [19-21]. Understanding

how to reactivate genes along the Xi might therefore give us insights regarding the mechanisms of some diseases that show a higher female prevalence, including Graves disease, Hashimoto's thyroiditis, and systemic lupus erythematosus. Furthermore, targeted reactivation of genes on the inactive X chromosome could represent a therapeutic approach in heterozygous females affected by X-linked diseases. This is perhaps best exemplified in the case of Rett syndrome, where in a mouse model, expression of a wild-type *Mecp2* allele was shown to be sufficient to rescue the disease and relieve the neurological symptoms [22, 23].

Based on the finding that pluripotent cells of the blastocyst have two active X chromosomes [24, 25], reprogramming of somatic cells to an embryonic-like pluripotent state represents a useful system to model Xi gene reactivation. In mouse, this system is well established and was used to describe a hierarchy of epigenetic marks that maintain silencing along the Xi [26]. In human, similar studies were attempted but have been more difficult to interpret, possibly because of inherent instability of human pluripotent stem cells *in vitro* [27]. Here, we review current understanding of human X chromosome inactivation during development and in embryonic stem (ES) cells, and outline efforts to reactivate human X-linked genes using different reprogramming strategies. The implication of our recent finding that cellular fusion can induce the reactivation of human X-linked genes ahead of cell division, is discussed. In addition, we highlight the generic use of cell fusion-mediated reprogramming to evaluate reactivation sensitivities of human Xi genes in specific cell types.

2. X CHROMOSOME INACTIVATION: DIFFERENCES BETWEEN HUMAN AND MOUSE

Much of our knowledge of X chromosome inactivation and its molecular mechanisms is derived from studies performed in the mouse, where the process is easily accessible *in vivo* and can be recapitulated by mouse ES cell differentiation *in vitro*. Recent studies however, have shown that XCI is remarkably different in rodents as compared to humans or other mammalian species [28, 29].

One of the major differences between mouse and human is the onset of XCI and its regulation in the early embryo. In mouse the paternally-inherited X chromosome is initially silenced at the four-cell stage [30] and subsequently reactivated in the embryonic lineage (i.e. the pluripotent epiblast) of the blastocyst, in which random inactivation is then established around implantation [31, 32]. Extra-embryonic cells maintain the paternal X chromosome as inactive throughout subsequent developmental stages. In human, instead, both X chromosomes are maintained in an active state throughout pre-implantation development and no parent-of-origin effect has been detected either in embryonic or extra-embryonic lineages [24, 25]. A recent single-cell RNA-seq study showed that in human embryos a progressive and chromosome-wide downregulation of X-linked genes takes place at the time of blastocyst formation but there is no evidence of mono-allelic expression and indeed X chromosome inactivation. A separate study showed that by choosing a more relaxed threshold for defining allelic expression a minor decrease in bi-allelic expression could be detected, but this was not until late blastocyst stages and only on a gene-by-gene basis [33]. This suggests that XCI is established later, and supposes that a distinct dosage compensation mechanism might be used during human preimplantation development.

Interestingly, human *XIST* RNA is initially expressed and coats both X chromosomes in the pre-implantation blastocyst concomitantly with bi-allelic expression of X-linked genes [24, 25]. Remarkably, *XIST* RNA shows a dispersed nuclear pattern in human embryos and it is not associated with enrichment of repressive histone modifications typically marking the Xi [24]. Whether dual *XIST* coating leads to downregulation of gene expression on both X-chromosomes or it instead represents an intermediate state that precedes human XCI awaits clarification. The reported different localization of *Xist*/*XIST,* that coats the Xi in mouse and both X chromosomes in human epiblast cells, may infer species-specific regulation. In mouse, *Xist* is antagonized by its antisense *Tsix* that is expressed from the active X chromosome (Xa) and inhibits *Xist* upregulation *in cis* [34, 35]. Furthermore, several pluripotency factors (e.g. Pou5f1, Nanog and Rex1) were reported to inhibit *Xist* and/or activate *Tsix,* thus coordinating the initiation of XCI with the onset of differentiation [36-39]. In human, the function of *TSIX* is not conserved [40] and the role of pluripotency factors has not been thoroughly dissected, due to the epigenetic instability of human ES cells with both X chromosomes active [27]. Recently another long non-coding RNA, named *XACT*, was reported to coat the active X chromosome in human ES cells, but not in mouse where no orthologue has yet been found [41]. As *XACT* coats the Xi in human ES cells that undergo epigenetic erasure of XCI and lose Xi-associated *XIST*, it has been proposed that *XACT* regulates *XIST* localization or function at the onset of XCI [42]. Supporting this hypothesis, it was shown that *XACT* transgenes in mouse ES cells prevent the accumulation of *Xist in cis* leading to inactivation of the non-transgenic X chromosome, whereas *XAC*T transgene downregulation rescued random XCI [33]. Recent studies in humans indicate that *XACT* is expressed in pre-implantation embryos [25] where it coats either one or two X chromosomes alongside with *XIST* [33]. Although *XIST* and *XACT* coat the same X chromosomes, they occupy distinct spatial domains suggesting that *XACT* may alter proper *XIST* localization and block silencing ahead of XCI initiation.

3. X CHROMOSOME REACTIVATION IN HUMAN ES AND iPS CELLS

Reversal of X chromosome inactivation was first observed following the fusion of mouse somatic and pluripotent embryonic carcinoma (EC) cells in hybrids that acquired the tumorigenicity and differentiation potential of EC cells [43]. As inactivation was not reversed in hybrid cells formed between two somatic cells, this suggested an association between pluripotent reprogramming and Xi reactivation. Further studies then confirmed that the mouse inactive X is reactivated when female somatic cells re-acquire a pluripotent state both *in vivo,* during primordial germ cell (PGCs) development [44, 45], and *in vitro,* upon reprogramming by ES cell fusion, nuclear transfer or induced pluripotent stem (iPS) cell methods [46-48] (reviewed in [49]). In addition, it was shown that several pluripotency-associated transcription factors (such as Oct4, Nanog, Sox2, Rex1, c-Myc and Klf4) regulate the expression of *Xist* and *Tsix* in mouse ES cells, thereby coordinating the onset of XCI with loss of pluripotency and susceptibility to differentiation [36-39]. Exogenous overexpression of some of these so-called Yamanaka factors by mouse somatic cells was sufficient to induce pluripotent reprogramming [50] and kinetic studies showed an ordered progression of Xi reactivation, revealing important mechanistic events in the process of Xi reactivation [26].

Comparable studies using human cells as targets for reprogramming have been hampered by the heterogeneity and instability of human ES and iPS cells when maintained in culture [51-56]. The status of the X chromosomes in these cells is apparently extremely sensitive to culturing conditions and a consensus about the factors that allow human ES cells with two active X chromosomes to be reliably propagated remains challenging [27]. Newly established human ES cell lines were shown to maintain both X chromosomes active when cultured in low oxygen but quickly inactivate one of the two X chromosomes upon stress [57]. In standard medium conditions (with TGFβ-activin and βFGF) most human ES cell lines already have one XIST-coated Xi and one Xa, and progressively undergo loss of *XIST* and partial gene reactivation upon further culture [42, 51, 52, 58]. Because reactivated genes cannot be silenced upon differentiation of these XIST-negative XaXi human ES cells such state is commonly referred to as 'erosion' [59]. Similarly, somatic cells reprogrammed to iPS cells in the standard culturing conditions have one Xa and one Xi that undergoes XCI erosion upon prolonged culture [55]. A recent study suggested that the human somatic Xi is initially reactivated during iPS reprogramming but this state is not adequately stabilised so that only XaXi iPS colonies are derived [60]. Several alternative culture conditions have been proposed that may help in isolating and maintaining human stem cells in a naïve state with two active X chromosomes, although these studies rely on indirect measures of X chromosome inactivation, such as the presence of a XIST-coated Barr body [61-63] or X:autosome expression ratios [64], or have directly analysed the expression of only a few X-linked genes [65, 66]. Two of these culture conditions [62, 63] are able to reprogram human ES and iPS cells with an inactive or eroded X chromosome to a state resembling human epiblast cells whereby X-linked genes are bi-allelically expressed and can be silenced upon differentiation [33, 67]. Nonetheless, allele-specific expression analysis showed that upon differentiation these XaXa human ES cells preferentially inactivate the same X chromosome that was silenced in their primed state. Furthermore, only a minority of ES cells (<10%) expressed *XIST* bi-allelically and showed dual X chromosome coating as observed in the human epiblasts, [67]. These differences between embryonic cells and experimentally derived 'naïve' ES cells with mono-allelic XIST expression and non-random XCI, underscore our need to find better culture conditions.

4. HUMAN X CHROMOSOME REACTIVATION UPON CELL FUSION-MEDIATED REPROGRAMMING

The lack of consensus about culturing conditions that allow stable propagation of human pluripotent cells with two active X chromosomes and difficulties in tracking rare reprogrammed cells made iPS reprogramming unsuitable for studying the reversal of X inactivation in human. As an alternative, we used cell fusion between human female fibroblasts and mouse ES cells to transiently reprogram the somatic nucleus and study the reactivation of the human Xi. This system allowed us to analyse early reprogramming events that occur in the absence of, or immediately following, cell division and therefore are much less likely to be dependent upon cell culture conditions [68]. Human pluripotency genes are in fact rapidly re-expressed 1-2 days after cell fusion when the majority of cells are pre-mitotic heterokaryons that precede nuclear fusion and hybrid formation. Single cell-based imaging techniques allowed us to discriminate pre- and post- mitotic reprogramming events by the respective analysis of heterokaryon and hybrid cells that can be easily distinguished by having spatially discrete, or fused human and mouse nuclei, respectively (Fig. 1). The first

event that we detected in heterokaryons 1-2 days after fusion was the delocalization of *XIST* RNA from the Xi that resulted in a dispersed RNA-FISH signal reminiscent of human epiblast cells [24] and was accompanied by a loss of H3K27me3 enrichment from the Xi. *XIST* delocalization preceded and was tightly associated with the bi-allelic expression of two X-linked genes, *ATRX* and *HUWE1* (Fig. 1). By day 3 after fusion, *XIST* was delocalized in the majority (>50%) of heterokaryons and hybrids, whereas X-linked bi-allelic expression was only detected in a subset of cells (30%) suggesting that loss of *XIST* is necessary but insufficient for Xi gene reactivation. Conversely, *XACT* RNA was not detected until day 6 and it was confined to a very restricted minority of hybrids (<1%) in which it showed no preferential association with the Xa or both X chromosomes. This suggests that *XACT* might be dispensable for *XIST* delocalization and Xi gene reactivation during pluripotent reprogramming. It was recently shown that *XACT* coats the Xi ahead of *XIST* delocalization in human ESCs during erosion or conversion from a XaXi primed to XaXa naïve state [33]. Altogether, these results suggest that *XACT* might help to stabilize X-linked gene expression in human pluripotent cells but is not fully active early during reprogramming. As *XACT* was not detected in heterokaryons, *XACT* re-expression may require several cell divisions or be dependent upon factors that are induced late in reprogramming. Interestingly, as *XIST* is delocalized independently of *XACT* in cell fusion-mediated reprogramming, this suggests that other factors may regulate dissociation in this context and offers an opportunity to dissect the contribution of recently discovered XIST-interacting factors [69, 70] in this process.

To investigate the extent of reactivation induced by cell fusion-mediated pluripotent reprogramming we developed an approach to allow gene expression from each of the two X chromosomes in female human fibroblasts to be discriminated based on SNP detection and RNA sequencing [71]. In this strategy (summarized in Figure 2) reciprocal single cell clones (type a or type b) were isolated from a source of primary human female fibroblasts. RNA sequence comparisons were performed on both cell types allowing the identification of around 350 SNPs differing between X^1 and X^2 and to construct haplotypes corresponding to each of two X chromosomes (Xa and Xi). After fusing individual human clones with mouse ES cells, allele-specific analysis of human RNA-seq reads allowed us to identify genes that were expressed from the Xi upon pluripotent reprogramming. Around 10% of Xi genes that were sampled showed stable reactivation at 4 and 6 days after fusion (green circles shown in Figure 3). As these reactivated genes were common to different clones, we concluded that reactivation is not due to haplotype-specific effects, such as sequence variations or mutations along gene regulatory elements, but rather reflected reliable differences in the susceptibility of certain genes to reactivation during pluripotent reprogramming. The majority of genes sensitive to reactivation clustered to two regions on the short Xp arm within the most recently added strata, and alongside genes that have previously been shown to escape from X inactivation [72, 73]. This observation may infer that such genes have evolved regulatory mechanisms that are somewhat distinct from the rest of the X chromosome. Surprisingly, histone modifications such as H3K9me3 and H3K27me3 that normally segregate along the human Xi [74] and define different functional heterochromatin compartments [75] did not predict Xi locus reactivation. Instead we observed that genes that were particularly susceptible to reactivation were also those showing variable Xi expression among different fibroblast clones ahead of reprogramming. This probably reflects an intrinsic predisposition to transcriptional activation within the somatic cells that may be harnessed upon reprogramming. Interestingly, a recent paper has

shown that immune-related genes along the Xi (i.e. *CD40L* and *CXCR3*) have a higher susceptibility to reactivation in mature naïve T and B lymphocytes whereby the Xi shows dispersed Xist/XIST patterns and loss of Xi-associated heterochromatin marks similarly to what we have observed upon fibroblast cell fusion mediated reprogramming [18]. As cell context might influence the predisposition of Xi genes to be reactivated, it will be important in the future to analyse stochastic Xi re-expression and reprogramming-mediated reactivation in different somatic cell types.

As DNA demethylation has previously been shown to induce stochastic Xi reactivation in somatic cells [76], we asked whether reactivation-sensitive genes were selectively responsive to DNA hypomethylation by culturing them in the presence of 5-aza-deoxycytidine. This did not induce the re-expression of these candidate genes, but pretreating human fibroblasts with 5-aza-deoxycytidine instead triggered the reactivation of a second class of genes upon fusion with mouse ES cells (highlighted in red, figure 3). This unexpected result has two important corollaries. Firstly, it suggests that cell fusion-mediated reprogramming, when combined with distinct chromatin-modifying drugs, could unveil subsets of Xi loci with different requirements for reactivation (figure 3, (+X) lower panel). Accordingly, if silencing of different Xi domains (or individual loci) in different cell types is controlled by distinct combinations of factors and chromatin states, it may be possible to use this approach to unravel successful strategies to reverse silencing. Secondly, as pluripotent reprogramming is known to initiate chromatin remodeling along the inactive X, leading to the loss of Xi-associated H3K27me3 and *XIST* delocalization, DNA methylation might be a limiting factor for the reactivation of some loci, while de-acetylation or de-ubiquitination might, for example, be required for others. Future studies that compare the sensitivity of different cell types to reprogramming-mediated Xi reversal and that screen the effects of pretreating cells with defined chromatin-modifying drugs will be required to test these possibilities.

Analysis of global expression profiles of human fibroblast clones upon cell fusion-mediated reprogramming showed that cell fusion-mediated reprogramming induces genes associated with both the primed and naïve human pluripotent states [62]. As heterokaryons and hybrids are heterogeneous in XIST delocalisation and Xi gene reactivation, it might be possible to segregate different pluripotent states at the single cell level. Of note, the majority of cells show a delocalised XIST pattern associated with persistent expression and loss of H3K27me3 as observed in the human naïve pluripotent state. As the Xi changes detected during cell fusion-mediated reprogramming occur within 3-4 days it is likely to reflect an intermediate state in the transition from somatic to pluripotent state rather than erasure of XCI reactivation. Supporting this hypothesis, RNA-FISH analysis showed bi-allelic expression of ATRX and HUWE1, two Xi genes that do not lose silencing in eroded human ES cells [59]. Single cell studies of the human X chromosomes in the cell fusion-reprogramming system might indeed give us the opportunity to dissect the conversion from the somatic to pluripotent state and further define human pluripotency.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Recent studies of the extent of human Xi expression induced by fusing mouse ESCs with human fibroblasts, have revealed that pluripotent conversion results in partial reactivation of the Xi chromosome by approximately 30% of cells, despite loss of focused *XIST* and

H3K27me3 signals by most fused cells [68, 71]. This suggests that although loss of *XIST* and H3K27me3 may be required to reactivate genes along the human Xi chromosome, these chromatin changes are insufficient for Xi re-expression. By comparing Xi loci that escaped XCI, were sensitive to reactivation, or resisted reactivation in this setting, we noted that many reactivation-sensitive loci reside within the same domains of spatial association (TADs) as genes known to escape XCI in human fibroblasts [71]. Whether this shared "accessibility" reflects characteristics of the underlying DNA sequence, and whether reactivation is enabled through spatial association, remains to be investigated. Whatever the outcome, it seems likely that studies of variable escape from XCI as well as reprogramming-mediated Xi reactivation, will be informative in understanding the basis of Xi chromosome expression and silencing at a locus-specific level. This may be informative in attempts to ameliorate X-linked human disease by re-expressing previously silenced alleles.

The epigenetic status of X chromosomes in human pluripotent cells has been the topic of much investigation and debate [27]. One unexpected outcome of reprogramming experiments between human female fibroblasts and mouse ES cells has been the induction of a repertoire of human transcripts, revealed by RNA-seq, with features of both primed and naive ES cells. Future single cell analysis may allow us to interrogate the basis of this mixed profile, and segregate different pluripotent states with distinct states of Xi silencing or reactivation. This information may be useful in trying to decipher the epigenetic states and transitions of individual human pluripotent cells *ex vivo* and within the epiblast of the embryo, as well as the mechanisms of dose compensation that operate before and after implantation.

COMPETING INTERESTS

We have no competing interests

AUTHORS' CONTRIBUTION

I.C. and A.G.F. conceived the study and wrote the manuscript

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FIGURE LEGENDS

Figure 1. Cell fusion-mediated human Xi reactivation. Schematic representation of female human fibroblast reprogramming induced by fusion with mouse ESCs. Upon fusion heterokaryons are formed in which human (white) and mouse (grey) nuclei remain spatially discrete (days 2 and 3) before the nuclei fuse and generate hybrid cells (days 4-6). Lower panels show confocal images of representative cells (%) showing *ATRX* RNA expression (red), *XIST* RNA (green), and stained with DAPI (blue). Before fusion *ATRX* expression is mono-allelic (from the presumed Xa) and *XIST* signal is compact (coating the presumed Xi). After fusion, *XIST* signal becomes diffuse or lost and *ATRX* expression is bi-allelic. In

hybrids, *ATRX* re-expression persists on newly replicated chromatids (arrows, days 4-6).

Figure 2. Strategy to detect Xi reactivation using allele-specific RNA-sequencing. Single cell clones were isolated from a human (X_1X_2) female fibroblast culture to generate homogenous lines that express either the X1 (X1 white, inactive X2 black; clone type a) or the X2 (X2 white, inactive X1 black; clone type b). RNA sequencing of reciprocal clones a and b allowed heterozygous SNPs to be identified, and alleles on the two different human X chromosomes to be discriminated. The extent of Xi reactivation following pluripotent reprogramming was assessed by comparing allelic expression (human-specific reads) in fibroblast clones before and after cell fusion.

Figure 3. Screening for chromatin modifiers that promote human Xi reactivation. The extent of expression of Xi genes in a representative clone before (hF) and after cell fusion-mediated reprogramming (hFxmESC) reveals that some genes (highlighted in green) are susceptible to reactivation following reprogramming (for details see [71]). As pre-treatment of cells with drugs that reduced DNA methylation (+5-aza-deoxyC) revealed a selective reactivation of additional 'Xi' genes (red dots), we propose to screen for candidate modifiers (+ agent) that restrict the expression of specific human X-linked genes. Grey shades indicate 10% of Xi expression versus total.

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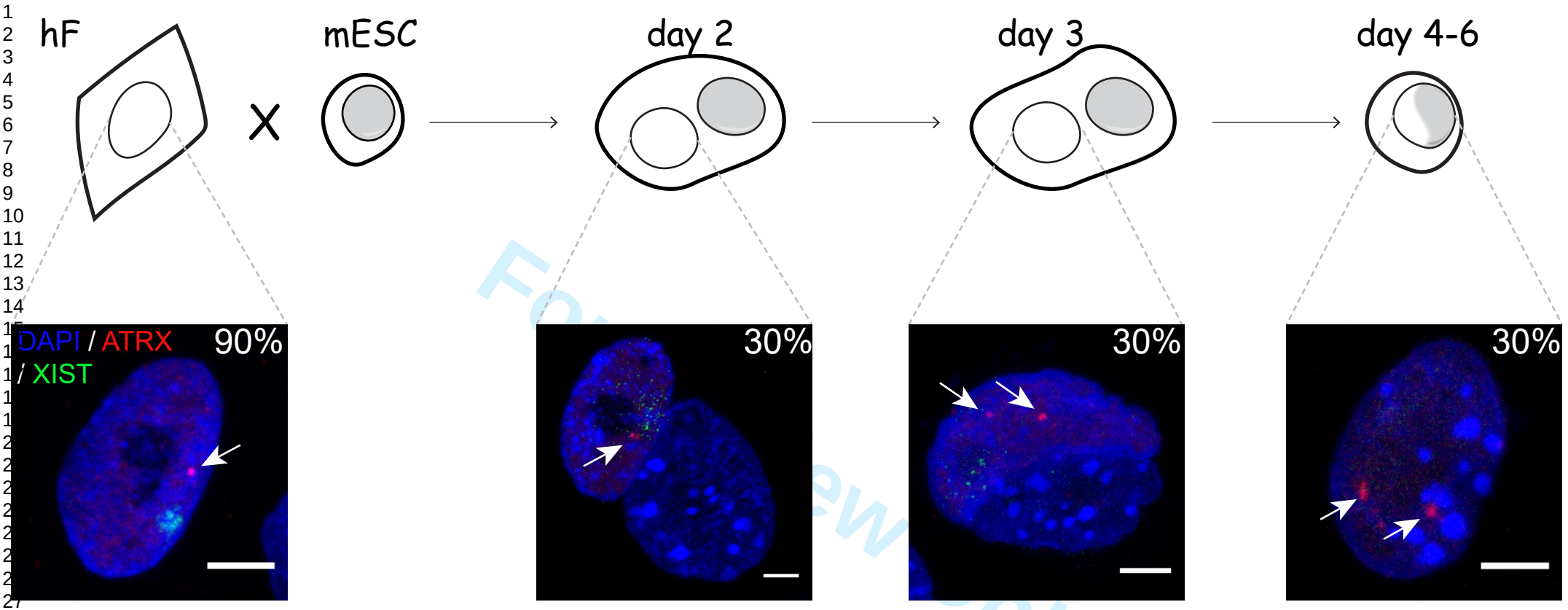

Figure 2