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REVIEW

Role of Long Non-coding RNAs in Reprogramming to Induced Pluripotency

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Abstract The generation of induced pluripotent stem cells through somatic cell reprogramming requires a global reorganization of cellular functions. This reorganization occurs in a multi-phased manner and involves a gradual revision of both the epigenome and transcriptome. Recent studies have shown that the large-scale transcriptional changes observed during reprogramming also apply to long non-coding RNAs (lncRNAs), a type of traditionally neglected RNA species that are increasingly viewed as critical regulators of cellular function. Deeper understanding of lncRNAs in reprogramming may not only help to improve this process but also have implications for studying cell plasticity in other contexts, such as development, aging, and cancer. In this review, we summarize the current progress made in profiling and analyzing the role of lncRNAs in various phases of somatic cell reprogramming, with emphasis on the re-establishment of the pluripotency gene network and **X chromosome reactivation**.

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Introduction

High-throughput sequencing techniques have demonstrated that mammalian genomes are ubiquitously transcribed [1]. The transcription of large numbers of non-coding RNAs (ncRNAs) [2] might help to explain interspecies differences despite limited variations in the number and sequence of coding genes. ncRNAs are classified based on their length into small RNAs (< 200 nucleotides), *e.g.*, microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs), and long ncRNAs (lncRNAs, > 200 nucleotides) [3,4].

lncRNAs are typically transcribed by RNA polymerase II, although this process occurs with different modalities and origins. They include circular RNAs (circRNAs) [5], enhancer RNAs (eRNAs) [6], antisense transcripts [7], and long intergenic ncRNAs (lincRNAs) [8,9]. These lncRNAs recruit epigenetic regulators to chromatin and serve as scaffolds to stabilize protein complexes or as decoys for proteins and miRNAs [10–12]. These diverse regulatory modes are due to the complementary base pairing of lncRNAs with both DNA and other RNAs, the ability of lncRNAs to interact with proteins, and the localization of lncRNAs in different cellular compartments (*e.g.*, cytoplasm, nucleus, or mitochondrion) [13,14]. Interestingly, a large fraction of lncRNAs also associate with ribosomes and, paradoxically, some previously annotated lncRNAs produce small peptides with biological functions [15,16]. The latter raises relevant questions regarding the true nature of some lncRNAs, but the general significance of these findings requires further investigation.

Although the functional relevance of lncRNAs has not yet been systematically explored, some are known to regulate key aspects of normal and pathological cellular functions such as proliferation [17], metabolism [18,19], and epithelial cytoarchitecture [20]. The recent discovery that lncRNAs display cell-specific and development-specific expression patterns has also suggested a pivotal role of lncRNAs in cell fate determination [21–23]. In support of this idea, suppression of several embryonic stem cell (ESC)-specific lincRNAs influences pluripotency maintenance/exit and early lineage commitment [21]. Similarly, the heart-associated lncRNA *Braveheart* serves as a key player in the activation of the core vascular gene network during mouse cardiac cell fate specification [22]. In addition, lincRNA Yin Yang 1 (*linc-YY1*), which is highly conserved in humans, regulates myogenesis through dislodgement of the YY1/polycomb repressive complex on target promoters, resulting in the activation of muscle gene expression [23].

Here, we summarize the current knowledge of lncRNA profiling and functions in an extreme scenario of cell fate transition, somatic cell reprogramming.

lncRNAs are new players in somatic cell reprogramming

Mammalian somatic cells can be reprogrammed to an ESC state and this has revolutionized stem cell research [24,25]. Originally, the reprogramming factor cocktail contained SOX2, KLF4, OCT4 (encoded by *Pou5f1 or POU5F1* gene in mice or humans, respectively), and c-MYC (SKOM), but other combinations of exogenous factors are also effective [26,27]. More recently, mouse reprogramming has been achieved using only chemicals [28]. Remarkably, induced pluripotent stem cells (iPSCs) provide *a priori* unlimited number of individual-specific stem cells that can be used for *in vitro* disease modeling, drug screening, and potential cell-based therapies [29,30]. Although producing iPSCs from different cell sources and mammalian species is in general no longer an issue [31–34], the underlying mechanisms remain unclear and clarifying them is important for improving iPSC quality [35].

Reprogramming occurs through a stepwise process that involves reorganization of most cell functions, culminating in the reactivation of the pluripotency gene program [36,37]. This conversion is characterized by several roadblocks and checkpoints. For example, reprogramming cells must overcome the senescence/apoptosis barrier to acquire the ability to proliferate indefinitely [38,39], switch their metabolism from oxidative phosphorylation to glycolysis [40], and undergo a mesenchymal-to-epithelial transition (MET) [41,42]. By the end of these events, under standard conditions, only a small percentage of the original population activates the endogenous pluripotency gene circuitry.

Since the first demonstration of somatic cell reprogramming, multiple regulatory factors have been identified. Among these, miRNAs play essential roles in creating or removing reprogramming roadblocks [43,44]. For example, components of miRNA cluster 302–367 suppress *Tgfbr2* to neutralize the pro-mesenchymal effects of TGFβ cytokines secreted by somatic cells or present in serum, facilitating the MET [45,46]. miRNA cluster 302–367 also targets genes encoding chromatin regulators (*e.g.*, *BAF170*) that prevent the activation of pluripotency genes in the late phase of reprogramming [46]. Conceivably, lncRNAs, which are present in much higher numbers than miRNAs and are in principle more versatile players in cell regulation, could be important regulators of reprogramming too. In this regard, lncRNAs also experience phase-dependent changes during reprogramming, and their regulation shares the same epigenetic mechanisms that drive mRNA changes in reprogramming [47,48]. These findings suggest a coordinated role between protein-coding and ncRNAs in reorganizing cellular functions. Studying lncRNAs is, therefore, important to understand reprogramming as a whole (**Table 1**). Such knowledge may also contribute to clarifying the role of lncRNAs in other contexts such as development, aging, and cancer.

p53-regulated lncRNAs in reprogramming

Proliferation facilitates the appearance of stochastic events needed for directing chromatin reorganization into a pluripotent state during reprogramming [38]. However, somatic cells have a limited life span/proliferation capacity, and reprogramming is a stressful process involving activation of senescence/-cell death pathways. Accordingly, suppressing p53, $p16^{Ink4a}$, or $p19^{Arf}$ enhances reprogramming efficiency by accelerating proliferation and/or reducing apoptosis [39,49,50].

Interestingly, several p53-regulated lncRNAs have strong impacts on reprogramming efficiency (**Figure 1**). For instance, expression of *lincRNA-RoR* (regulator of reprogramming) [51] and *lincRNA-p21* [52,53] is induced by p53 [54,55], which can influence reprogramming in positive and negative manner, respectively. Similarly, expression of p53-repressed

Table 1 lncRNAs and their functions in somatic cell reprogramming

Name	Function or mechanism	Refs.
lincRNA-RoR	Inhibiting p53 translation and acting as a miR-145 sponge to promote reprogramming efficiency	[51,58]
lincRNA-p21	Activating *p21* expression and inhibiting expression of pluripotency genes by recruiting SETDB1 or DNMT1 to derail reprogramming	[52,53]
LNCPRESS1	Acting as a decoy for histone deacetylase SIRT6	[56]
lincRNA-1463	Inhibiting pre-iPSC to iPSC conversion	[52]
lincRNA-1526	Inhibiting pre-iPSC to iPSC conversion	[52]
lincRNA-1307	Promoting pre-iPSC to iPSC conversion	[52]
Zeb2-NAT	Maintaining *Zeb2* expression and inhibiting the MET	[66]
Gas5	Maintaining expression of *Tet1* and pluripotency genes, and protecting *NODAL* mRNA from microRNA-mediated degradation	[70,71]
Snhg14	Binding to the promoter of *Sox2* to enhance its expression, promoting reprogramming, and maintaining pluripotency in iPSCs	[75]
Peblr20	Promoting reprogramming by activating endogenous *Pou5f1* in a *trans* manner, and recruiting TET2 to the enhancer region of *Pou5f1* to activate eRNAs	[76]
Ladr49 and *Ladr83*	Inhibiting expression of muscle-related genes	[47]
Ladr86 and *Ladr91*	Promoting expression of mitochondria-associated genes and blocking the metabolic switch	[47]
Xist	Impairing XCR, promoting MET, and inhibiting pre-iPSC to iPSC conversion	[87,93]

Note: iPSC, induced pluripotent stem cell; XCR, X chromosome reactivation; MET, mesenchymal-to-epithelial transition; lncRNA, long non-coding RNA; *lincRNA-RoR*, long intergenic non-coding RNA-regulator of reprogramming; SETDB1, SET domain bifurcated histone lysine methyltransferase 1; DNMT1, DNA methyltransferase 1; *LNCPRESS1*, lncRNA p53-regulated and ESC specific 1; SIRT6, sirtuin 6; *Zeb2-NAT*, zinc finger E-box binding homeobox 2-natural antisense transcript; *Gas5*, growth arrest specific 5; TET2, ten-eleven translocation 2; *NODAL*, nodal growth differentiation factor; *Snhg14*, *Sox2* promoter-interacting lncRNA 14 (also known as *Spilr14*); *Sox2*, SRY-box transcription factor 2; *Peblr20*, *Pou5f1* enhancer-binding lncRNA 20; eRNA, enhancer RNA; *Ladr49,* long non-coding RNA activated during reprogramming 49; *Xist*, X-inactive specific transcript.

LNCPRESS1 (lncRNA p53-regulated and ESC-associated 1) is robustly induced during reprogramming and activates the pluripotency network [56]. Given these circumstances, it would be useful to systematically profile lncRNAs during reprogramming in the presence or absence of p53 or other pro-senescence regulators.

lincRNA-RoR

lincRNA-RoR (2603 nucleotides) is located on chromosome 18q21.31. Its expression is induced by p53 [54] and facilitates human reprogramming by suppressing the p53-mediated transcriptional response to oxidative stress and DNA damage [51]. However, overexpression or knockdown of *lincRNA-RoR* does not influence cell growth at the early stages of reprogramming. In cancer cells, *lincRNA-RoR* suppresses p53 translation through heterogeneous nuclear ribonucleoprotein I (hnRNP I), a classical RNA-binding protein (RBP) involved in splicing [54]. In this regard, a fraction of hnRNP I is localized in the cytoplasm, where it promotes the translation of p53 through binding to internal ribosome entry sites. Apart from suppressing the p53 pathway, *lincRNA-RoR* sequesters pro-differentiation miRNAs, including miR-145, to maintain the expression of the core pluripotency transcription factors OCT4, SOX2, and NANOG in human ESCs [57,58]. Although these two latter mechanisms have not yet been tested in reprogramming, it is likely that they also contribute to the effects of *lincRNA-RoR* in this process. Notably, *lincRNA-RoR* is more highly expressed in human iPSCs than in ESCs [51], which may be a consequence of a selective advantage conferred during reprogramming.

lincRNA-p21

lincRNA-p21 (3121 nucleotides, located on chromosome 6p21.2) resides 5 kb upstream of *p21*. It was originally discovered as an executioner of the p53-mediated apoptotic response [55]. Expression of *lincRNA-p21* is induced by p53 and impairs mouse reprogramming, but different *cis* or *trans* modes of action have been proposed [52,53]. The *cis*-acting model is based on the observation that conditional excision of the *lincRNA-p21* promoter and first exon reduces the expression of *p21* mRNA, resulting in enhanced proliferation and reprogramming efficiency based on alkaline phosphatase activity (an early marker of reprogramming) [53]. However, it is important to note that removal of the *lincRNA-p21* locus leads to the loss of multiple enhancers that control the transcription of nearby genes, including *p21* itself, independently of *lincRNA-p21* [59]. In fact, removal of the *lincRNA-p21* locus alters the expression of nearby genes even in tissues with no detectable *lincRNA-p21* transcript. Conversely, the *trans*-acting model of *lincRNA-p21* in reprogramming proposes that *lincRNA-p21* impairs reprogramming independently of proliferation by binding to pluripotency loci (*e.g.*, *Nanog*) and blocking their reactivation [52]. In this model, *lincRNA-p21* represses pluripotency loci by recruiting the histone 3 lysine 9 (H3K9) methyltransferase SETDB1 or the maintenance DNA methyltransferase DNMT1. Binding of *lincRNA-p21* to these epigenetic regulators is mediated by the classical RBP hnRNP-K. Hence, suppressing hnRNP-K also results in enhanced reprogramming efficiency, although it is unlikely that it acts exclusively through *lincRNA-p21*. The idea that expression of p53-induced lncRNAs such as *lincRNA-p21* prevents reprogram-

ming independent of proliferation or apoptosis is attractive, as this may have implications for the control of cell fate when stem cells are under stress or in aging and in p53-negative cancers.

In a screen for lncRNAs modulating the conversion of pre-iPSCs to iPSCs, *lincRNA-p21* was identified as a negative regulator of reprogramming [52]. Pre-iPSCs are stable but incompletely reprogrammed clones, and their conversion to iPSCs is commonly used as a proxy for the late phase of reprogramming [60]. The same screen also identified *lincRNA-1463* and *lincRNA-1526* as negative regulators of the conversion of pre-iPSCs to iPSCs. However, it is unclear whether expression of these two lncRNAs is also regulated by p53.

LNCPRESS1

Another screening study conducted on differentiating human ESCs led to the discovery of *LNCPRESS1* (832 nucleotides, located on chromosome 7q22.1). *LNCPRESS1* is a p53-repressed lncRNA that positively regulates the pluripotency gene network in human ESCs [56]. *LNCPRESS1* acts as a decoy for the histone deacetylase SIRT6, thereby enriching the activating H3K56/K9 acetylation marks at pluripotency loci. Interestingly, expression of *LNCPRESS1* is strongly induced during reprogramming [56], suggesting a potential role of *LNCPRESS1* in improving reprogramming efficiency.

Other lncRNAs regulating reprogramming

In addition to lncRNAs regulated by p53, several other lncRNAs have been identified which can regulate somatic cell reprogramming through MET, metabolic switch, or reactivation of the pluripotency gene network (**Figure 1**). ESCs are epithelial-like, therefore, reprogramming of mesenchymal-like cells such as fibroblasts to iPSCs unavoidably involves the acquisition of an epithelial phenotype. This occurs in the early phase of reprogramming through MET [41,42,61]. Since pluripotent cells and somatic cells have different metabolic profiles, reprogramming, logically, also requires metabolic remodeling. iPSCs exhibit a metabolic shift from oxidative phosphorylation to glycolysis, which is associated with a decreased number and complexity of mitochondria. Importantly, blocking MET or the metabolic switch derails reprogramming [41,42,62], but not every reprogramming

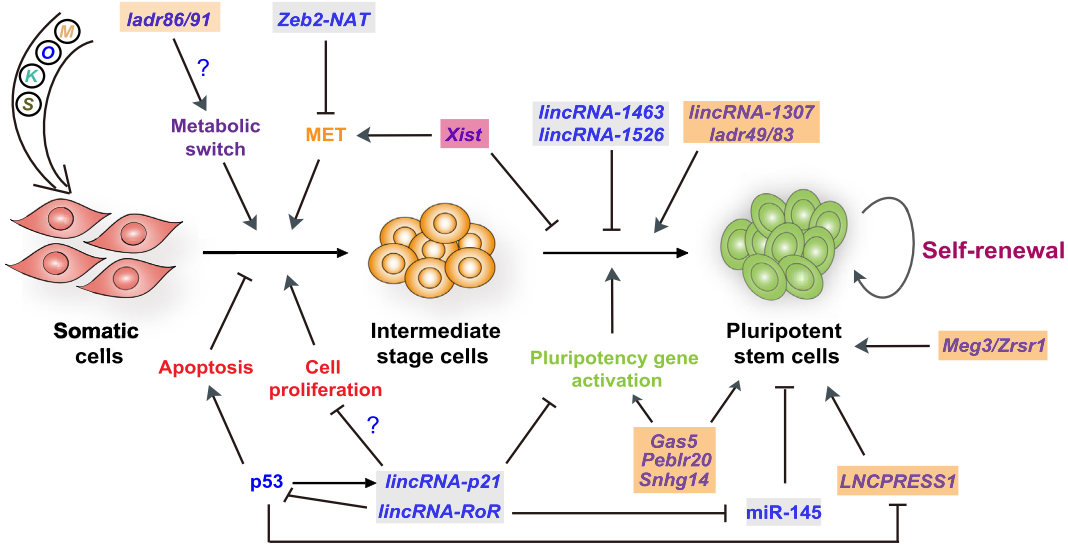

Figure 1 lncRNAs regulating somatic cell reprogramming

Schematic depiction of lncRNAs regulating different stages of mouse or human reprogramming with exogenous factors. In the early phase of conversion from somatic cells to reprogramming intermediates, *Xist* is involved in the acquisition of an epithelial phenotype and *Iadr86/91* is involved in the metabolic switch from an aerobic to an anaerobic production of energy [47]. *lincRNA-p21* is involved in the early as well as the late phases (reactivation of the pluripotency gene network) of reprogramming through suppression of cell proliferation and pluripotency gene activation, respectively. Similarly, *lincRNA-RoR* regulates the early phase of reprograming by suppressing p53 (a negative regulator of cell survival), and the late phase by acting as a sponge for miR-145, which targets multiple pluripotency gene transcripts. *lincRNA-1463* and *lincRNA-1526* negatively regulate the activation of pluripotency genes in reprogramming, whereas *lincRNA-1307* and *Iadr49/83* are positive regulators. *Gas5*, *Peblr20*, *Snhg14*, and *LNCPRESS1* positively regulate the activation of pluripotency genes in reprogramming and/or in ESCs. Expression of the imprinted lncRNAs *Meg3/Zrsr1* is related to the acquisition of full pluripotency in mouse iPSCs. Repressive ncRNAs are marked in a gray box, promotive ncRNAs in a yellow box, and ncRNAs with a dual function in a red box. MET, mesenchymal-to-epithelial transition; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; lncRNA, long non-coding RNA; *Xist*, X-inactive specific transcript; *Iadr86*, lncRNA activated during reprogramming 86; *lincRNA-ROR*, long intergenic non-coding RNA-regulator of reprogramming; *LNCPRESS1*, lncRNA p53-regulated and ESC associated 1; *Meg3*, maternally expressed 3; *Zrsr1*, lncRNA zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1; *Zeb2-NAT*, zinc finger E-box binding homeobox 2-natural antisense transcript; *Gas5*, growth arrest specific 5; *Peblr20*, *Pou5f1* enhancer-binding lncRNA 20; *Snhg14*, also known as *Spilr14*, *Sox2* promoter-interacting lncRNA 14; S, SRY-box transcription factor 2 or SOX2; K, Kruppel like factor 4 or KLF4; O, OCT4; M, c-MYC.

intermediate that successfully passes through these checkpoints achieves full activation of the pluripotency gene network. Likewise, even when somatic cells have been successfully reprogrammed to iPSCs, their DNA methylation patterns often do not faithfully reflect those of ESCs. For example, iPSCs can show aberrant DNA methylation of imprinted regions, which alters the expression of lncRNAs encoded by those regions [63–65].

Zeb2-NAT

Zinc finger E-box binding homeobox 2-natural antisense transcript (*Zeb2-NAT*, 430 nucleotides, located on chromosome 2q22.3) is a natural antisense transcript for the transcription factor ZEB2 [66]. ZEB2 is a master regulator of the epithelial-to-mesenchymal transition (EMT) that represses the expression of many epithelial genes [67]. *Zeb2-NAT* is involved in maintaining *Zeb2* expression by preventing the splicing of the *Zeb2* 5′-untranslated region (5′-UTR) [66]. Interestingly, *Zeb2-NAT* is highly expressed in fibroblasts from aged mice [68], which are known to be less amenable to reprogramming [69]. The reprogramming efficiency of these aged fibroblasts is enhanced when expression of *Zeb2-NAT* is reduced. Given that aging promotes the accumulation of DNA damage, thereby activating p53, it is plausible that expression of *Zeb2-NAT* is also induced by p53 in aged fibroblasts and during reprogramming. However, this speculation has not yet been tested.

Gas5

Expression of the lncRNA growth arrest specific 5 (*Gas5*, 656 nucleotides, located on chromosome 1q25.1) is controlled by pluripotency transcription factors. *Gas5* plays a pivotal role in reprogramming and self-renewal of mouse ESCs by maintaining the expression of genes encoding key pluripotency factors and ten-eleven translocation 1 (*Tet1*) [70]. The effect of *Gas5* on *Tet1* expression suggests that *Gas5* regulates active DNA demethylation in reprogramming. Its human ortholog, *GAS5*, also controls human ESC pluripotency by protecting *NODAL* mRNA from miRNA-mediated degradation [71], a mechanism that may also participate in human reprogramming. *GAS5* is also a well-known regulator of cell proliferation and apoptosis in various cell contexts, including breast cancer, lung cancer, and differentiating mouse ESCs [70,72,73].

Ladr lncRNAs

Single-cell transcriptomic analysis of mouse reprogramming has unveiled numerous lncRNAs that are activated or repressed during this process [47]. Among them, knockdown of two lncRNAs activated during reprogramming 49 and 83 (*Ladr49* and *Ladr83*), showed modest effects on reprogramming efficiency, but led to the upregulation of muscle-related genes in reprogramming intermediate cells. Interestingly, these two lncRNAs have been previously shown to physically associate with polycomb repressive complex 2 (PRC2) [21,74]. On the other hand, depletion of *Ladr86* and *Ladr91*, which show upregulated expression in reprogramming, represses mitochondria-associated genes, suggesting a role in the metabolic switch during reprogramming.

Promoter/enhancer-interacting lncRNAs

A recently devised approach chromatin-RNA *in situ* reverse-transcription sequencing (CRIST-seq) took advantage of the specificity of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system for DNA to profile pluripotency-specific lncRNAs that interact with the promoter of the core pluripotency genes *Sox2* and *Pou5f1* [75]. Among the identified lncRNAs interacting with the *Sox2* promoter, 59 of them were differentially expressed in reprogramming. Notably, overexpression of one of these differentially expressed lncRNAs, *Sox2* promoter-interacting lncRNA 14 (*Spilr14*, also known as *Snhg14*), led to significant enhancement of reprogramming efficiency, whereas its knockdown caused loss of pluripotency in iPSCs. Another lncRNA, *Pou5f1* enhancer-binding lncRNA 20 (*Peblr20*), was identified utilizing a strategy combining RNA reverse transcription-associated capture sequencing (RAT-seq) and RNA sequencing [76]. *Peblr20* is expressed at higher levels in iPSCs than in mouse embryonic fibroblasts and promotes reprogramming by activating endogenous *Pou5f1* in *trans* through the recruitment of TET2 to the enhancer region, which enhances the expression of eRNAs.

Imprinted lncRNAs

Maternally expressed 3 (*Meg3*, also known as *Gtl2*) is localized at the imprinted *Dlk1–Dio3* gene cluster on mouse chromosome 12qF1, which also encodes numerous miRNAs [77,78]. ncRNAs harbored in this region are maternally expressed in mammals. Interestingly, they are strongly repressed in most mouse iPSCs compared to ESCs, which is responsible for the failure to support the development of all-iPSC mice [63,79]. This inability can be reversed through reactivation of the imprinted *Dlk1–Dio3* locus in reprogramming using the histone deacetylase inhibitor valproic acid or ascorbic acid (vitamin C, Vc) [63,80,81]. Vc facilitates the conservation of imprinting at this locus by interfering with reprogramming factor-induced loss of H3K4 methylation, which prevents the recruitment of DNA methyltransferase 3A (DNMT3A). Importantly, *MEG3*, which is encoded in the *DLK1–DIO3* locus, is also frequently silenced in human iPSCs [82]. Similarly, hypomethylation of the maternally expressed imprinted lncRNA zinc finger (CCCH type), RNA binding motif, and serine/arginine rich 1 (*Zrsr1*) is associated with reduced pluripotency in mouse iPSCs, which could not be rescued by treating reprogramming cells with Vc or PD0325901 and CHIR99021 (inhibitors of MEK1/2 and GSK3, respectively) [64]. It remains to be clarified how these imprinted lncRNAs regulate the reprogramming process.

X chromosome reactivation during reprogramming

In the late stage of reprogramming, another important process involving lncRNAs is X chromosome reactivation (XCR) (**Figure 2**). During the development of mice and other mammals, one of the two X chromosomes in females is randomly silenced in somatic cells shortly after implantation to maintain the dosage equivalence between the sexes [83,84]. X chromosome inactivation (XCI) is initiated by the

expression of X-inactive specific transcript (*Xist*), a 17,918-nucleotide lncRNA localized in the X chromosome inactivation center (XIC) [85]. *Xist* coats the X chromosome, which gradually removes RNA polymerase II and active histone marks such as H3K4 trimethylation. This is followed by a sequential gain of diverse repressive marks including H3K27 trimethylation (H3K27me3), macroH2A.1 histone (macroH2A), and DNA methylation on the inactivated X chromosome (Xi). Consequently, Xi is silenced throughout life with remarkable stability. Despite possessing a stable nature, Xi can be reactivated in specific contexts such as primordial germ cell differentiation, somatic cell nuclear transfer (SCNT), and somatic cell reprogramming [86]. Due to its ease and reproducibility, reprogramming provides an unprecedented tool for characterizing the events involved in XCR [87,88].

A relevant question in the field of reprogramming is the order of the epigenetic events leading to XCR. High-resolution single-cell time course analyses of reprogramming using immunofluorescence and RNA fluorescence *in situ* hybridization (FISH) [87,89] demonstrate that XCR occurs in the following order: (1) recruitment of H3K27 methyltransferase EZH2 to Xi (Xi^{EZH2+}) in E-cadherin (CDH1) positive ($CDH1^{+}$) cells, (2) activation of NANOG in Xi^{EZH2+} cells, (3) loss of EZH2, H3K27me3, *Xist* coating, and macroH2A1 on Xi in $NANOG^{+}$ cells, and (4) removal of DNA methylation on Xi and activation of the transcribed antisense to *Xist* (*Tsix*). Therefore, except for DNA demethylation, the events of XCR in reprogramming follow the inverse order of the events of XCI in development. Notably, the removal of DNA methylation during XCR in reprogramming is TET-independent and hence passive, and the expression of *Tsix* is paradoxically dispensable.

Conspicuously, despite the initiation of XCR in $NANOG^{+}$ cells, full reactivation of the core pluripotency circuitry precedes XCR in reprogramming. Accordingly, XCR serves as one of the crucial standards for *bona fide* mouse iPSC identification [90]. This is consistent with the observation that the pluripotency factor PRDM14 represses *Xist* expression [91]. Although ectopic expression of *Xist* impairs XCR in reprogramming, its depletion does not affect XCR or reprogramming efficiency [87]. This is inconsistent with the observations in SCNT, the efficiency of which is greatly improved following *Xist* elimination [92]. A possible explanation could be that *Xist* plays opposite roles at different stages of somatic cell reprogramming, as SCNT has no obvious phases. In support of this idea, *Xist* depletion impairs the MET in the early phase of reprogramming but significantly improves the conversion of pre-iPSCs to iPSCs [93]. Interestingly, the reprogramming booster Vc [81] promotes XCR in reprogramming by preventing the relocalization of macroH2A onto Xi [93]. This is likely mediated by the boosting effect of Vc on H3K27me3 demethylases [94,95]. It remains to be determined whether Vc also acts through unrelated mechanisms, for example by decreasing the levels of N^6-methyladenosine (m^6A) deposition on *Xist*, as Vc is also a cofactor for another two dioxygenases, the alpha-ketoglutarate-dependent dioxygenase fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5), which are responsible for erasing this epitranscriptomic mark [96–98].

Notably, the aforementioned mechanisms have been investigated in mouse cell reprogramming, but it is unclear whether the same principles apply to human cells. In this regard, XCR is either absent or unstable in female donors when human iPSCs are generated using standard culture conditions [99], but is present in reprogramming to naïve pluripotency [100]. Further studies are needed to understand the differences and similarities between XCR in mouse and human cell reprogramming.

Perspectives

It is becoming increasingly evident that lncRNAs are critical players in cell fate regulation. Few lncRNAs, mostly related to p53 or cell senescence, have been well studied thus far, but the repertoire of lncRNAs that regulate pluripotency/reprogramming is likely large. Systematic profiling is needed

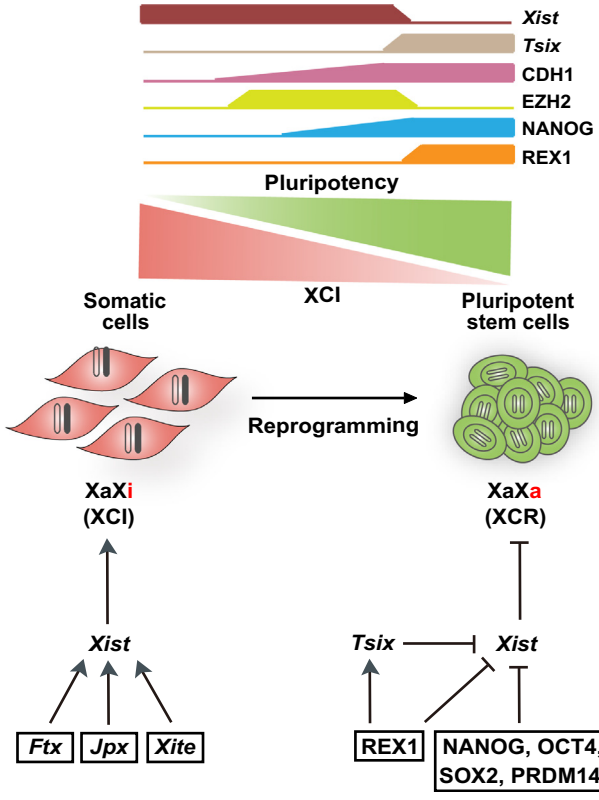

Figure 2 X chromosome reactivation in somatic cell reprogramming

Both X chromosomes are active in mammalian female blastocysts and inactivation of one of them is an epigenetic hallmark of embryonic development. *Xist* is transcribed from XIC and is the main responsible for carrying out XCI during development. The Xi is reactivated during reprogramming of somatic cells to iPSCs through repression of *Xist*. In ESCs and iPSCs, *Xist* is regulated by different pluripotency factors including NANOG, OCT4, PRDM14, and REX1, along with *Tsix*. XCR, X chromosome reactivation; XCI, X chromosome inactivation; XIC, X-chromosome inactivation center; Xa, active X chromosome; Xi, inactive X chromosome; *Tsix*, *Xist* antisense RNA; CDH1, cadherin 1 (E-cadherin); EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; NANOG, nanog homeobox; *Ftx*, five prime to *Xist*; *Jpx*, just proximal to *Xist*; *Xite*, X-inactivation intergenic transcription element; PRDM14, PR/SET domain 14.

for functional characterization of these lncRNAs. This could be facilitated by developing highly efficient/deterministic reprogramming systems [101], as low-efficiency protocols are prone to cell heterogeneity and the cell reprogramming kinetics are asynchronous, which complicates the interpretations. In addition, specialized high-throughput RNA sequencing approaches such as global nuclear run-on sequencing (GRO-seq) and sequencing with RNase R treatment are necessary to enrich for certain lncRNAs such as eRNAs and circRNAs [102,103], respectively, since they are rarely detected using conventional RNA sequencing methodologies. In this regard, several circRNAs exhibit human iPSC/ESC-specific expression [104,105]. Among them, *circBIRC6* and *circCORO1C* positively regulate pluripotency maintenance and reprogramming [104]. *circBIRC6* acts as a sponge for differentiation-mediated miRNAs, whereas the mechanism underlying *circCORO1C* regulation is unknown. An important consideration is that functional lncRNA screens with short hairpin RNAs (shRNAs) or small interfering RNAs (siRNAs) have inevitable limitations because many lncRNAs are localized in the nucleus, where shRNAs/siRNAs cannot effectively silence transcripts. As a solution, CRISPR/Cas9-based screening systems assisted by comprehensive computer prediction algorithms could be used to increase on-target efficiency and minimize off-target effects. This approach requires eliminating the whole DNA fragment (rather than introducing a frame shift for coding genes), potentially in the promoter region, to suppress expression/functionality of lncRNAs without affecting the nearby genes [106]. CRISPR/Cas13 and CRISPR interference are potential alternatives, as they can be used to edit RNA or repress gene expression without altering the genome [107,108]. Collectively, the characterization of lncRNAs in reprogramming will not only help to uncover new layers of regulation for the diverse pathways modulating reprogramming but may also have implications in other types of cell fate transitions.

Competing interests

The authors have declared no competing interests.

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