A study on X chromosome reactivation using iXist

Lai, Lan Tian

2017

Lai, L. T. (2017). A study on X chromosome reactivation using iXist. Master's thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/72782>

<https://doi.org/10.32657/10356/72782>

A Study on X Chromosome Reactivation Using iXist

LAI LAN TIAN

School of Biological Sciences

A thesis submitted to the Nanyang Technological University in
fulfilment of the requirement for the degree of Master of
Science

2017

ACKNOWLEDGEMENTS

I thank my supervisor, Dr. Zhang Li-Feng for his patience, advice and guidance. I would also like to thank my ex-colleagues, Rosi Chelliah, for her work in establishing the iXist cell line that was used in my project. Besides, I appreciate the helps and discussions I received from my colleagues, Norbert Ha, Dr. Hong Ru, Zhen Yashu, Mehnaz Huq, Siti Nandirah and Dr. Tani Agarwal. Last but not least, I would like to thank Vanessa Seet for her help during her FYP in the preliminary screening for candidates.

TABLE OF CONTENTS

Acknowledgements	i
Table of Contents	ii
List of Figures	iv
Abbreviations	v
Abstract	vii
1. Introduction	1
2. Materials and Methods	
2.1 Plasmids	6
2.2 Cell Culture	6
2.3 Cell Lines	7
2.4 Induction and differentiation cell surviving assay	7
2.4 Induction cell surviving assay	8
2.5 Induction cell surviving assay with AP staining	8
2.6 Cytospin and immuno-RNA FISH	9
2.7 Reverse transcription PCR and qPCR	10
2.8 Microscopy and image collection	11
2.9 Image Analysis	11
2.10 Statistical Analysis	12

3. Results	
3.1 Establishing a system for XCR study	13
3.2 Knock-down of HAT candidates leads to more cell death caused by induced XCI in undifferentiated ES cells	17
3.3 iXist excludes Kat8 and H4K16ac more efficiently in differentiating cells	22
4. Discussion	25
5. Conclusion	31
6. References	32
7. Author's Publications	38

LISR OF FIGURES

Figure 1 XCI and XCR events during embryonic development	2
Figure 2 Design of iXist	14
Figure 3 Experimental design for XCR study using iXist	16
Figure 4 Preliminary screening for HATs candidate as XCR players	18
Figure 5 Expression profile of <i>Kat8</i> and <i>Msl2</i> in the knock-down cell lines	19
Figure 6 Cell survival assessment in the knock-down cell lines using AP staining	20
Figure 7 Pluripotency expression profile of the iXist shRNA <i>Kat8</i> or shRNA <i>Msl2</i> knock-down cell lines	21
Figure 8 Comparison of the morphological changes between iXist and iXist carrying shRNA against <i>Kat8</i> or <i>Msl2</i>	22
Figure 9 Immuno-RNA FISH targeting <i>Kat8</i> or H4K16ac followed by <i>Xist</i> cloud detection in iXist	24

ABBREVIATIONS

2i	2 inhibitors (CHIR99021 and PD0325901)
AA	Ascorbic acid
BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
Dox	Doxycycline
ESC	Embryonic stem cell
FBS	Fetal Bovine Serum
FISH	Fluorescence in situ hybridization
H2AK119ub	Histone 2 Lysine 119 ubiquitination
H3K27me3	Histone 3 lysine 27 trimethylation
H4K16ac	Histone 4 Lysine 16 acetylation
HDAC3	Histone deacetylase 3
Hr(s)	Hour(s)
iPSC	Induced pluripotent stem cell
LIF	Leukemia inhibitory factors
lncRNA	Long non-coding RNA
MOF/Kat8	Males absent on the first/Lysine acetyltransferases 8
MSL	Male Specific Lethal
NEO	neomycin-resistance gene
OSKM	Oct4, Sox2, Klf4 and Myc
PGC	Primordial germ cell
Pol II	RNA Polymerase II

PRC2	Polycomb repressive complex 2
rtTA	Reverse tetracycline transactivator
RT	Room temperature
TRE	Tetracycline response element
XCI	X chromosome inactivation
XCR	X chromosome reactivation
Xa	Active X
Xi	Inactive X
<i>Xic</i>	X inactivation center
<i>Xist</i>	X inactive specific transcript

ABSTRACT

Female mammals inactivate one of the two X chromosomes in each somatic cell in order to balance the X-linked gene dosage between females and males (X chromosome inactivation, XCI). This process is mediated by non-coding RNA X inactive specific transcript (*Xist*). Here, I show that, when embryonic stem (ES) cells were cultured under undifferentiated condition, inducible *Xist* was sufficient to silence genes *in vitro*. Furthermore, the induced XCI was counteracted by the endogenous capability of X chromosome reactivation (XCR) in the pluripotent ES cells. Thus, perturbing XCR players should tip the balance towards stronger gene silencing effects. Using this experimental system, we show that shRNA knock-down of histone acetyltransferase Kat8 and its associated protein from Male Specific Lethal (MSL) complex, Msl2, significantly enhanced the gene silencing effect of induced XCI in undifferentiated ES cells. Interestingly, Kat8 and Msl2 are involved in *Drosophila* dosage compensation by up-regulating X-linked gene expression from the single X chromosome in males. Through immuno-RNA FISH, we also show that induced XCI caused Kat8 exclusion from the X chromosome territory. Moreover, the exclusion also occurred to Histone 4 Lysine 16 hypoacetylation (H4K16ac), the histone modification associated to Kat8 enzyme activity. Taken together, our results show that Kat8 and Msl2 are involved in mammalian XCR.

INTRODUCTION

Eutherian mammals, such as mice or human, have evolved over the course of evolution to have different number of X chromosomes between the two sexes. This bias is however balanced by epigenetically inactivating one of the X chromosomes in female cells through a process called X chromosome inactivation (XCI) (Lyon, 1961).

Since XCI was first proposed in 1961 (Lyon, 1961), the field has been much advanced (Gendrel and Heard, 2014; Lessing et al., 2013). There are two rounds of XCI and X chromosome reactivation (XCR) during mouse embryogenesis (Huynh and Lee, 2003; Okamoto et al., 2004) (Fig 1). First wave of XCI takes place at 4-cell stage. During this process, paternal X is always inactivated due to the genetic imprints inherited from the parents (Kay et al., 1994). Therefore, this form of XCI is called imprinted XCI. At the late blastocyst stage between embryonic day 3.5 (E3.5) and E4.5, the epiblast cell lineage, which eventually forms the embryo proper, undergoes paternal XCR, whereas the cell lineages, which eventually form the extra-embryonic tissues, continuously maintain the imprinted XCI (Mak et al., 2004; Okamoto et al., 2004). Shortly after implantation between E5.5 and E6.5, a second wave of XCI occurs in the epiblast cell lineage (Rastan, 1982; Takagi et al., 1982). During this process, one X is randomly silenced in each cell. Once the random XCI is established, the XCI status is clonally maintained in all progenies. The second wave of XCR occurs between E8.5 and E12.5 in the primordial germ cells (PGCs) that eventually become the germline cells (Chuva de Sousa Lopes et al., 2008; Sugimoto and Abe, 2007).

Figure 1. XCI and XCR events during embryonic development.

First wave of XCI takes place at 4-cell stage and the imprinted Xi are reactivated at blastocyst stage (first wave of XCR). Shortly after implantation between E5.5-6.5, second wave of XCI is initiated at the epiblast cell lineage. Second wave of XCR occurs in PGCs that eventually develop into oocytes. Abbreviations: XCI, X chromosome inactivation; XCR, X chromosome reactivation; PGC, primordial germ cell

In the mouse, a 18 kb long non-coding RNA (lncRNA) called *Xist* (X inactive specific transcript) (Borsani et al., 1991; Brockdorff et al., 1991), plays an important role in XCI. The *Xist* gene resides at a X-linked locus named X inactivation center (*Xic*) (Rastan, 1983; Rastan and Robertson, 1985). As its name suggests, *Xic* contains other regulators of XCI in addition to *Xist*. One of the important regulators is the antisense transcript of *Xist*, *Tsix*, which functions as *Xist* repressor (Debrand et al., 1999; Lee et al., 1999; Mise et al., 1999). Upon XCI initiation, *Tsix* on the future Xi is allele specifically downregulated, which, in consequence, leads to *Xist* upregulation. *Xist* transcripts then coat the X chromosome territory in *cis* and trigger XCI (Brockdorff et al., 1992). *Xist* RNA coating is essential for establishing XCI (Marahrens et al., 1997; Penny et al., 1996). The coating of *Xist* RNA along Xi can be visualized as a “cloud” signal (the *Xist* cloud) by RNA fluorescence in situ hybridization (FISH). Recent studies have begun to identify a few *Xist*-binding proteins which shed light on the mechanism of epigenetic silencing triggered by *Xist* (Chu et al., 2015; McHugh et al., 2015; Minajigi et al., 2015; Moindrot et al., 2015). For example, Spen/SHARP was identified to interact with the co-repressors to deacetylate X chromosome as well as exclude RNA polymerase II (Pol II) (McHugh et al., 2015). Subsequently, more chromatin-modifiers are recruited to establish multi-layer epigenetic modifications to achieve chromosome-wide gene silencing. Once the XCI is established, Xi displays various heterochromatin markers including enrichment of H3K27me3, H2AK119ub and macroH2A that can be visualized by immunofluorescence (Costanzi and Pehrson, 1998; Fang et al., 2004; de Napoles et al., 2004; Plath et al., 2003).

The random XCI process can be recapitulated *in vitro* by *in vitro* differentiation of mouse embryonic stem cells (ESCs). ESCs are derived from the inner cells mass of E3.5 to E4.5 blastocysts. Similar to the epiblast cell lineage, female mouse ESCs have two active X chromosomes (Xa). When cultured in medium containing inhibitors to ERK1/ERK2 and GSK3 β signaling (termed 2i + LIF conditions) (Nichols and Smith, 2009; Ying et al., 2008), ESCs are capable of indefinite self-renewal and maintain the potential to differentiate into different types of cells. Upon removal of 2i and LIF from the medium, the ESCs undergo *in vitro* differentiation and lose the pluripotency. Random XCI occurs during this process. Therefore, mouse ESCs is a useful tool for studying the process of XCI.

XCR, on the other hand, had been shown to be coupled with nuclear cloning (Eggan et al., 2000) and cell fusion (Ying et al., 2002) when pluripotency is artificially achieved. The milestone discovery of induced pluripotent stem cells (iPSCs) in mice (Takahashi and Yamanaka, 2006) and later in human cells (Park et al., 2008; Takahashi et al., 2007) not only grants regenerative medicine great promises, but also open a door for the XCR field. By forced expressing certain pluripotent factors such as Oct4, Sox2, Klf4 and c-Myc (OSKM), somatic cells can be dedifferentiated back to the naïve pluripotency state. It is no coincidence that the random XCI in somatic cells is reversed during this reprogramming process, showing XCR status is tightly linked to the pluripotency state of the cells (Maherali et al., 2007; Pasque and Plath, 2015). Therefore, XCR study has become increasingly important as studying XCR benefit the clinical application of iPSCs.

XCR, however, remains largely unknown as compared to XCI due to the absence of tools to recapitulate the two XCR events during embryogenesis *in vitro*

(Ohhata and Wutz, 2013). Although iPSC culture protocols are established, the process of iPSCs formation is a slow, stochastic and highly heterogeneous process (Hanna et al., 2009), making it a less favorable tool for XCR study. Nonetheless, recent study on single cell reveals that during the iPSCs reprogramming, several epigenetic events occur in the inverse order of XCI (Pasque et al., 2014). In fact, *Tsix* and *Prdm14*, two genes previously known to repress *Xist*, were confirmed to be involved in XCR (Payer et al., 2013). PRDM14 was shown to indirectly repress *Xist* by silencing the *Xist* activator RNF12. However, both *Tsix* and PRDM14 do not possess epigenetic enzyme activities. How does the Xi coated with multi layers of heterochromatin mark get reactivated remains largely mysterious. Thus, more effort is required to unearth other players and the detailed mechanism of XCR.

Understanding XCR could provide insights in the epigenetic reprogramming that is useful for the regenerative medicine. Xi reactivation could also be potential treatment for various X-linked diseases (Franco and Ballabio, 2006). Therefore, in this project, we aim to establish a ESC system that can be used to identify XCR players.

MATERIALS AND METHODS

Plasmids

shRNA Plasmid Construction using pSUPER RNAi system

Forward and reverse oligos targeting *Kat8* (5'-GTGATCCAGTCTCGAGTGA-3'), *Msl1* (5'-GTACCTTTCCACCACAGAAAT-3'), *Msl2* (5'-CCCAGTCTCTTAGCCATAATG-3'), *Msl3* (5'-GCTGCGTTCAAGAAAGGAAAT-3') and *Kat7* (5'-CCTCGAACTCCAACCGGAAAT-3') were designed following pSUPER RNAi system manual (OligoEngine). Oligos were annealed and ligated to the BglII linearized pSUPER vectors. Ligated products were transformed into DH5 α *E. coli* and individual colonies were picked and shRNA targeting sequences were confirmed by sequencing.

Cell Culture

ES medium contains 15% Fetal Bovine Serum (FBS) (Hyclone), DMEM (Gibco), penicillin streptomycin (Gibco), sodium bicarbonate, HEPES (Gibco), Glutamax (Gibco), β -mercaptoethanol (Sigma-Aldrich), nonessential amino acids (Gibco). ESCs were cultured in ES medium with 2 inhibitors, namely CHIR99021 (Stemgent) and PD0325901 (Stemgent), plus leukemia inhibitory factor (2i + LIF) as described (Ying et al., 2008). For differentiating culture condition used in the immuno-RNA FISH, ESCs were cultured in ES containing 50 μ g/mL ascorbic acid (AA) (Sigma-Aldrich).

Cell Lines

Ainv15, iXist and shRNA Knock-down Cell Lines

Ainv15 was a gift from Dr. Chen from Nankai University, China. Our former colleague, Rosi Chelliah, established iXist, originally named as Av3.1.12, by co-transfecting a plasmid containing full length *Xist*, tdTomato and a promoter-start codon together with pSALK-CRE, a CRE recombinase-expression plasmid, into Ainv15 cells. To knock down *Kat8*, *Msl1*, *Msl2*, *Msl3* and *Kat7* in iXist, iXist cells were transfected with shRNA plasmids targeting *Kat8*, *Msl1*, *Msl2*, *Msl3* and *Kat7* respectively using Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions. Selection was begun 48-hour post transfection using 2i + LIF medium with 300 µg/mL Hygromycin B (Gibco) and lasted up to two weeks. Surviving colonies were picked and expanded followed by quantitative real time PCR (qPCR) to confirm the knock-down level.

Induction and Differentiation Cell Surviving Assay

ESCs colonies were dissociated into single-cell suspension and 1.0×10^6 cells were plated in a bacteria petri dish in ES medium containing 50 µg/mL AA and 400 µg/mL G418 (Gibco) in the presence or absence of 2 µg/mL Dox (Clontech) starting from day 0 (D0). Embroid bodies (EBs) started to form and they were transferred to a new petri dish on the following day. Forty-eight hours later, EBs were transferred to a T75 flasks coated with 0.2% gelatin (Sigma-Aldrich). Medium was refreshed when necessarily thereafter. Cells were harvested and counted on D6 to assess the percentage of cells survived.

Induction Cell Surviving Assay

ESCs were cultured until 60- 70% confluency. After trypsinization, 1.0×10^5 cells were seeded into each well of the 6-well plate. In addition, 1.0×10^4 cells were seeded into each well of another 6-well plate as the control. The next day, on D0, culture medium was changed to 2i + LIF medium containing 2 $\mu\text{g/mL}$ Dox and 400 $\mu\text{g/mL}$ of G418. For the control plate, 2i + LIF medium containing 400 $\mu\text{g/mL}$ G418 without Dox was used. Medium was refreshed daily. On D6, the cells were harvested and counted to determine the number of surviving cells. Cells were stained with Trypan Blue and counted using the CountessTM Automated Cell Counter (Invitrogen).

For p300 inhibition, experimental design was similar except inhibitors for p300, 10 μM of L002 (Sigma) or 25 μM of C646 (Sigma) was added into the medium in addition to Dox and G418.

Induction Cell Surviving Assay with Alkaline Phosphatase (AP) Staining

ESC colonies were trypsinized and 10×10^3 cells were seeded into each well of the 12-well plate on D-1. On D0, medium was changed to 2i containing 300 $\mu\text{g/mL}$ G418 in the presence or absence of 1 $\mu\text{g/mL}$ Dox. Cells were fixed on D4 in 4% PFA for 10 min followed by AP staining (Vector Laboratories) following manufacturer's manual. The plates were then scanned at 1200 dpi (Epson) and individual wells were cropped out from the scanned images. The cropped images were then subjected to the CellProfiler (Carpenter et al., 2006) pipeline for analyzing the number of surviving colonies.

Cytospin and Immuno-RNA FISH

Cytospun slides were prepared and Immuno-RNA FISH was carried out as described previously (Lai et al., 2013). In brief, colonies were trypsinized and resuspended in PBS at 8×10^5 cells/mL. 100 μ L of cell suspension was loaded into each cytofunnel and were cytospun onto glass slide at 1000 rpm for 10 min using cytofunnels in a Shandon Cytospin 4 centrifuge (Fisher Scientific). Slides were then dried for 3 min followed by a wash in PBS for 5 min. Subsequently, slides were fixed in 4% PFA at RT for 5 min. Slides were subjected to permeabilization in PBS 0.5% Triton for 30 min before immunostainings were carried out.

Immunostainings were performed with antibodies diluted in PBS containing 0.2% Tween-20 and 0.5% Bovine Serum Albumin (BSA). Primary incubations were carried out at 4°C overnight. After incubation, slides were washed in PBS containing 0.2% Tween-20 for 5 min, three times, at RT. Secondary incubations were performed at RT for 1 hr followed by three times of PBS plus 0.2% Tween-20 washes at RT. Primary antibodies used were: rabbit anti-Kat8 (1:1500, Abcam, Cat# ab200660) and rabbit anti-H4K16ac (1:400, Abcam, Cat# ab109463). Goat anti-rabbit Alexa 488 (1:1000) (Abcam, Cat# ab150077) was used as secondary antibody.

Slides were post-fixed in 4% PFA for 10 min before RNA FISH. After fixation, slides were rinsed in PBS containing 0.2% Tween-20 for 3 times, 5 min each. Slides were then dehydrated through 80%, 90% and 100% ethanol sequentially for 2 min each and dried for 2 min at RT. Denatured probe was applied onto slides and hybridization was carried out at 42°C for 3 hr in a humid and dark environment. Afterwards, slides were washed in 50% formamide, 2 x SSC at 45°C

for three times, 5 min each followed by three washes in 2 x SSC at 45°C, 5 min each. Slides were mounted in Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories) and coverslips were sealed with nail polish. Mouse *Xist* DNA probe was generated using Sx9, a P1 DNA construct containing a 40 kb genomic fragment covering the mouse *Xic* region via nick translation (Roche) following manufacturer's manual.

Reverse Transcription PCR and qPCR

Total RNA was isolated using 1 mL of Trizol (Life Technologies) following the manufacturer's instructions. RNA was dissolved in nuclease-free water followed by DNase I (Promega) treatment at 37°C for 1 hour. Subsequently, phenol chloroform was added in 1:1 ratio for phenol chloroform extraction followed by precipitation using LiCl at a final concentration of 0.5M. One microgram of total RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-rad). For qPCR, 1 µL of cDNA from reverse transcription was used as template in each qPCR reaction for gene expression profiling following SsoAdvanced™ Universal SYBR® Green Supermix's protocol (Bio-Rad, USA) in the Bio-Rad CFX Connect Real Time PCR Detection System. Primers used were as follow: *Actb* (F:5'-ACTGCCGCATCCTCTTCCTC-3', R: 5'-CCGCTCGTTGCCAATAGTGA-3'); *Gapdh* (F:5'-CCAATGTGTCCGTCGTGGAT-3', R: 5'-TGCCTGCTTCACCACCTTCT-3'); *Nanog* (F:5'-TCGAATTCTGGGAACGCCTC-3', R: 5'-GTCTTCAGAGGAAGGGCGAG-3'); *Sox2* (F:5'-TTCGAGGAAAGGGTTCTTGCTG-3', R: 5'-TCCTTCCTTGTTTGTAACGGTCCT-3'); *Oct4* (F:5'-

TGTTCCCGTCACTGCTCTGG-3', R: 5'-TTGCCTTGGCTCACAGCATC-3');
Gata4 (F:5'-CCTGGAAGACACCCCAATCTC-3', R: 5'-
GGTAGTGTCCCGTCCCATC-3'); Fgf5 (F:5'-CCTTGCGACCCAGGAGCTTA-
3', R: 5'-CCGTCTGTGGTTTCTGTTGAGG-3'); Kat8 (F:5'-
AACCAGAAGTCACCGTGGAG-3', R: 5'-TCCTGGTCATTCACTCGAGAC-
3'); Msl2 (F:5'-CCCGGTGACTCTCTTTTGCT-3', R: 5'-
GCTTCCAAGTTTGGCTGCAA-3').

Microscopy and Image Collection

Phase contrast and fluorescence images of cells were collected on Eclipse Ti microscope (Nikon) equipped with non-oil (4x, NA0.13 and 20x, NA0.50) and oil objective lens (60x, NA1.40) and a digital camera Clara Series model C01 (Andor). Dichroic mirrors and filters in filter turrets were optimized for DAPI, Cy2/Alexa Fluor 488 and Cy3. Images were captured using NIS Elements AR imaging software (Nikon) and contrast of images was processed in Photoshop (Adobe).

Image Analysis

Image analysis was performed in NIS Elements AR imaging software. The exclusion pattern of Kat8 or H4K16ac was determined by examining the signal through all the collected focal planes in a z-series. Any ambiguous "exclusion", will be scored as no exclusion in order to avoid bias to increase the significance level of exclusion.

Statistical Analysis

Student's t-test was used in Microsoft Excel for statistical significance analysis.

Statistical significance level was set at p -value < 0.05 .

RESULTS

Establishing a System for XCR Study

Previous study reported that induction of a *Xist* cDNA transgene on the X chromosome in a male mouse ESC line was sufficient to cause X-linked gene silencing (Wutz and Jaenisch, 2000). A male mouse ESC line, named iXist, carrying an inducible full length *Xist* transgene on the X chromosome was generated from Ainv15 cells in our lab previously. Ainv15 cells harbors reverse tetracycline transactivator (rtTA) at the Rosa26 locus of Chromosome 6 and a tetracycline responsive element (TRE), loxP site and a neomycin gene without promoter and start codon at the *Hprt* locus of Chromosome X (Kyba et al., 2002). Using CRE recombinase, a transgene cassette including a full length *Xist*, a red fluorescence protein tdTomato and a PGK promoter with start codon was recombined onto the loxP site. This resulted in the iXist cell line which carries an inducible X-linked, full-length *Xist* transgene, a red fluorescence reporter as well as the resistance to neomycin (Fig 2).

Figure 2. Design of iXist.

Schematic drawings of the *rtTA* at the *Rosa26* locus on the chromosome 6 and TRE-loxP site at the *Hprt* locus on the X chromosome of Ainv15 cells. Cre-mediated recombination allows transgene to be specifically inserted downstream of a TRE, restoring the expression of G418-resistant gene (NEO). In iXist, a full length *Xist*, a tdTomato reporter were recombined into the *Hprt* locus.

Abbreviations: *rtTA*, reverse tetracycline transactivator; TRE, tetracycline response element; Δ NEO, truncated neomycin resistance gene; pPGK, phosphoglycerokinase promoter; ATG, methionine initiation codon

We observed that when iXist was induced by Dox under differentiating condition for 6 days, almost 100% of the cells were killed as a result of ectopic gene silencing of the X chromosome (Fig 3A). This result is significantly different from the induction of *Xist* under undifferentiated condition, where the cell death effect was clearly not as severe as the cell death effect in differentiating cells (Fig 3A). Earlier studies on cell fusion of ESCs and somatic cells show that somatic cell could be dedifferentiate back to pluripotent state and the process is coupled with XCR of the Xi from the somatic cell (Cowan et al., 2005; Tada et al., 2001). Besides, under undifferentiated condition, gene silencing caused by the inducible *Xist* cDNA is reversible upon removal of Dox from the ESCs culture medium (Wutz and Jaenisch, 2000). These observations and studies show that ESCs possess endogenous XCR capacity and therefore the induced XCI status in ECSs is a balanced outcome of the XCI and XCR counteracting forces (Fig 3B). Thus, we proposed that induction of XCI in ESCs could be used as a unique tool to study both XCI and XCR. With the established experimental system, in this project, we aim to identify novel XCR players. Perturbing the XCR forces should tip the balance leading to a stronger cell killing effect (Fig 3B).

Figure 3. Experimental design for XCR study using iXist.

(A) Cell survival rate of iXist cultured under undifferentiated condition versus differentiating condition in the presence of 2 $\mu\text{g/mL}$ of Dox for 6 days followed by cell count. Cell survival rate was plotted as percentage of the cell number of +Dox over -Dox samples. P-value was calculated using one-tailed paired Student's t-test (***, $p < 0.001$). Data are shown as mean $\pm$ SEM of biological replicates ($n = 3$). **(B)** Schematic depiction of the experimental design. Under differentiating condition, induction of *Xist* leads to close to 100% cell death. Under undifferentiated condition, however, only some cell death was observed when *Xist* is induced as an outcome of the counteracting forces between XCI and XCR. Therefore, knocking down of XCR players should tip the balance towards XCI and lead to more cell death.

Spen/SHARP, an *Xist*-binding protein was shown to recruit SMRT and in turn activate histone deacetylase 3 (HDAC3) (McHugh et al., 2015). This had led to us to hypothesize that histone acetyltransferases (HATs) are involved in XCR. We shortlisted a few HATs which are expressed in ESCs to examine their role in XCR. One of the candidates, Males absent on the first/Lysine acetyltransferases 8 (MOF/Kat8) is known to be the key member of the Male Specific Lethal (MSL) complex that is involved in the X chromosome dosage compensation in *Drosophila* (Kelley et al., 1995; Morales et al., 2005; Scott et al., 2000). Therefore, three Kat8 associated proteins from the MSL complex were also included in this study.

Knock-down of HAT Candidates Leads to More Cell Death Caused by Induced XCI in Undifferentiated ES cells

To test whether the candidate HATs are involved in XCR, we inhibited or knocked down HATs in iXist cells in the presence of Dox. Due to the balance of XCI and XCR counteracting forces, knocking down of XCR players should tip the balance towards XCI therefore leading to more cell death. Inhibition of p300 using either L002 or C646 at high concentration did not lead to more cell death when compared to the control iXist which was treated with only DMSO (Fig 4A). For Kat8 and Kat7, we established stable knock-down cell lines using the shRNA approach. Besides, known members from the MSL complex, namely *Msl1*, *Msl2* and *Msl3* were also included in the knock-down study. In the absence of Dox, no obvious growth defect or cell death was observed in the knock-down cell lines. In the presence of Dox, induction of *Xist* leads to gene silencing in iXist cells and knocking down of XCR candidates deteriorate the cell death. The preliminary

screening based on hemocytometer cell count shows that knocking down of *Kat8* and *Msl2* leads to significantly more cell death although only *Kat8* is significant (Fig 4B). We decided to further validate *Kat8* and *Msl2*.

Figure 4. Preliminary screening for HATs candidate as XCR players.

(A) iXist cells were treated with DMSO (control) or p300 inhibitors, L002 or C646 in the presence or absence of 1 μ g/mL of Dox for 4 days followed by cell count to access the cell survival rate. Cell survival rate was plotted as percentage of the cell number of +Dox over -Dox samples. P-value was calculated using two-tailed paired Student's t-test. Data are shown as mean $\pm$ SEM of biological replicates (n = 3). **(B)** iXist cells were stably transfected with shRNAs targeting different HATs or HAT-associated proteins. Cells were then cultured in the presence or absence of Dox for 4 days followed by cell count to access the cell survival rate. Cell survival rate was plotted as percentage of the cell number of +Dox over -Dox samples. P-value was calculated using two-tailed Student's t-test with equal variance. Data are shown as mean $\pm$ SEM of biological replicates (n =

Decreased expression level of *Kat8* and *Msl2* in the knock-down cell lines were confirmed using qPCR. Results show that knock-down level of *Kat8* and *Msl2* are at least 60% (Fig 5). To further confirm the roles of *Kat8* and *Msl2* in XCR, *Xist* was induced in both knock-down cell lines for 4 days followed by an AP staining in order to more accurately access the cell survival rate. Result confirms *Kat8* and *Msl2* knock-down significantly reduced the number of surviving colonies when XCI was induced in undifferentiated iXist cells (Fig 6A and 6B).

Figure 5. Expression profile of *Kat8* and *Msl2* in the knock-down cell lines.

Knock-down of *Kat8* and *Msl2* was confirmed by qPCR. Data are shown in relative fold expression and are normalized using *Actb* and *Gapdh*. P-value was calculated using two-tailed Student's t-test with equal variance (***, $p < 0.001$). Data presented are mean $\pm$ SEM of three independent experiments.

Figure 6. Cell survival assessment in the knock-down cell lines using AP staining.

(A) Representative images from AP staining of the control and knock-down cell lines. (B) iXist and knock-down cell lines were induced for 4 days in 2i medium in the presence of 1 $\mu\text{g/mL}$ of Dox followed by AP staining. Cell survival rate was plotted as percentage of the colony number of +Dox over -Dox samples. P-value was calculated using one-tailed Student's t-test with equal variance. Data presented are mean $\pm$ SEM of biological replicates ($n = 3$).

To rule out the possibility that lower cell survival rate in the knock-down cell lines was caused by the loss of ES cell pluripotency, we investigated the pluripotency gene expression levels in the knock-down cell lines. Knocking down *Kat8* and *Msl2* did not affect the expression of key pluripotency-related genes in iXist cells (Fig 7). Consistent with the gene expression profile, there was also no obvious colony morphological changes observed in the knock-down cells (Fig 8).

Figure 7. Pluripotency expression profile of the iXist shRNA *Kat8* or shRNA *Msl2* knock-down cell lines.

Pluripotency of iXist carrying shRNAs against *Kat8* or *Msl2* was checked using qPCR targeting pluripotency markers *Nanog*, *Sox2*, *Oct4*. *Gata4* and *Fgf5* are expressed in low level in undifferentiated cells were included as negative control. *Nanog* expression level in iXist was plotted for a direct comparison. Relative fold of expression was shown and was normalized to *Actb* and *Gapdh*. Error bar indicate SEM of technical replicates (n = 3). No statistical analysis was performed as only one round of qPCR was performed.

Figure 8. Comparison of the morphological changes between iXist and iXist carrying shRNA against *Kat8* or *Msl2*.

Representative phase contrast images of iXist, *Kat8* knock-down and *Msl2* knock-down cells. Cells were imaged in ES medium. Scale bar: 100 μ M.

iXist Excludes Kat8 and H4K16ac More Efficiently in Differentiating Cells

Next, we performed immuno-RNA FISH to investigate the effect of induced *Xist* on the subcellular localization of *Kat8* as well as the acetylation pattern of H4K16ac, a histone mark for active genes known to be specifically acetylated by *Kat8*, in the iXist cells. iXist cells were first cultured in undifferentiated condition (2i + LIF medium). Medium was switched to differentiating condition (ES + AA) or remained unchanged. Dox treatment was carried out for 3 days. Immunostaining of *Kat8* followed by RNA FISH detecting *Xist* reveals that some *Xist* positive cells show *Kat8* exclusion resembling an empty pocket where the *Xist* cloud resides (Fig

9A). Quantification shows that when cultured in differentiating condition, 75% of the cells (n = 51) with *Xist* clouds shows Kat8 exclusion compared to only 53% (n = 56) when the cells were cultured in undifferentiated condition (Fig 9B). Similarly, the percentage of cells showing H4K16ac “hole” is higher in differentiating condition (90%) (n = 57) when compared to undifferentiated condition (63%) (n = 43) (Fig 9B).

Figure 9. Immuno-RNA FISH targeting Kat8 or H4K16ac followed by *Xist* cloud detection in iXist.

(A) Immuno-RNA FISH analysis shows *Xist* clouds with or without Kat8 or H4K16ac exclusion. The yellow arrowheads show the Kat8/H4K16ac hole. Focused images of merged z-series are shown. **(B)** Population of *Xist* cloud positive cells with or without Kat8/H4K16ac exclusion. Scale bar: 5 μ M.

DISCUSSION

Mouse ESCs has long been established as a useful tool for XCI study. Here, we have established an inducible ESCs system and extended the use of ESCs system to XCR study. In our preliminary screening for XCR players, two candidates appeared to be involved in XCR. We decided to confirm the two candidate genes by accessing the cell survival rate using AP staining after inducing the knock-down cell lines. Indeed, the result is reproducible despite different method was used. Our result shows that knocking down of Kat8 or Msl2 shifts the balance of the induced XCI force and the build in XCR force in iXist, thus leading to more cell death.

Kat8, also known as Male absent on the first (Mof), belongs to the MYST family of HATs which is specific for H4K16ac (Akhtar and Becker, 2000; Smith et al., 2005; Taipale et al., 2005). Originally found in *Drosophila* to be responsible for X chromosome dosage compensation, Kat8 was found to be essential for embryonic development, normal chromatin architecture, DNA damage repair as well as stem cell pluripotency in mammals (Gupta et al., 2008; Li et al., 2012; Thomas et al., 2008). Msl2 was given its name after *Msl2* mutants in *Drosophila* were found to cause male-specific lethality. Together with other proteins namely Msl1, Msl3, Mle and Kat8, Msl2 form the MSL complex that is responsible for X chromosome dosage compensation in *Drosophila* (Kelley et al., 1995; Morales et al., 2004, 2005; Scott et al., 2000). Unlike eutherian mammals, *Drosophila* compensate the X-linked gene dosage by transcriptionally upregulating the single X in the males (Akhtar and Becker, 2000; Conrad et al., 2012; Larschan et al., 2011). This makes the complex an X chromosome “activator”. Although mammalian cells have adopted a

fundamentally different X chromosome compensation system, the MSL complex is conserved in mammals (Mendjan et al., 2006; Smith et al., 2005). Mammals retain the orthologs of MSL complex members but the function of the complex is less studied. Therefore, it would be interesting to find out the role of MSL complex in mammalian cells. Compared to *Kat8*, *Msl2* knock-down has led to a more severe cell death when XCI was induced in iXist. This suggests that *Msl2* has a more vital role in XCR than *Kat8* despite the fact that *Kat8* is the catalytic unit of the MSL complex. In fact, it was demonstrated that ectopic expression of *Msl2* is sufficient to recruit other dosage compensation players to the female X chromosomes therefore showing that *Msl2* is the key regulator for dosage compensation in *Drosophila* (Kelley et al., 1995). *Msl2* consists of a cysteine-rich domain (CXC), a nucleic acid binding domain, which enables *Msl2* to bind to X chromosome in *Drosophila* (Fauth et al., 2010). *Msl2* then acts as a recruiter to assemble other members of the MSL complex. As *Msl2* acts at the upstream of the dosage compensation process and is essential for MSL complex assembly in *Drosophila*, this may explain why knocking down *Msl2* has a more severe effect in the iXist cells when compared to *Kat8* knock-down. Nonetheless, these studies were done in *Drosophila*. Therefore, whether mammals employ similar mechanism to “activate” X chromosome remains to be investigated. Notably, the MSL complex in *Drosophila* specifically recognizes some high-affinity sites (HAS) on the X chromosomes (Alekseyenko et al., 2008; Straub et al., 2008). However, previous study shows that under undifferentiated condition, inducible XCI can also be reversed upon removal of Dox even if the *Xist* transgene was inserted into autosomes (Wutz and Jaenisch, 2000). This shows that the endogenous reactivation

force in mouse ESCs is a global effect and therefore is probably different from the X chromosome-specific hyperactivating force in *Drosophila* to a certain extent.

It was previously reported that homozygous deletion of *Kat8* disrupts ES cell self-renewal and pluripotency although heterozygous deletion resulted in a only moderate decrease in AP staining and the heterozygous mutant animals were phenotypically normal (Li et al., 2012). To rule out the possibility that the increased cell death in the knock-down cell lines was due to the disruption of pluripotency, we examined the pluripotency expression profile of the knock-down cell lines. Our result shows knock-down cell lines expressed comparable level of pluripotency markers compared to the control cell line iXist, indicating the cell death is indeed caused by disruption of XCR but not self-renewal.

A recent study on MSL complex links *Msl2* to *Xist* repression by regulating *Tsix* expression (Chelmicki et al., 2014). *Tsix* is known to repress *Xist* by its antisense transcription through *Xist* (Luikenhuis et al., 2001; Shibata et al., 2004). In the iXist cell line, the *Xist* transgene was inserted at the *Hprt* locus that is ~50 Mb away from the endogenous *Tsix* and the *Xist* transgene does not contain the promoter as well as the 5' region of *Tsix*. Although we could not rule out *Msl2*'s indirect involvement in *Xist* repression thus plays a role in XCI repression or XCR, our results show that *Kat8* and *Msl2* are directly involved in XCR but not through the action of *Tsix*. We further investigated *Kat8* and H4K16ac acetylation pattern between the undifferentiated cells and differentiating cells. Our results further demonstrate that under undifferentiated condition, iXist cells display lower percentage of *Kat8* and H4K16ac exclusion from the *Xist* clouds region. This supports the notion that undifferentiated cells have the endogenous XCR capacity to

counteract with the induced XCI. However, the image analysis was done using simple scoring of presence or absence of exclusion. To improve the quality of image analysis, co-localization tools like Coloc from ImageJ or Colocalization pipeline from CellProfiler could be used in future studies.

Although our system successfully identified Kat8 and Msl2 as the potential XCR players, there are few things could be improved further. Firstly, we demonstrated the knock-down efficiency of the Kat8 and Msl2 via qPCR at mRNA level but not protein level. Although the same shRNAs were used and knock-down of Kat8 and Msl2 at the protein levels were confirmed via Western Blot in previous studies (Chelmicki et al., 2014; Zhou et al., 2009), our study has not performed Western Blot due to time constraint. Understanding that subsequent experiments and data interpretation are highly dependent on the successful silencing of protein expression, this knock-down level will be confirmed using Western Blot in future studies. Secondly, the lack of scrambled shRNA in our knock-down system which may raise the concern of target specificity. We argue that growth rate and pluripotency gene expression changes were not observed in the knock-down cell lines. Besides, knocking down of two different proteins from the same protein complex give rise to mutants with similar phenotypes. Thus, it is unlikely that these two candidates were picked up due to knocking down of non-specific genes that cause higher cell death. Nonetheless, it would be ideal to have a different shRNA targeting the same gene to address the off-target concerns. Lastly, our system was designed to fish out XCR candidates if there is more severe cell death as a result of tip of balance towards stronger cell killing effect. This is considered a stringent test and only individual genes that play more important roles in XCR may be picked up

by the screening. In other words, genes that are involved in XCR but require synergistic interactions may be overlooked by this system. As a result, knocking down of multiple “weak” XCR players simultaneously are required to achieve a significant increase in cell death but this may be technically cumbersome.

Future Work

iXist cell line enabled us to study XCR and suggests that Kat8 and Msl2 are involved in XCR. However, more work need to be done to provide direct evidence for their roles in XCR. Immediate experiment would be knocking down *Kat8* and *Msl2* using different shRNA sequences to address the off-target concern. We have demonstrated that knocking down the *Kat8* or *Msl2* will lead to more cell death in the iXist cells. Conversely, using the same system, overexpressing Kat8 or Msl2 in the knock-down cell lines should rescue the XCR and therefore revert the cell killing effect. Besides, our lab has established a system where proteins could be artificially tethered to the *Xist*. This is another useful tool to study the effect of XCR players when they are brought into the vicinity of the *Xist*. Similar tool has been utilized to rescue XCI gene silencing by artificially tether XCI effector to the *Xist* mutant (Chen et al., 2016; Patil et al., 2016). It is well established that various heterochromatin markers such as H3K27me3, H2AK119ub, macroH2A are enriched on Xi. We could compare various Xi markers in the knock-down cell lines to the iXist cells to gain further insights of the role of Kat8 and Msl2 in XCR. In addition, we could compare the X-linked genes expression between the knock-down cell lines and iXist cells to access level of XCI and XCR using RNA FISH to detect nascent transcripts.

Additionally, we could also make use of our fibroblast cell line that has a EGFP on the Xi (EGFP-negative cells). Following iPSC formation, we could check XCR efficiency by assessing the percentage of EGFP-positive cells via FACS by comparing the wildtype and the XCR knock-down or knockout cell lines.

CONCLUSION

Using iXist cells, we have successfully established a system that allow us to study the XCR. With this system, our work suggests that the mammalian orthologs of *Drosophila* dosage compensation complex, Kat8 and its associated protein Msl2 are involved in the XCR.

REFERENCES

- Akhtar, A., and Becker, P.B. (2000). Activation of transcription through histone H4 acetylation by MOF, an acetyltransferase essential for dosage compensation in *Drosophila*. *Mol. Cell* 5, 367–375.
- Alekseyenko, A.A., Peng, S., Larschan, E., Gorchakov, A.A., Lee, O.-K., Kharchenko, P., McGrath, S.D., Wang, C.I., Mardis, E.R., Park, P.J., and Kuroda, M.I. (2008). A Sequence Motif within Chromatin Entry Sites Directs MSL Establishment on the *Drosophila* X Chromosome. *Cell* 134, 599–609.
- Borsani, G., Tonlorenzi, R., Simmler, M.C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., Willard, H.F., Avner, P., and Ballabio, A. (1991). Characterization of a Murine Gene Expressed from the Inactive X-Chromosome. *Nature* 351, 325–329.
- Brockdorff, N., Ashworth, A., Kay, G.F., Cooper, P., Smith, S., McCabe, V.M., Norris, D.P., Penny, G.D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. *Nature* 351, 329–331.
- Brockdorff, N., Ashworth, A., Kay, G.F., McCabe, V.M., Norris, D.P., Cooper, P.J., Swift, S., and Rastan, S. (1992). The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* 71, 515–526.
- Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I., Friman, O., Guertin, D.A., Chang, J., Lindquist, R.A., Moffat, J., Golland, P., and Sabatini, D.M. (2006). CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7, R100.
- Chelmicki, T., Dündar, F., Turley, M.J., Khanam, T., Aktas, T., Ramírez, F., Gendrel, A.-V., Wright, P.R., Videm, P., Backofen, R., Heard, E., Manke, T., and Akhtar, A. (2014). MOF-associated complexes ensure stem cell identity and Xist repression. *Elife* 3, e02024.
- Chen, C.-K., Blanco, M., Jackson, C., Aznauryan, E., Ollikainen, N., Surka, C., Chow, A., Cerase, A., McDonel, P., and Guttman, M. (2016). Xist recruits the X chromosome to the nuclear lamina to enable chromosome-wide silencing. *Science* 354, 468–472.
- Chu, C., Zhang, Q.C., da Rocha, S.T., Flynn, R.A., Bharadwaj, M., Calabrese, J.M., Magnuson, T., Heard, E., and Chang, H.Y. (2015). Systematic Discovery of Xist RNA Binding Proteins. *Cell* 161, 404–416.
- Chuva de Sousa Lopes, S.M., Hayashi, K., Shovlin, T.C., Mifsud, W., Surani, M.A., and McLaren††, A. (2008). X Chromosome Activity in Mouse XX Primordial Germ Cells. *PLoS Genet.* 4, e30.
- Conrad, T., Cavalli, F.M.G., Vaquerizas, J.M., Luscombe, N.M., and Akhtar, A. (2012). *Drosophila* dosage compensation involves enhanced Pol II recruitment to male X-linked promoters. *Science* 337, 742–746.

- Costanzi, C., and Pehrson, J.R. (1998). Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. *Nature* 393, 599–601.
- Cowan, C.A., Atienza, J., Melton, D.A., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science* 309, 1369–1373.
- Debrand, E., Chureau, C., Arnaud, D., Avner, P., and Heard, E. (1999). Functional analysis of the DXPas34 locus, a 3' regulator of Xist expression. *Mol. Cell. Biol.* 19, 8513–8525.
- Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W., Yanagimachi, R., and Jaenisch, R. (2000). X-Chromosome inactivation in cloned mouse embryos. *Science* 290, 1578–1581.
- Fang, J., Chen, T., Chadwick, B., Li, E., and Zhang, Y. (2004). Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X inactivation. *J. Biol. Chem.* 279, 52812–52815.
- Fauth, T., Müller-Planitz, F., König, C., Straub, T., and Becker, P.B. (2010). The DNA binding CXC domain of MSL2 is required for faithful targeting the Dosage Compensation Complex to the X chromosome. *Nucleic Acids Res.* 38, 3209–3221.
- Franco, B., and Ballabio, A. (2006). X-inactivation and human disease: X-linked dominant male-lethal disorders. *Curr. Opin. Genet. Dev.* 16, 254–259.
- Gendrel, A.-V., and Heard, E. (2014). Noncoding RNAs and Epigenetic Mechanisms During X-Chromosome Inactivation. *Annu. Rev. Cell Dev. Biol.* 30, 561–580.
- Gupta, A., Guerin-Peyrou, T.G., Sharma, G.G., Park, C., Agarwal, M., Ganju, R.K., Pandita, S., Choi, K., Sukumar, S., Pandita, R.K., Ludwig, T., and Pandita, T.K. (2008). The mammalian ortholog of *Drosophila* MOF that acetylates histone H4 lysine 16 is essential for embryogenesis and oncogenesis. *Mol. Cell. Biol.* 28, 397–409.
- Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C.J., Creighton, M.P., van Oudenaarden, A., and Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature* 462, 595–601.
- Huynh, K.D., and Lee, J.T. (2003). Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos. *Nature* 426, 857–862.
- Kay, G.F., Barton, S.C., Surani, M.A., and Rastan, S. (1994). Imprinting and X chromosome counting mechanisms determine Xist expression in early mouse development. *Cell* 77, 639–650.
- Kelley, R.L., Solovyeva, I., Lyman, L.M., Richman, R., Solovyev, V., and Kuroda, M.I. (1995). Expression of Msl-2 causes assembly of dosage compensation regulators on the X chromosomes and female lethality in *Drosophila*. *Cell* 81, 867–877.
- Kyba, M., Perlingeiro, R.C.R., and Daley, G.Q. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 109, 29–37.

- Lai, L.T., Lee, P.J., and Zhang, L.F. (2013). Immunofluorescence protects RNA signals in simultaneous RNA-DNA FISH. *Exp. Cell Res.* *319*, 46–55.
- Larschan, E., Bishop, E.P., Kharchenko, P. V., Core, L.J., Lis, J.T., Park, P.J., and Kuroda, M.I. (2011). X chromosome dosage compensation via enhanced transcriptional elongation in *Drosophila*. *Nature* *471*, 115–118.
- Lee, J., Davidow, L.S., and Warshawsky, D. (1999). Tsix, a gene antisense to Xist at the X-inactivation centre. *Nat. Genet.* *21*, 400–404.
- Lessing, D., Anguera, M.C., and Lee, J.T. (2013). X chromosome inactivation and epigenetic responses to cellular reprogramming. *Annu. Rev. Genomics Hum. Genet.* *14*, 85–110.
- Li, X., Li, L., Pandey, R., Byun, J.S., Gardner, K., Qin, Z., and Dou, Y. (2012). The histone acetyltransferase MOF is a key regulator of the embryonic stem cell core transcriptional network. *Cell Stem Cell* *11*, 163–178.
- Luikenhuis, S., Wutz, A., and Jaenisch, R. (2001). Antisense transcription through the Xist locus mediates Tsix function in embryonic stem cells. *Mol. Cell. Biol.* *21*, 8512–8520.
- Lyon, M.F. (1961). Gene Action in the X-chromosome of the Mouse (*Mus musculus* L.). *Nature* *190*, 372–373.
- Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K., and Hochedlinger, K. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* *1*, 55–70.
- Mak, W., Nesterova, T.B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A.P., and Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. *Science* *303*, 666–669.
- Marahrens, Y., Panning, B., Dausman, J., Strauss, W., and Jaenisch, R. (1997). Xist-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes Dev.* *11*, 156–166.
- McHugh, C.A., Chen, C.-K., Chow, A., Surka, C.F., Tran, C., McDonel, P., Pandya-Jones, A., Blanco, M., Burghard, C., Moradian, A., Sweredoski, M.J., Shishkin, A.A., Su, J., Lander, E.S., Hess, S., Plath, K., and Guttman, M. (2015). The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. *Nature* *521*, 232–236.
- Mendjan, S., Taipale, M., Kind, J., Holz, H., Gebhardt, P., Schelder, M., Vermeulen, M., Buscaino, A., Duncan, K., Mueller, J., Wilm, M., Stunnenberg, H.G., Saumweber, H., and Akhtar, A. (2006). Nuclear pore components are involved in the transcriptional regulation of dosage compensation in *Drosophila*. *Mol. Cell* *21*, 811–823.
- Minajigi, A., Froberg, J., Wei, C., Sunwoo, H., Kesner, B., Colognori, D., Lessing, D., Payer, B., Boukhali, M., Haas, W., and Lee, J.T. (2015). Chromosomes. A comprehensive Xist interactome reveals cohesin repulsion and an RNA-directed chromosome conformation. *Science* *349*, aab2276–aab2276.

- Mise, N., Goto, Y., Nakajima, N., and Takagi, N. (1999). Molecular Cloning of Antisense Transcripts of the Mouse Xist Gene. *Biochem. Biophys. Res. Commun.* 258, 537–541.
- Moindrot, B., Cerase, A., Coker, H., Masui, O., Grijzenhout, A., Pintacuda, G., Schermelleh, L., Nesterova, T.B., and Brockdorff, N. (2015). A Pooled shRNA Screen Identifies Rbm15, Spen, and Wtap as Factors Required for Xist RNA-Mediated Silencing. *Cell Rep.* 12, 562–572.
- Morales, V., Straub, T., Neumann, M.F., Mengus, G., Akhtar, A., and Becker, P.B. (2004). Functional integration of the histone acetyltransferase MOF into the dosage compensation complex. *EMBO J.* 23, 2258–2268.
- Morales, V., Regnard, C., Izzo, A., Vetter, I., and Becker, P.B. (2005). The MRG domain mediates the functional integration of MSL3 into the dosage compensation complex. *Mol. Cell. Biol.* 25, 5947–5954.
- de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M., Koseki, H., and Brockdorff, N. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev. Cell* 7, 663–676.
- Nichols, J., and Smith, A. (2009). Naive and Primed Pluripotent States. *Cell Stem Cell* 4, 487–492.
- Ohhata, T., and Wutz, A. (2013). Reactivation of the inactive X chromosome in development and reprogramming. *Cell. Mol. Life Sci.* 70, 2443–2461.
- Okamoto, I., Otte, A.P., Allis, C.D., Reinberg, D., and Heard, E. (2004). Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* 303, 644–649.
- Park, I.-H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley, G.Q. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451, 141–146.
- Pasque, V., and Plath, K. (2015). X chromosome reactivation in reprogramming and in development. *Curr. Opin. Cell Biol.* 37, 75–83.
- Pasque, V., Tchieu, J., Karnik, R., Uyeda, M., Sadhu Dimashkie, A., Case, D., Papp, B., Bonora, G., Patel, S., Ho, R., Schmidt, R., McKee, R., Sado, T., Tada, T., Meissner, A., and Plath, K. (2014). X chromosome reactivation dynamics reveal stages of reprogramming to pluripotency. *Cell* 159, 1681–1697.
- Patil, D.P., Chen, C.-K., Pickering, B.F., Chow, A., Jackson, C., Guttman, M., and Jaffrey, S.R. (2016). m6A RNA methylation promotes XIST-mediated transcriptional repression. *Nature* 537, 369–373.
- Payer, B., Rosenberg, M., Yamaji, M., Yabuta, Y., Koyanagi-Aoi, M., Hayashi, K., Yamanaka, S., Saitou, M., and Lee, J.T. (2013). Tsix RNA and the germline factor, PRDM14, link X reactivation and stem cell reprogramming. *Mol. Cell* 52, 805–818.
- Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for Xist in X chromosome inactivation. *Nature* 379, 131–137.
- Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H.,

- de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. *Science* 300, 131–135.
- Rastan, S. (1982). Timing of X-chromosome inactivation in postimplantation mouse embryos. *J. Embryol. Exp. Morphol.* 71, 11–24.
- Rastan, S. (1983). Non-random X-chromosome inactivation in mouse X-autosome translocation embryos--location of the inactivation centre. *J. Embryol. Exp. Morphol.* 78, 1–22.
- Rastan, S., and Robertson, E.J. (1985). X-chromosome deletions in embryo-derived (EK) cell lines associated with lack of X-chromosome inactivation. *J. Embryol. Exp. Morphol.* 90, 379–388.
- Scott, M.J., Pan, L.L., Cleland, S.B., Knox, A.L., Heinrich, J., and Kuroda, M. (2000). MSL1 plays a central role in assembly of the MSL complex, essential for dosage compensation in *Drosophila*. *EMBO J.* 19, 144–155.
- Shibata, S., Lee, J.T., Geske, R., Bradley, A., Li, M., Dani, C., Robertson, M., Chambers, I., Simth, A., Khosravi, R., and al., et (2004). Tsix transcription- versus RNA-based mechanisms in Xist repression and epigenetic choice. *Curr. Biol.* 14, 1747–1754.
- Smith, E.R., Cayrou, C., Huang, R., Lane, W.S., Côté, J., and Lucchesi, J.C. (2005). A human protein complex homologous to the *Drosophila* MSL complex is responsible for the majority of histone H4 acetylation at lysine 16. *Mol. Cell. Biol.* 25, 9175–9188.
- Straub, T., Grimaud, C., Gilfillan, G.D., Mitterweger, A., and Becker, P.B. (2008). The Chromosomal High-Affinity Binding Sites for the *Drosophila* Dosage Compensation Complex. *PLoS Genet.* 4, e1000302.
- Sugimoto, M., and Abe, K. (2007). X chromosome reactivation initiates in nascent primordial germ cells in mice. *PLoS Genet.* 3, 1309–1317.
- Tada, M., Takahama, Y., Abe, K., Nakatsuji, N., and Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr. Biol.* 11, 1553–1558.
- Taipale, M., Rea, S., Richter, K., Vilar, A., Lichter, P., Imhof, A., and Akhtar, A. (2005). hMOF histone acetyltransferase is required for histone H4 lysine 16 acetylation in mammalian cells. *Mol. Cell. Biol.* 25, 6798–6810.
- Takagi, N., Sugawara, O., and Sasaki, M. (1982). Regional and temporal changes in the pattern of X-chromosome replication during the early post-implantation development of the female mouse. *Chromosoma* 85, 275–286.
- Takahashi, K., and Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* 126, 663–676.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872.
- Thomas, T., Dixon, M.P., Kueh, A.J., and Voss, A.K. (2008). Mof (MYST1 or

KAT8) is essential for progression of embryonic development past the blastocyst stage and required for normal chromatin architecture. *Mol. Cell. Biol.* 28, 5093–5105.

Wutz, A., and Jaenisch, R. (2000). A Shift from Reversible to Irreversible X Inactivation Is Triggered during ES Cell Differentiation. *Mol. Cell* 5, 695–705.

Ying, Q.-L., Nichols, J., Evans, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. *Nature* 416, 545–548.

Ying, Q.-L., Ying, Q.-L., Wray, J., Wray, J., Nichols, J., Nichols, J., Battle-Morera, L., Battle-Morera, L., Doble, B., Doble, B., Woodgett, J., Woodgett, J., Cohen, P., Cohen, P., Smith, A., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453, 519–523.

Zhou, Y., Schmitz, K.-M., Mayer, C., Yuan, X., Akhtar, A., and Grummt, I. (2009). Reversible acetylation of the chromatin remodelling complex NoRC is required for non-coding RNA-dependent silencing. *Nat. Cell Biol.* 11, 1010–1016.

AUTHOR'S PUBLICATION

Book Chapter

Lan-Tian Lai, Zhenyu Meng, Fangwei Shao, Li-Feng Zhang. (2016) Simultaneous RNA-DNA FISH. pp 135–145

Peer-reviewed Journals

Ru Hong, Bingqing Lin, Xinyi Lu, **Lan-Tian Lai**, Xin Chen, Amartya Sanyal, Huck-Hui Ng, Kun Zhang, Li-Feng Zhang. (2017) High-resolution RNA allelotyping along the inactive X chromosome: evidence of RNA polymerase III in regulating chromatin configuration. *Sci Rep* 7:45460. doi: 10.1038/srep45460

Reelina Basu*, **Lan-Tian Lai***, Zhenyu Meng, Jun Wu, Fangwei Shao, Li-Feng Zhang. (2014) Using amino-labeled nucleotide probes for simultaneous single molecule RNA-DNA FISH. *PLoS One* 9:e107425. doi: 10.1371/journal.pone.0107425

Lan-Tian Lai, Pin Jie Lee, Li-Feng Zhang (2013) Immunofluorescence protects RNA signals in simultaneous RNA-DNA FISH. *Exp Cell Res* 319:46–55.

*co-first authors