Nanog regulates chromatin organization in mouse stem cells

by

Calvin Chun Man Tang

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Biochemistry
University of Toronto

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Department of Biochemistry

University of Toronto

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Abstract

Mouse embryonic stem cells (ESCs) are known to possess an “open” global chromatin architecture characterized by dispersed chromatin fibres throughout the nucleus that differs from the organization in differentiated cell types, where chromatin generally congregates into numerous compact domains. The unique chromatin organization in ESCs may be partly attributed to core transcription factors that regulate many genes involved in maintaining pluripotency, including those encoding chromatin-remodeling complexes. My hypothesis is that Nanog, one of these core transcription factors, functions in maintaining an “open” chromatin organization in mouse ESCs. Through electron spectroscopic imaging, an inverse correlation was identified between Nanog expression level and the chromatin fibre density of constitutive heterochromatic regions in ESCs. Furthermore, the global chromatin structure in the more differentiated epiblast stem cells became less compact upon Nanog overexpression. Together, these findings support the idea that Nanog plays a role in maintaining dispersed chromatin in mouse ESCs.
Acknowledgments

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<tr>
<td>Bone morphogenetic protein 4</td>
<td>BMP4</td>
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<tr>
<td>Charge-coupled device</td>
<td>CCD</td>
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<tr>
<td>Complementary DNA</td>
<td>cDNA</td>
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<tr>
<td>Chromodomain-helicase-DNA-binding protein 1</td>
<td>Chd1</td>
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<tr>
<td>Chromatin immunoprecipitation</td>
<td>ChIP</td>
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<tr>
<td>Dulbecco’s modified eagle medium</td>
<td>DMEM</td>
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<tr>
<td>2,4,6-Tri(dimethylaminomethyl) phenol</td>
<td>DMP-30</td>
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<tr>
<td>Deoxyribonucleic acid</td>
<td>DNA</td>
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<tr>
<td>Doxycycline</td>
<td>Dox</td>
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<td>Electron microscopy</td>
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<td>Embryonic day 3.5</td>
<td>E3.5</td>
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<td>Electron spectroscopic imaging</td>
<td>ESI</td>
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<td>ESCs</td>
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<td>Epiblast stem cells</td>
<td>EpiSCs</td>
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<td>Fetal bovine serum</td>
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<td>Term</td>
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<tr>
<td>Fibroblast growth factor</td>
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<td>Fluorescent <em>in-situ</em> hybridization</td>
<td>FISH</td>
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<tr>
<td>Green fluorescent protein</td>
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<td>Immunofluorescence</td>
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<td>Jak2</td>
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<td>Leukemia inhibitory factor</td>
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<td>Nonenyl succinic anhydride</td>
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<tr>
<td>Phosphorus buffered saline</td>
<td>PBS</td>
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<tr>
<td>Primitive endoderm</td>
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<td>Term</td>
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<tr>
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<td>Sox2</td>
</tr>
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<td>SRY (sex determining region Y)-box 2</td>
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<tr>
<td>Signal transducer and activator of transcription 3</td>
<td>Stat3</td>
</tr>
<tr>
<td>Trophoderm</td>
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Chapter 1

1 Introduction

1.1 Chromatin and early development

Chromatin is often defined as a complex of DNA, histone, and nonhistone proteins that constitute chromosomes. Two types of chromatin are often distinguished during interphase of the cell cycle, namely euchromatin and heterochromatin. Euchromatin has been described as dispersed and transcriptionally active chromatin and is enriched in histone modifications such as H4 acetylation and H3K4me3 (Grewal et al, 2007). Heterochromatin is often defined as compact chromatin associated with silenced and gene-poor regions of the genome. It is involved in the proper functioning of centromeres as well as the maintenance of genomic integrity by preventing potentially hazardous recombination events between repetitive DNA sequences (Allshire et al, 1995; Grewal et al, 2007). Heterochromatin is often divided into two types: facultative and constitutive heterochromatin. Facultative heterochromatin is cell type dependent and will undergo changes in chromatin state in response to cellular signals (Grewal et al, 2007). An example of such heterochromatin is seen during X-chromosome inactivation where some cells inactivate the paternal X-chromosome while others inactivate the maternal one. Constitutive heterochromatin is not cell type dependent and DNA sequences packaged into this type of heterochromatin are the same in each cell of the organism. It includes regions enriched in repetitive sequences such as centromeres that are marked with histone modifications such as H3K9me3 and H4K20me3 (Guenatri et al, 2004).

Recent research has revealed a relationship between chromatin architecture and development in mice (Ahmed et al, 2010). Development of a mouse embryo begins at the unicellular zygotic stage and progresses by subsequent cleavage events to form a more matured embryo consisting of distinct cell types. Through electron spectroscopic imaging, which will be described in more detail later in the introduction, changes in chromatin organization have been observed during the transition from the one-cell to early post-implantation stage embryo. In one-cell stage embryos, chromatin is organized as dispersed 10 nm fibres distributed evenly in both the male and female pronuclei. This is in contrast to most differentiated cell types such as hepatocytes, where significant chromatin accumulation is observed at the nuclear envelope and
nucleolar periphery. In the two-cell stage, chromatin becomes more structured as compact chromatin accumulates along the nuclear periphery to form a rim measuring from 30 nm to 60 nm in thickness. Furthermore, the chromatin tends to organize into compact domains throughout the nucleoplasmic space. As the embryo further progresses through development, embryonic and extraembryonic tissues become established by embryonic day 3.5 (E.3.5). This stage in development offers the chance to examine and compare the chromatin organizations in pluripotent and lineage-commited cells. Embryonic tissue is composed of pluripotent epiblast cells, whereas extraembryonic tissue consists of the lineage-restricted trophectoderm (TE) and primitive endoderm (PE) cells. TE cells form the outer epithelial layer of the blastocyst and will eventually form the placental interface between mother and child. As for PE cells, they form the extraembryonic endoderm layers of the yolk sac and together with TE cells will support the growth of the fetus in the uterine environment. In epiblast cells from E3.5 embryos, chromatin assumes a state characterized by highly dispersed 10 nm fibres throughout the nucleoplasmic space, which has been suggested to be a hallmark of pluripotency. This is in contrast to TE and PE cells, where compact chromatin is present along the nuclear envelope and relatively fewer dispersed chromatin fibres are found within the interior of the nucleus. This may serve to create gene-silencing domains required for these particular cell types. These data provide evidence that chromatin organization is not random but differs depending on the cell type. In post-implantation embryo at embryonic day 5.5 (E5.5), epigenetic changes such as X-chromosome inactivation are present in epiblast cells that may contribute to the observed decrease in developmental potential, such that the ability to contribute to blastocyst chimeras is lost (Nichols et al, 2009). In other words, if E5.5 epiblast cells are injected into a blastocyst derived from another mouse, the cells will not contribute to the cell population in the adult mouse. With regards to chromatin organization, epiblast cells that underwent implantation possess a significantly different chromatin architecture. In contrast to the dispersed chromatin state in E3.5 epiblast cells, chromatin in E5.5 epiblast cells forms compact domains throughout the nucleoplasmic space and along the nuclear envelope (Ahmed et al, 2010). The precise mechanism by which this transition occurs still requires further investigation. During all these different stages of development, there is deposition of chromatin-associated proteins, exchange of histone variants, and changes in epigenetic state; all of which might contribute to the changes in chromatin organization and also demonstrate that chromatin remodeling is important during development.
1.2 Mouse pluripotent stem cells

To gain insight into the mechanisms of chromatin remodeling, we can begin by asking how an “open” global chromatin architecture, which is characterized by highly dispersed chromatin fibres throughout the nucleoplasmic space, is maintained in cells such as E3.5 epiblast cells. Factors that maintain this chromatin state may be important for pluripotency since the loss of certain chromatin remodeling factors affects the proliferation and differentiation of epiblast cells (Gaspar-Maia et al, 2009). Therefore, chromatin organization and pluripotency are intimately related and identifying regulators of chromatin structure will further our understanding of the pluripotent state.

In this study, mouse stem cells were chosen as the model system to identify factors that affect the chromatin configuration of stem cells. These cells, which include embryonic and epiblast stem cells, can be derived from mouse embryos and were chosen for study instead of their equivalent in vivo cells because of feasibility from an experimental point-of-view. Culturing stem cells in vitro enables the growth of a large number of cells and there is less heterogeneity in terms of growth environment and genomic differences between cells. Mouse stem cells have been shown to retain essential properties of their in vivo counterparts including similar chromatin organization and developmental potential, providing support that these cells are biologically relevant (Ahmed et al, 2010).

Mouse embryonic stem cells (ESCs) are derived from the inner cell mass of E3.5 pre-implantation blastocysts and were the first pluripotent cells isolated from embryos (Evans and Kaufman, 1981). These cells retain attributes of E3.5 epiblast cells and can be reincorporated into the embryo and resume normal development to generate soma and germ cells (Nichols et al, 2009). Furthermore, ESCs can be microinjected into blastocysts derived from other mice and generate chimaeras, which can be confirmed through methods such as the use of genetic coat color markers (Smith, 2001). As expected from pluripotent cells, ESCs can differentiate into the three germ layers: mesoderm, ectoderm, and endoderm, which will eventually give rise to every cell type within the adult organism (Evans and Kaufman, 1983). However, ESCs cannot produce extraembryonic cell types such as trophectoderm cells, which are involved in uterine implantation among other roles (Smith, 2001). ESCs can self-renew indefinitely and are maintained in vitro in media supplemented with two factors, bone morphogenetic protein 4
(BMP4) and leukemia inhibitory factor (LIF) (Ying et al, 2003). LIF is a cytokine that functions to activate the transcription factor STAT3 to inhibit ESC differentiation and enhance proliferation. BMP4 is a growth factor that functions to induce the expression of Id genes through the Smad pathway, which in turn blocks expression of lineage-specific factors. With respect to epigenetics, the presence of two active X-chromosomes is a distinguished feature of female ESCs and changes in the epigenetic landscape and transcription levels of self-renewal- and differentiation-associated genes that accompany the loss of pluripotency initiate X-chromosome inactivation (Nichols et al, 2009). In terms of biological relevance, the activation of both X chromosomes in E3.5 epiblast cells is necessary for random inactivation that occurs following implantation of the embryo. Using whole genome tiling arrays, the genome of ESCs has been shown to be globally transcriptionally active compared to differentiated cells and large regions including repetitive sequences and lineage-specific genes are transcribed at a low level (Efroni et al, 2008). Consistent with this, many lineage-specific genes in ESCs are bivalent marked with the transcriptionally repressive histone mark H3K27 and the euchromatin associated mark H3K4me3, which may permit low-transcription of the genes as well as priming them for activation (Bernstein et al, 2006). Furthermore, upregulation of chromatin remodeling proteins and the global transcriptional machinery has been observed in ESCs (Efroni et al, 2008). This may be critical for maintaining a dispersed chromatin state as well as pluripotency since the loss of certain chromatin remodeling factors interferes with ESC proliferation and differentiation.

ESCs also show hyperdynamic binding of histones and chromatin-associated proteins such as linker histone H1 and the heterochromatin protein HP1 (Meshorer et al, 2006). The presence of a loosely bound fraction of chromatin-architectural proteins in ESCs has been suggested to contribute to the maintenance of the pluripotent state and is necessary for restructuring the global chromatin organization during differentiation. Examples of such reorganization is seen in heterochromatic regions, where chromatin is relatively dispersed in ESCs and become more compact in ESC-derived neuronal precursor cells. All these observations indicate an intimate connection between chromatin organization and pluripotency in mouse ESCs.

Epiblast stem cells (EpiSCs) are derived from epiblast cells of E5.5 post-implantation mouse embryos and are transcriptionally similar to their in vivo counterparts as confirmed by gene expression microarrays (Brons et al, 2007). In addition to derivation from embryos, EpiSCs can also be produced through in vitro differentiation of ESCs by culturing them in media
containing fibroblast growth factor (FGF) and activin (Guo et al, 2009). Furthermore, the reverse conversion, from EpiSCs to ESCs, can be achieved by overexpressing the transcription factor Klf4 in EpiSCs and culturing the cells in the presence of LIF and BMP4. In terms of developmental potential, EpiSCs are pluripotent and possess the ability to generate all three germ layers in vitro (Brons et al, 2007). However, unlike ESCs, EpiSCs cannot contribute to mouse chimaeras through injection into blastocysts and therefore possess a relatively lower developmental potential. With respect to growth characteristics, mouse EpiSC colonies show a more flattened morphology compared to the more domed ESC colonies. Furthermore, the doubling time of ESCs is 10-14 hours compared to 18 hours for EpiSCs, indicating a longer cell cycle in EpiSCs (Pauklin, 2011). As for epigenetics, X-chromosome inactivation occurs in EpiSCs and can serve as a marker to distinguish between ESCs and EpiSCs (Nichols et al, 2009).

Although both ESCs and EpiSCs are pluripotent, there are differences in growth conditions, developmental potential, and etc. Because of this, ESCs and EpiSCs are said to be in “naïve” and “primed” pluripotent states, respectively where the “naïve” state is associated with a higher developmental potential.

This difference in pluripotency between mouse ESCs and EpiSCs is also reflected in chromatin organization. Similar to E3.5 epiblast cells, mouse ESCs have highly dispersed and fairly uniform chromatin with few compact regions (Fussner et al, 2011). This may be attributed to high expression levels of chromatin-remodeling factors and hyperdynamic binding of chromatin architectural proteins (Meshorer et al, 2006; Efroni et al, 2008). On the other hand, chromatin in EpiSCs forms numerous compact domains throughout the nucleus (Ahmed et al, 2010). Based on previous studies that will be introduced later, various factors are involved in the regulation of chromatin organization and possibly contribute to the differences seen between ESCs and EpiSCs. This study is mainly interested in identifying transcription factors that affect chromatin organization in ESCs, specifically the ones maintaining the global “open” chromatin state. From this investigation, we hoped to gain further insight into the relationship between chromatin structure and transcription factors. Using both light and electron microscopy techniques, I examined the effects of transcription factors on the chromatin architecture in ESCs and EpiSCs.
1.3 Imaging

1.3.1 Light microscopy

For decades, light microscopy has been instrumental in furthering our knowledge on cellular structure and function. It is therefore no surprise that many discoveries regarding nuclear structure and chromatin organization were made through light microscopy studies. An example is the finding that individual chromosomes occupy discrete volumes in the nucleus, forming what are known as “chromosome territories” (Rapkin et al, 2011). Furthermore, the relative spatial arrangement of chromosome territories with respect to each other is not random and depends on factors such as cell type and transcription level of certain genes. These findings were made with the use of whole chromosome fluorescent “paints”, where probes that hybridize along a single chromosome are tagged with a particular fluorescent dye. With electron microscopy (EM), the separation of chromosomes cannot be seen since chromatin fibres intermingle at the boundaries of individual chromosome territories. Another finding made with light microscopy is that Giemsa-stained chromosomes in metaphase show a linear arrangement of alternating light and dark bands that reveal the macroscale organization of different parts of an individual chromosome (Craig et al, 1993). Light regions are gene-rich and early-replicating, while the dark regions are gene-poor and late-replicating. The pattern of dark and light bands is specific and reproducible for each chromosome and can be used for its identification. In another experiment, the conformation of a 4 Mb segment of mouse chromosome 14 containing gene clusters separated by gene deserts was studied (Shopland et al, 2006). The gene clusters and deserts were labeled with green and red fluorescent probes, respectively, and configurations of that segment of the genome within the nucleus were observed. Various conformations were seen, ranging from a linear “striped” arrangement to a cluster organization, demonstrating that the conformation of a genomic region is flexible. Light microscopy is also useful for identifying subnuclear structures. Through the use of fluorophore-tagged antibodies against specific histone marks and proteins, we can locate structures such as constitutive heterochromatic regions and PML bodies, which allows us to compare the nuclear organization between cell types.

In relation to the study of chromatin, light microscopy has both advantages and disadvantages when compared to electron microscopy. A major advantage is that living cells can be examined with a light microscope and thus dynamic processes such as changes in nuclear
organization can be studied. For electron microscopy, the sample must be dehydrated and thus live specimens are not possible. In addition, subnuclear structures such as heterochromatic regions can be identified and located with light microscopy using fluorophore-tagged antibodies against specific histone marks or proteins. Furthermore, multiple fluorophores can be used to examine the distribution of several compounds in the same sample, which is useful for determining colocalization. Although immuno-gold labeling can be used to pinpoint the location of specific proteins at the EM level, the procedure is more complicated. Immunofluorescence (IF) microscopy can also be used to visualize the expression levels of various factors within the cell, which can then be correlated to changes in chromatin organization. For example, expression levels of transcription factors as determined by fluorescence signal intensity can be correlated with changes in chromatin compaction. In terms of cost, light microscopes are relatively cheap compared to electron microscopes, which require high maintenance costs and occupy large areas of space.

With respect to disadvantages, the spatial resolution of light microscopy is relatively low compared to electron microscopy. Imposed by the wavelength of light, the theoretically maximum resolution of light microscopes is around 200 nm (Rapkin et al, 2012). Electron microscopes have much higher resolution, to the extent that individual nucleosomes can be distinguished and therefore can provide more detailed images of chromatin organization within the nucleus (Bazett-Jones et al, 1999). When using fluorophores for light microscopy studies, photobleaching can be problematic especially for low expression proteins since signal intensity decreases over time due to photon-induced chemical damage and covalent modification to fluorophores (Lichtman et al, 2005). This can occur through interaction of fluorophores with free radical oxygen species, which will cause loss of fluorescence. Furthermore, quenching through short-range interactions between fluorophores and their local environment can also reduce fluorescence intensity.

1.3.2 Electron microscopy

Electron microscopy (EM) enables one to examine biological structures at the sub-micron scale and has been instrumental in providing insight into chromatin structure. For example, EM first showed that chromatin assumes a beads-on-a-string nucleosomal arrangement (Woodcock et
al, 1976) and has provided information on the spatial organization of nucleosomes. EM studies showed that the conformation of oligonucleosomes depends on the surrounding ionic environment (Woodcock et al, 1997). Chromatin adopts an open zigzag configuration in low ionic conditions, while increasing the ionic strength induces compaction due to less repulsion between DNA strands by charge shielding. EM also provided an early description of heterochromatin as compact chromatin along the nuclear periphery and nucleolus (Rapkin et al, 2012). Although EM has provided high resolution data, it has limitations that must be noted. In order to resolve individual nucleosomes using conventional EM methods, chromatin often needs to be isolated from cells (Woodcock et al, 1997). This results in the loss of information regarding chromatin organization within the nucleus. Furthermore, evidence has indicated that the process of extracting chromatin alters the in situ conformation and thus extracted chromatin may not be a good representation of chromatin in vivo. In conventional EM, heavy atom contrast reagents such as uranium acetate are used to visualize biological materials due to poor electron scattering (Bazett-Jones et al, 2008). However, these stains do not bind each biological component with equal affinity such that some structures within the cell are stained disproportionately compared to others. Because of this, the signal intensity of cellular regions does not serve as a good representation of mass density. Furthermore, heavy atom stains form large precipitates that coat the sample and thereby limit the resolution of images. To circumvent some of the aforementioned limitations, an alternative form of EM was utilized to examine chromatin.

Electron spectroscopic imaging (ESI) is a form of transmission electron microscopy that generates images with electrons that have inelastically interacted with the sample (Bazett-Jones et al, 2008). The underlying physical principle is that some incident electrons passing through a sample will lose energy through ionization of the electrons within the atoms of the specimen. In the case of core electron ionizations, the energy loss events are element-specific. An electron spectrometer attached to the microscope will separate the electrons based on energy loss and generate an energy-loss spectrum that reflects the elemental content of the specimen. Element-specific maps can then be produced from electrons that lost specific amounts of energy. A phosphorus map can reveal the distribution of nucleic acids within the nucleus since the backbone of these molecules is enriched in phosphorus, while a nitrogen map shows the organization of both nucleic acid and protein. Since the atoms within the specimen itself serve as contrasting agents in ESI, no heavy atom stains are needed and thus higher resolution can be
achieved compared to conventional EM. As a result of this, individual chromatin fibres and nucleosomes can be distinguished using ESI without extracting chromatin from the nucleus, enabling one to obtain detailed information on chromatin organization in vivo. With respect to mass loss associated with analyzing a sample with the electron microscope, the rates of this process for elements found within biological specimens are similar (Dellaire et al, 2004). For this reason, information from ESI analysis is quantitative and nuclear structures can be distinguished based on their nitrogen to phosphorus ratios. For example, nucleoli have higher nitrogen to phosphorus ratios compared to heterochromatic regions. Because of the advantages it offers, ESI is suitable for imaging chromatin and its organization in the nucleus. In this study, the unique advantages of ESI were combined with IF to visualize specific chromatin domains within the nuclei of mouse pluripotent stem cells at high resolution. In correlative IF/ESI, cells are labeled with antibodies against a marker of interest such as the constitutive heterochromatin marker HP1α (Fig. 1). IF images of the nuclei are overlaid onto the corresponding low magnification EM images to identify regions rich in the marker. Higher magnification ESI micrographs are then taken in those regions to reveal the underlying chromatin structure.
Principle behind correlative IF/ESI. An immunofluorescence image of the nucleus of a mouse embryonic fibroblast cell labeled with antibody against the constitutive heterochromatin-associated protein HP1α (A). Low magnification ESI micrograph of the same nucleus (B). An overlap of the immunofluorescence image and ESI micrograph (C). The white circle marks the heterochromatic region shown in the ESI-generated image (D). Compact chromatin is seen in the heterochromatic region. Chromatin and protein-based structures are colored yellow and blue respectively.
1.4 ESI and chromatin

In terms of applications, ESI provides the spatial resolution to study the composition of nucleoprotein complexes that may be too large to be examined through X-ray crystallography or magnetic resonance spectroscopy (Bazett-Jones et al, 1999). ESI can be used to distinguish and measure the phosphorus and nitrogen contents of nucleoprotein complexes, which will provide information on the stoichiometric ratio of nucleic acid:protein within the complexes. ESI has also been proven useful for examining the chromatin organization within different cell types (Rapkin et al, 2012) (Fig. 2). Although the genomes of different mouse cell types are similar in sequence, the chromatin configuration differs significantly which demonstrates that chromatin organization is not solely dictated by sequence. In mouse ESCs (Fig. 2A) chromatin fibres are highly dispersed throughout the nucleus with little or no regions of compact chromatin (Fussner et al, 2011). As mentioned previously, structural chromatin proteins such as HP1 and linker histone in ESCs have been shown to associate with chromatin in a loose and hyperdynamic way (Meshorer et al, 2006). Furthermore, the genome in ESCs is globally transcriptionally active such that normally silent regions and tissue-specific genes are transcribed at low levels (Efroni et al, 2008). All of these characteristics may enable ESCs to easily adopt new chromatin configurations during cell differentiation. EpiSCs, which is more differentiated relative to ESCs, have compact chromatin clusters scattered throughout the nucleoplasmic space (Fig. 2B). At a higher stage of chromatin compaction, chromatin in hepatocytes (Fig. 2C) and kidney epithelial cells (Fig. 2D) forms compact domains along the nucleolus and nuclear periphery with large areas of the nucleus devoid of chromatin. In the extreme case of chromatin compaction, chromatin in mature B cells (Fig. 2E) is mainly packaged into large blocks of heterochromatin. From the above observations, it appears that the chromatin landscape is cell-type dependent. Chromatin in the large nuclei of differentiated cells appears to form numerous compact domains that leave large portions of the nuclear volume devoid of chromatin. However, chromatin forms few or no compact regions and occupies a higher percentage of the relatively small nuclear volume in ESCs. For example, the percentage of the nuclear volume occupied by chromatin for ESCs and heptocytes are 37% and 14%, respectively (Rapkin et al, 2012). These observations support the notion that chromatin compaction is independent of cell volume and also led us to rethink the long-believed role of chromatin compaction as a solution for fitting the genome inside the nucleus. Rather, a more important role of heterochromatin might be to serve as
functional or structural domains. Among the different cell types, the chromatin organization in mouse ESCs appears to be unique in that few or no regions of compact chromatin are present and an investigation into the factors that contribute to this characteristic chromatin organization will help us better understand the pluripotent state of these cells.
Figure 2. Chromatin organization in different mouse cell types

Images were contributed by the Bazett-Jones lab. In the ESI-generated images, chromatin and protein-based structures are colored yellow and blue respectively. Mouse ESCs possess a chromatin organization characterized by highly dispersed chromatin fibres with few compact domains (A). EpiSCs have compact chromatin clusters distributed throughout the nucleoplasmic space (B). Chromatin in heptocytes and kidney epithelial cells form compact domains along nucleoli and the nuclear periphery (C, D). In mature B cells, chromatin is mostly situated within large compact domains with large area of the nucleoplasmic space devoid of chromatin (E).
1.5 Core transcription factors

It is likely that numerous factors influence chromatin structure in ESCs. One possible approach to identify these factors would be to first examine the effects of main transcription factors on chromatin organization and then further explore downstream chromatin-associated complexes. Key regulators in ESCs known as the core transcription factors, which include Oct4, Sox2, and Nanog, function together to form an autoregulatory loop by positively regulating their own promoters and exert their influence mainly by promoting the expression of factors required to maintain pluripotency as well as repressing lineage-specific transcription factors (Jaenisch et al, 2008). Furthermore, these transcription factors share a large fraction of their target genes, indicating they generally do not regulate their targets independently but instead function together. Each of the core transcription factors and their connections to chromatin in ESCs will be introduced below.

Oct4 is a member of the POU family of transcription factors and an essential component for maintaining pluripotency (Niwa et al, 2000). Ortholog genes of this factor share a high level of sequence conservation and have been found in species such as human, mouse, and bovine (Pesce et al, 2001). At least in mice, its expression begins at the blastomere stage and is later restricted to pluripotent stem cells such as cells within the inner cell mass of embryos. Deficiency of Oct4 in ESCs will result in differentiation into trophectoderm cells and subsequent cell death, while overexpression will promote differentiation into primitive endoderm and mesoderm cells (Niwa et al, 2000). Therefore, the expression level of Oct4 needs to be within a certain range to maintain pluripotency. Furthermore, the reprogramming of somatic cells into induced pluripotent stem cells requires the forced expression of various transcription factors including Oct4, further demonstrating the importance of this factor in pluripotency (Takahashi et al, 2006). Oct4 can act as a suppressor of differentiation as well as be involved in the transactivation of certain genes. Transactivation can occur by the linking of Oct4 and the transcriptional machinery through certain coactivators (Pesce et al, 2001). Oct4 has also been shown to heterodimerize with Sox2 to regulate numerous genes in ESCs. For example, dimerization of Oct4 and Sox2 is required for binding to the enhancer of FGF-4 gene needed for transcriptional activation (Ambrosetti et al, 1997).
Sox2 is a SRY-related HMG box transcription factor that is necessary to maintain pluripotency and is also required for the generation of induced pluripotent stem cells (Rizzino, 2009). Unlike Oct4 and Nanog, the expression of Sox2 is not limited to pluripotent cells and is also seen in early neural cells (Eminli et al, 2008). With respect to the importance of Sox2 during development, inactivation of the Sox2 gene results in the death of the embryo at the peri-implantation stage (Avilion et al, 2008). Furthermore, knock down of Sox2 by RNA interference causes ESCs to differentiate primarily into trophectoderm-like cells through inactivation of Oct4, indicating that Sox2 has a role in regulating Oct4 expression level (Chew et al, 2005). Sox2 is known to form a heterodimer with Oct4 in ESCs to regulate their own genes as well as numerous others involved in self-renewal and pluripotency (Rizzino, 2009). However, the mechanism through which the Oct4-Sox2 regulates transcription still remains unclear.

Nanog is a homeodomain-containing protein that promotes the maintenance of the pluripotent state in ESCs (Mitsui et al, 2003). In terms of structure, Nanog consists primarily of three domains, namely the N-terminal, homeodomain, and C-terminal domains (Pan et al, 2007). The serine-rich N-terminal domain is comprised of 96 amino acids and the C-terminal contains a well-conserved tryptophan repeat domain that functions as a trans-activator. Nanog is crucial for mouse development since Nanog-null embryos do not develop beyond implantation (Mitsui et al, 2003). Expression of Nanog in mouse embryo begins within the interior cells of the morulae stage and is then later restricted to epiblast cells at the blastocyst stage (Pan et al, 2007). ESCs overexpressing this factor are less prone to differentiation and can propagate in the absence of LIF, a factor usually required for culturing wild-type ESCs (Chambers et al, 2003). In the absence of Nanog, ESCs are more prone to differentiate into primitive endoderm cells. Although Nanog is not part of the minimal cassette of transcription factors required to reprogram mouse somatic cells to iPSCs, the expression of the endogenous Nanog gene during the final stage of reprogramming is imperative for achieving “ground-state” pluripotency (Silva et al, 2009). With respect to functions in ESCs, Nanog binds to >90% of promoter regions that are also bound by Sox2 and Oct4, demonstrating the intimate connection between the core transcription factors (Pan et al, 2007). Nanog can promote pluripotency by upregulating the expression of pluripotency-associated factors such as Rex1 (Shi et al, 2006) and can prevent ESC differentiation through the repression of factors such as the endoderm-associated GATA6 (Mitsui et al, 2003). Although the mechanism through which Nanog promotes pluripotency is
still unclear, ongoing research on transcription networks and interaction partners of Nanog will shed light on this matter.

Regarding their importance on chromatin structure, the core transcription factors are known to interact with chromatin-remodeling complexes that may be involved in the maintenance of an open chromatin state in ESCs. An example is the esBAF complex, which possesses ATP-dependent chromatin remodeling activity and occupies the promoters of numerous genes encoding pluripotency-associated factors such as Klf4 and Sall4 (Ho et al, 2009). Subunits of the BAF complex are known to interact with the core transcription factors and bind to their overlapping regions. Another factor known to be regulated by the core transcription factors is the ATP-dependent helicase Chd1. ESCs depleted of Chd1 show an increased number of heterochromatic regions, as marked by H3K9me3 (Gaspar-Maia et al, 2009). Through unknown mechanisms, possibly involving the aforementioned chromatin-associated factors, the expression levels of core transcription factors were discovered to correlate with chromatin dispersion. From light microscopy studies, high Nanog expression levels have been correlated with more open chromatin structures in constitutive heterochromatic domains in pluripotent ESCs and embryos (Fussner et al, 2011). In other words, high Nanog-expressing cells possess more disrupted heterochromatin compared to low Nanog-expressing cells. Similar to the in vitro ESCs, epiblast cells from early mouse blastocysts have chromatin that is generally dispersed throughout the nucleoplasmic space (Ahmed et al, 2010). However, the corresponding cells from Oct4-null embryos lose pluripotency and chromatin forms numerous compact domains, indicating that Oct4 and the maintenance of the pluripotent state are crucial for maintaining dispersed chromatin structures in ESCs. From these data, one can deduce a close connection between core transcription factors and chromatin organization in ESCs.

1.6 Summary and hypothesis

The chromatin organization of mouse ESCs seems to be unique among the different cell types since it is characterized by dispersed global chromatin with few compact regions. Although both ESCs and EpiSCs are pluripotent, their chromatin organizations are strikingly different and numerous factors may contribute to this difference (Fig. 3). The identification and elucidation of these factors would enable us to better understand the relationship between chromatin and
pluripotency. Through light microscopy studies (Fussner et al, 2011), Nanog expression was shown to correlate with heterochromatin dispersion in mouse ESCs, leading us to believe that Nanog is involved in the regulation of chromatin organization. However, due to the resolution restriction imposed by light, the underlying chromatin organization at those heterochromatic regions is still uncertain unless one is able to visualize the arrangement of chromatin fibres. In this study, the chromatin organization within Nanog-null, heterozygous, and overexpressing mouse ESCs were studied at the sub-micron level with ESI to determine the relationship between chromatin configuration and Nanog expression. Furthermore, Nanog was also overexpressed in EpiSCs to see whether the factor can disperse chromatin in cells further down the developmental pathway. My hypothesis is that the core transcription factor Nanog is involved in maintaining the characteristic “open” chromatin architecture in ESCs.
Figure 3. Difference in chromatin organizations between ESCs and EpiSCs

Both images were ESI-generated. Chromatin and protein-based structures are colored yellow and blue respectively. Mouse embryonic stem cells have an “open” chromatin organization characterized by highly dispersed chromatin fibres. Through changes in the growth media, ESCs can differentiate into EpiSCs and chromatin will cluster into compact domains. Research in identifying factors responsible for maintaining the dispersed chromatin architecture in ESCs is still under progress.
Chapter 2

2 Materials and Methods

2.1 Cell culture

Sall-KO, Nanog-null, heterozygous, and overexpressing ES cells were grown on feeders (mitomycin C treated MEFs day 15.5) in DMEM supplemented with 10% FBS, L-glutamine, penicillin-streptomycin antibiotics, sodium pyruvate, beta-mercaptoethanol, non-essential amino acids and recombinant LIF. EpiSCs were grown in chemically-defined media supplemented with activin A and FGF2. EpiSCs with an integrated Dox-inducible Nanog construct were made to overexpress Nanog through treatment with Dox for 48 hrs.

2.2 Correlative immunofluorescence microscopy and electron spectroscopic imaging

All the procedures were done at room temperature unless stated otherwise. Fresh cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences (EMS)) in phosphate buffered saline (PBS) for 20 minutes. Cells were washed three times with PBS and stored in the buffer overnight at 4°C. Samples were washed in PBS and permeabilized with PBS containing 0.5% Triton X-100 for 5 minutes. This was followed by PBS washes and an hour incubation with PBS containing 5% donkey serum. The following primary antibodies were used: mouse anti-HP1α (1:200, Upstate), rabbit anti-H3K9me3, and rabbit anti-Nanog (1:200, ReproCELL) overnight at 4°C. Samples were washed in PBS followed by an 1 hour incubation with the secondary antibodies of Cy3-labelled donkey anti-mouse/rabbit (1:400) and Cy5-labelled donkey anti-mouse/rabbit (1:200). Cells were washed in PBS and post-fixed with PBS containing 1% glutaraldehyde for 5 minutes. Samples were washed in PBS and then with distilled water. For dehydration, cells were incubated in a series of ethanol steps of 30, 50, 70, and 90% for 30 minutes in each step. Samples were placed in 100% ethanol overnight at 4°C. Cells were washed twice with 100% ethanol, incubated in Quetol 651 resin (EMS) for 1 hour, 3.5 hours in Quetol mix (described below), and incubated at 65-70°C for at least 24 hours. To prepare 200 mL of Quetol mix, add 70 mL of Quetol 651 resin, 108 mL of NSA (EMS), 24 mL of NMA (EMS), 4 mL of DMP-30 (EMS), and stir the contents for at least half an hour. Colonies of cells were cut
out from blocks of embedded samples and cut into 70 nm sections with an Ultracut UCT microtome (Leica Microsystems Inc.). Immunofluorescence images were obtained from the cells on sections as described below (under Immunofluorescence Imaging). Sections were carbon coated with 3 nm carbon films and examined with the electron microscope. Electron spectroscopic images were collected from these cells as described below (under electron spectroscopic imaging).

2.3 Immunofluorescence imaging

A Leica DMRA2 microscope with a CCD camera (Hamamatsu) was used to collect fluorescence images of cells. The software used to operate the microscope was OpenLab 3.5.1. Images collected were processed with Adobe Photoshop CS3 and used for correlative IF/ESI.

2.4 Electron spectroscopic imaging

Electron micrographs were collected with a transmission electron microscope (Tecnai 20, FEI) equipped with a post-column energy filter (Gatan Inc.). To obtain elemental-specific maps, images have to be collected before and at the ionization edge of the element on the energy loss spectrum (Bazett-Jones et al, 1999). Using nitrogen as an example, an image collected at the ionization edge of nitrogen is enhanced for that specific element but also contains background signal derived from electrons that did not interact with nitrogen atoms. To eliminate this background signal, a mathematical comparison between the pre- and post-edge (close to the edge) images can be performed to generate a nitrogen-specific map. To obtain nitrogen maps, pre- and post-edge images were collected with the imaging filter set at 385 and 415 eV, respectively. As for phosphorus maps, pre- and post-edge images were collected at 120 and 155 eV, respectively. Low magnification images of whole cells were also taken at 155 eV for correlation with immunofluorescence images. To better visualize the distribution of different elements relative to one another, phosphorus and nitrogen maps can be combined into a single image. To achieve this, phosphorus maps were subtracted from nitrogen maps to generate net-nitrogen maps with signals in chromatin-overlapping regions equal to zero and thus shows the distribution of proteins not associated with chromatin. Phosphorus images were then colored
yellow and net-nitrogen images colored blue. In the last step, phosphorus images were overlaid onto the net-nitrogen images for presentation. The resultant images have yellow regions that represent nucleic acid-rich areas and blue regions that are enriched in proteins that are not associated with chromatin. Softwares used to process the images are Digital micrograph (Gatan), ImageJ, and Adobe Photoshop CS3.

2.5 Cluster analysis

Cluster analysis is used to measure chromatin cluster sizes of global chromatin (devoid of constitutive heterochromatic marks such as HP1α). Raw phosphorus maps were high-passed in Adobe Photoshop CS3 to minimize the uneven illumination found in many images, possibly due to problems such as imperfect alignment of the electron beam in the microscope. Images were then filtered using the median option in ImageJ with the radius set to 2 pixels (Fig. 4). This reduced the noise in the images by replacing the value of each pixel with the median intensity value of neighboring pixels. Background signals were removed through auto-thresholding (MaxEntropy option) that sets the threshold levels based on the image’s histogram. The Maxentropy thresholding option was chosen since it removed enough background noise to give discrete chromatin clusters. The phosphorus maps were then subsequently binarized to generate black and white images that show chromatin clusters with discrete boundaries. Using the “analyzed particles” function in ImageJ, the average chromatin cluster size (in pixels) was measured in regions of the nucleus outside of heterochromatic regions, nucleoli, and the nuclear periphery. The minimum particle size considered to be a cluster was set at 100 pixels to ignore individual nucleosomes that may skew the results due to their large numbers. Cluster sizes in pixels were then converted to units of nm² (1 pixel = 1.43 nm²) for presentation. To show statistical significance between the cluster sizes of two cell types, the non-parametric Mann-Whitney test was used with the null hypothesis rejected when P<0.05. The Mann-Whitney test was chosen because it does not assume the data to have a particular probability distribution and thus is more widely applicable compared to parametric methods such as the Student’s t-test.
Figure 4. Cluster analysis

A schematic showing the steps in cluster analysis. The first step involves removing the background signal in high-passed phosphorus maps with the “filter” and “auto-threshold” functions in ImageJ. Although there are several options for auto-threshold such as the default option, the max entropy option was chosen because it removed enough background noise to give discrete chromatin clusters. Threshold-images were then binarized and, using the “analyzed particles” function, the average chromatin cluster size (in pixels) was measured in regions that exclude heterochromatic regions, nucleoli, and the nuclear periphery. Average chromatin cluster sizes can then be converted to units of nm$^2$ and be compared between cell types.
2.6 Phosphorus density analysis

Phosphorus density analysis was used to calculate the compaction within heterochromatic regions (enriched in marks such as HP1α). Phosphorus maps were high-passed to minimize uneven illumination within the images. The following steps were performed with ImageJ. Images were median-filtered with the radius set to 2 pixels to minimize noise (Fig. 5). Average signal intensity values were measured for heterochromatic regions (enriched in HP1α) as well as background regions that are devoid of chromatin. The ratio between heterochromatin and background values were calculated to obtain an indication of chromatin fibre density in heterochromatic regions after taking into account the background noise.
Figure 5. Phosphorus density analysis

A schematic showing the steps in phosphorus density analysis. The first step involves determining the heterochromatic region (marked by white circle) within the nucleus using both low-magnification ESI-generated image and an IF image showing signals from HP1α-enriched sites. Next involves smoothing out the high-passed phosphorus map of the heterochromatic region with the “filter” function in ImageJ. The average phosphorus intensities within the heterochromatic region (marked by red line) and also in the background (area devoid of chromatin) were measured and the ratio between the two was calculated to give a measure of the chromatin fibre density of heterochromatin.
Chapter 3

3 Results

Dr. P. Rugg-Gunn and Uglijesa Djuric generated and cultured the cell lines examined in the following experiments. Dr. P. Rugg-Gunn performed the IF and western blot experiments shown in figure 7. Uglijesa Djuric provided the western blot results shown in figure 6.

3.1 Chromatin compaction in heterochromatic regions correlates inversely with the Nanog expression level in mouse embryonic stem cells

A connection between Nanog and chromatin organization has been shown from a previous study based on fluorescence microscopy, where the chromatin compaction level in heterochromatic regions and the Nanog expression level in mouse ESCs were shown to have an inverse relationship (Fussner et al, 2011). In other words, cells expressing high levels of Nanog have less compact heterochromatin. For that study, the DAPI signal intensity of heterochromatic foci relative to background was used as the measure for chromatin compaction. DAPI is a fluorescent stain that binds preferentially to A-T rich regions and is known to form foci at constitutive heterochromatic regions when used in mouse cells. Although DAPI staining does provide a rough measure of chromatin compaction, a more accurate method to determine chromatin compaction would be to directly visualize the chromatin fibre organization at the sub-micron level. With correlative IF/ESI, the underlying chromatin structure at sites enriched in particular factors or histone marks can be studied at a resolution where individual nucleosomes can be distinguished. To study the relationship between Nanog and the chromatin organization in ESCs, I examined Nanog-null, heterozygous, and overexpressing cells with ESI. Nanog-heterozygous and null ESCs were generated through the replacement of exons 2-4 of one or both Nanog alleles, respectively, with drug resistance genes. Nanog-overexpressing ESCs were generated through random integration of a Nanog cDNA transgene. Through western blot analysis (Fig. 6i), it was confirmed that Nanog expression is absent in Nanog-null cells and substantial in Nanog-overexpressing cells. From qualitative examination of the phosphorus maps generated through ESI, the difference in the chromatin organization between the ESCs was in the compaction of constitutive heterochromatic regions, indicated by an enrichment of H3K9me3 as
determined by IF (Fig. 6ii). To quantify the compaction of heterochromatic regions, phosphorus density analysis was chosen to measure the average intensity of phosphorus signal within the region. The phosphorus intensity values in heterochromatic regions were then divided by the intensity values in background regions (areas devoid of chromatin) to obtain ratio values that can be used to compare the chromatin compaction in heterochromatic regions between different images and cell types. The analysis revealed that the chromatin density within constitutive heterochromatic regions differs between cells expressing varying levels of Nanog. Both Nanog null and heterozygous ESCs have the most compact heterochromatin with median chromatin density values around 2.40, followed by wild-type cells at 2.07, and Nanog-overexpressing cells have the least compact heterochromatin at 1.76 (Fig. 6iii). These differences were shown to be statistically significant (P < 0.05) with the Mann-Whitney test. From these results, an inverse relationship between the Nanog expression level and heterochromatin compaction is seen and this agrees with previous studies performed with light microscopy. This supports my hypothesis that Nanog is involved in maintaining the characteristic open chromatin configuration of ESCs.
Fibre Density of Heterochromatin

Wild-type

Nanog-overexpressing

Nanog-null

**ii**

**iii**

![Graph showing fibre density of heterochromatin](image)

- EpSC
- ESC
- Nanog-null ESC
- Nanog-heterozygous ESC
- Nanog-overexpressing ESC

* indicates a significant difference.
Figure 6. Inverse relationship between Nanog expression level and compaction of heterochromatin in mouse ESCs

Western blot showing the expression of Nanog in Nanog-null, heterozygous, and overexpressing cells (i). IF images and ESI micrographs of heterochromatin in ESCs expressing different levels of Nanog (ii). The left column shows IF images of nuclei with heterochromatic loci marked by fluorophore-tagged antibodies against H3K9me3. The middle column shows low-magnification ESI images of nuclei with the corresponding IF images overlapped on top and white circles indicate the specific heterochromatic regions shown on the right. The right column shows ESI images of the encircled heterochromatic regions with chromatin and protein-based structures colored in yellow and blue, respectively. Box plots of chromatin density in heterochromatic regions in ESCs expressing different levels of Nanog (iii). Phosphorus density analysis was used to quantify chromatin density and 20 cells were analyzed for each cell type. Nanog-null & heterozygous cells have more compact heterochromatin compared to wild-type ESCs and Nanog-overexpressing cells have less compact heterochromatin relative to wild-type ones. The asterisk denotes statistically significant differences between all the cell types except between Nanog-heterozygous and -null cells. The Asterisk also indicates P < 0.05 as determined by the Mann-Whitney test.

3.2 Dispersed global chromatin in Nanog-overexpressing epiblast stem cells

Although both ESCs and EpiSCs are derived from embryos close in terms of developmental stage, major differences have been found between the two cell types. Regarding growth characteristics, EpiSCs form relatively flat colonies and have a longer doubling time compared to ESCs (Pauklin, 2011). Furthermore, EpiSCs are grown in culture media supplemented with different factors, namely activin and Fgf (Guo et al, 2009). As for developmental potential, EpiSCs cannot contribute to chimaeric mice and are thus considered further down the developmental pathway than ESCs (Brons et al, 2007). All of these differences allow us to distinguish between two types of pluripotency, which are the “naïve” and “primed”
states (Nichols et al, 2009). The difference in pluripotency may also be reflected in chromatin organization since Ahmed and colleagues (2010) showed by ESI that chromatin in EpiSCs is organized into many compact domains throughout the nucleus, whereas chromatin in ESCs is more uniformly dispersed. This difference may be due to the lower expression level of Nanog in EpiSCs compared to ESCs (Han DW et al, 2010). If Nanog is involved in maintaining open chromatin, then an overexpression of the transcription factor in EpiSCs would theoretically decrease the compaction of chromatin. To test this idea, Nanog-overexpressing EpiSCs were generated by integrating doxycycline-inducible constructs containing Nanog and GFP genes into the genome of EpiSCs (Fig. 7i). Upon addition of doxycycline (Dox), the Nanog and GFP genes will be transcribed due to activation through the binding of the tetracycline transactivator to the TetO operator sequence. GFP was used as a marker for the expression of the genes within the construct. Immunofluorescence microscopy experiment showed that upon the addition of Dox to the construct-integrated EpiSCs, expression of GFP and Nanog were induced (Fig. 7ii). This indicates that overexpression of Nanog is primarily from transcription of the Nanog gene within the construct rather than the endogenous gene. However, no or low expression of GFP and Nanog were seen in Dox-untreated cells. A western blot was also performed to ensure expression of Nanog upon treatment with Dox (Fig. 7iii). Nanog expression in Dox-untreated and wild-type EpiSCs are minimal, as shown by the faint bands detected with an α-Nanog antibody. However, upon the addition of Dox, we see a thick band indicating high expression of Nanog. The expression of Oct4 was present in all cell types, ensuring that the cells were maintaining their pluripotent status. These results confirm that the Dox-inducible system is functioning as expected.
Figure 7. Nanog overexpression in EpiSCs upon treatment of Doxycycline

Data contributed by Dr. Peter Rugg-Gunn. Doxycycline-inducible construct containing Nanog and GFP genes (i). Upon the addition of Dox, the genes will be activated due to the binding of tetracycline transactivator to the TetO operator sequence. Immunofluorescence microscopy experiment showing that treatment with doxycycline (Dox) for 48 hrs results in overexpression of Nanog in EpiSCs with Dox-inducible constructs containing the Nanog and GFP genes (ii). The same result is revealed in a Western blot analysis (iii).
Through analysis by ESI, +Dox EpiSCs were shown to possess compact chromatin at constitutive heterochromatic regions (Fig. 8i-G), which is no different from -Dox EpiSCs (Fig.8i-C). However, the chromatin outside of heterochromatic regions, which will be called global chromatin, in +Dox EpiSCs appears to be dispersed and fairly uniform (Fig.8i-H). This is in contrast to the chromatin configuration seen in -Dox EpiSCs, where even global chromatin forms compact clusters (Fig.8i-D). To quantify the difference in chromatin compaction of global chromatin, cluster analysis was used to calculate and compare the sizes of chromatin clusters between +Dox and -Dox EpiSCs. In this analysis, background signal is filtered from phosphorus maps and the images are subsequently binarized. Chromatin cluster sizes were then measured in regions outside of heterochromatic regions and nucleoli. From the analysis (Fig. 8ii), chromatin clusters in EpiSCs treated with Dox are significantly smaller than clusters in -Dox cells as determined by the Mann-Whitney test (P<0.005). As a control, chromatin clusters in GFP-overexpressing EpiSCs were also analyzed and are not significantly different from control cells. These results indicate that there is an increase in global chromatin dispersion in Nanog-overexpressing EpiSCs, which support my hypothesis that Nanog is involved in the maintenance of an “open” chromatin architecture in ESCs.
i

EpiSCs (-Dox)

EpiSCs (+Dox)

Heterochromatin

Global chromatin

ii

Cluster Size (nm$^2$)

ESC  EpiSCs  -Dox EpiSCs  +Dox EpiSCs  -GFP EpiSCs  +GFP EpiSCs

*
Figure 8. Nanog overexpression correlates with dispersal of non-heterochromatin in EpiSCs

EpiSCs with integrated Dox-inducible Nanog constructs were immunofluorescently labeled with antibodies against the constitutive heterochromatin marker HP1α (i).

Immunofluorescence images showing enrichment of the marker in heterochromatic regions in the nuclei of both -Dox (A) and +Dox EpiSCs (E). Regions (red) enriched in the marker are marked on the low magnification EM images of the nuclei (B,F). White and dotted circles indicate heterochromatic and non-heterochromatin regions, respectively, seen in the ESI-generated images (C,D,G,H). Chromatin and protein-based structures are colored yellow and cyan, respectively. In –Dox EpiSCs, i.e. no Nanog overexpression, heterochromatin is highly compact (C) and domains of compact chromatin are present throughout the nucleoplasmic space (D).

Although heterochromatin is also compact in +Dox EpiSCs (G), global chromatin is dispersed rather than congregated into compact domains (H). Box plots of global chromatin cluster sizes for different cell types (ii). GFP EpiSCs are cells with Dox-inducible constructs containing a GFP gene and were used as controls. EpiSCs treated with Dox possess smaller global chromatin cluster sizes compared to control cells. Asterisk indicates P =0.0004 (< 0.005) as determined by the Mann-Whitney test.

3.3 Knockout of Sall1 increases global chromatin compaction in mouse ESCs

From data presented above, it appears that Nanog plays a role in maintaining open chromatin in mouse ESCs. To further explore other factors that may affect chromatin organization in ESCs, I examined the multi-zinc finger transcription factor Sall1. This factor is important for kidney organogenesis during mouse development and is known as a transcriptional repressor that can bind to major satellite sequences within constitutive heterochromatic regions (Nishinakamura et al, 2001; Yamashita et al, 2007). Furthermore, Sall1 can physically interact with Nanog and Sox2 and is involved in the regulation of Nanog expression since depletion of Sall1 leads to down-regulation of Nanog (Karantzali et al, 2011). Genome-wide promoter ChIP-
on-chip analysis revealed that Nanog and Sall1 share a large number of common target genes, many of which are related to self-renewal and differentiation. Since Sall1 interacts with core transcription factors and binds heterochromatin, it is a potential player in the regulation of chromatin organization in mouse ESCs. To explore the connection between Sall1 and chromatin, Sall1-Knockout ESCs were examined through ESI and were compared to wild-type ESCs. Global chromatin had higher compaction levels in Sall1-KO cells compared to wild type ones and this was confirmed with cluster analysis (Fig. 9). The median chromatin cluster size in Sall1-KO cells is 397 nm² compared to 289 nm² in wild-type cells, which is significantly different as determined by the Mann-Whitney test (P < 0.005). This result supports our notion that Sall1 is involved in the regulation of chromatin organization, possibly in conjunction with Nanog.
Figure 9. Mouse Sall1 KO ESCs possess more compact global chromatin relative to wild-type ESCs

ESI-generated images of global chromatin distribution within the nuclei of wild-type and Sall1 KO ESCs (i). Chromatin and protein-based structures are colored yellow and cyan, respectively. Global chromatin forms more compact domains in Sall1 KO ESCs compared to wild-type cells. This has been verified with cluster analysis (ii). Asterisk represents statistical significance (P value < 0.005, Mann-Whitney Test).
Chapter 4

4 Discussion

Studies have shown that the core transcription factors in ESCs interact with chromatin remodeling factors and that the chromatin architecture in mouse ESCs is drastically different from that of other cell types in that very few compact chromatin domains are present. These findings support the idea that there is a close connection between pluripotency and chromatin, which still remains largely unexplored and clarification of this area may help us understand more about the pluripotent state. Previous research has shown that the expression level of Nanog, a core transcription factor, is inversely correlated to the heterochromatin density in mouse ESCs (Fussner et al, 2011). This observation was further explored in this study by examining chromatin organization at a sub-micron level using ESI. Furthermore, the effect of Nanog overexpression on the chromatin configuration in EpiSCs was examined to test whether Nanog can promote chromatin dispersion in cells at a later developmental stage. Through these experiments, we can gain a better understanding on the relationship between factors important for pluripotency and chromatin architecture in ESCs.

Through quantitative examination of ESI-generated images with phosphorus density analysis, Nanog expression level in ESCs was shown to have an inverse relationship with the chromatin compaction level at heterochromatic regions. In Nanog-overexpressing ESCs, the chromatin at heterochromatic regions is more dispersed compared to wild-type cells. On the other hand, in Nanog-null/heterozygous cells, chromatin forms relatively compact domains at heterochromatic regions. These observations support my hypothesis that Nanog promotes the maintenance of an “open” chromatin architecture in ESCs. Variegation of Nanog expression is known to occur in ESC populations, where there are high Nanog-expressing cells with enhanced self-renewal capacity and low-Nanog expressing cells that are more susceptible to differentiation (Mitsui et al, 2003; Chambers et al, 2007). Together with my findings, this implies that a population of ESCs consists of cells at various levels of pluripotency, where some cells are more “naïve” pluripotent with high Nanog expression level and disperse heterochromatin and others that are less “naïve” pluripotent with low Nanog expression level and compact heterochromatin. The significance of this is that it shows a link between a core transcription factor and large-scale chromatin organization in mouse ESCs. Although the importance of heterochromatin structure
on the pluripotent state in stem cells is still unclear, a hypothesis might be that dispersed heterochromatin may be more accessible to transcriptional machinery and might allow the transcription of loci that are normally silent in more differentiated cell types. For example, transcription of LINEs, SINEs, retrotransposons, and satellite repeat sequences is notably higher in ESCs compared to differentiated cell types such as MEFs and C2C12 muscle cells (Efroni et al, 2008). This contributes to the elevated global genome transcription in ESCs and could be important for pluripotency.

Although the mechanism through which Nanog affects chromatin organization remains unclear, one can suggest several possible mechanisms. One possibility is that chromatin dispersion might be the result of upregulation of chromatin-remodeling activities through expression of Nanog and chromatin-modifying factors. The euchromatin-associated ATP-dependent helicase Chd1 is known to target genes involved in transcription and chromatin organization (Gaspar-Maia et al, 2009). In addition, it is required for the maintenance of open chromatin in ESCs. Regions rich in the heterochromatin mark H3K9me3 were significantly increased in Chd1 RNAi ESCs compared to wild-type ESCs. Furthermore, from fluorescence recovery after photobleaching (FRAP) experiments, the exchange of histone H1 decreased significantly in heterochromatin of Chd1 RNAi ESCs compared to wild-type cells, possibly indicating a more compact structure. It is possible that Nanog overexpression will cause a corresponding increase in the expression level of Chd1, which will antagonize the formation of heterochromatin and maintain open chromatin in ES cells. Nanog has also been linked to the kinase Jak2 (Griffiths et al, 2011). The enzyme is known to phosphorylate tyrosine 41 on histone 3 and this modification is known to interfere with the binding of HP1α to chromatin. HP1α is a chromatin-binding protein enriched in constitutive heterochromatin and can potentially mediate chromatin compaction via homodimerization through its chromoshadow domain thereby linking nearby nucleosomes or by recruiting chromatin-modifying factors (Grewal et al, 2007). It is possible that Nanog overexpression in ESCs upregulates Jak2, which will phosphorylate histones in heterochromatic regions and decrease the amount of HP1α binding leading to chromatin dispersal. Overall, the main idea is that Nanog might promote chromatin dispersal in heterochromatin through a single or combination of pathways such as upregulation of chromatin-remodeling enzymes and promotion of dissociation of structural proteins from chromatin.
EpiSCs generally possess a chromatin architecture characterized by compact chromatin clusters scattered throughout the nucleoplasmic space (Ahmed et al, 2010). Through ESI analysis, Nanog-overexpressing EpiSCs were shown to have a more dispersed global chromatin structure characterized by highly scattered chromatin fibres. A possible explanation is that an increase in histone acetylation levels induced by Nanog overexpression would disperse the numerous compact chromatin clusters normally seen in the nucleoplasmic space of EpiSCs. Acetylation on histones affects chromatin structure directly by removing positive charges from lysine residues, which reduces the neutralization of negative charges on DNA by histones and therefore promotes the dispersal of chromatin fibres. In agreement with this, a study has shown that an increased level of histone acetylation is correlated with an increase in plasticity and protein dynamics in euchromatin (Melcer et al, 2012). In contrast to global chromatin, heterochromatin is still composed of compact chromatin in Nanog-overexpressing EpiSCs. No chromatin dispersal occurred maybe because the dynamics of chromatin-associated factors such as HP1α and histone H1 were not increased due to counteraction by unknown factors present in EpiSCs. It is possible that EpiSCs require compact heterochromatin to silence certain genomic loci to remain in their “primed” pluripotent state.

In addition to Nanog, another factor involved in chromatin organization in mouse ESCs is Sall1. In Sall1 KO ESCs, chromatin clusters into compact domains throughout the nucleoplasmic space, which shows that Sall1 is required to maintain an “open” global chromatin architecture in ESCs. Since Nanog-null ESCs did not display compaction of global chromatin, this implies that Sall1 might affect chromatin organization through a different mechanism, possibly involving histone acetylation. Regarding heterochromatin structure, since Sall1 can bind to major satellite sequences, it might have an effect on the organization of heterochromatin but further study is required to shed light on this matter.

5 Conclusion

Mouse ESCs possess a chromatin organization characterized by highly dispersed chromatin fibres, which may be important for the maintenance of the pluripotent state. Although the mechanism for the formation of this chromatin architecture still requires further elucidation,
the data presented here provides evidence that the transcription factors Nanog and Sall1 can impact global chromatin structure. Loss of Nanog expression in ESCs is correlated with an increase in compaction of heterochromatin and overexpression of Nanog in EpiSCs promotes global chromatin dispersion. Furthermore, knockdown of Sall1 in ESCs was correlated with increased compaction of global chromatin. These observations support my hypothesis that Nanog is involved in the maintenance of an “open” chromatin organization in mouse ESCs.

6 Future Directions

In the future, hopefully more experiments will be performed to further support my hypothesis that Nanog plays an important role in the regulation of chromatin organization in mouse ESCs. From ESI analysis, Nanog-null ESCs displayed more compact heterochromatin compared to wild-type cells and it will be interesting to test whether restoring Nanog expression will cause chromatin dispersion at these sites. This can be accomplished by either inserting a construct containing the Nanog gene into the genome or by transfecting cells with a plasmid containing the gene.

To gain more insight into the mechanisms for compaction of heterochromatin in ESCs, the expression levels of various chromatin-associated factors such as Chd1 and Jak2 can be measured in Nanog-null ESCs through microarrays. Downregulation of particular factors in the absence of Nanog might indicate that Nanog acts through these proteins to influence chromatin configuration. The next step would be to downregulate those factors in wild-type ESCs and observe whether heterochromatin will become compact or not. If chromatin does compact, then it may indicate that the factor acts downstream of Nanog to affect chromatin organization. Previous research has also shown that histone modifications such as acetylation may be involved in the modulation of chromatin compaction. Therefore, using ESI to visualize the chromatin organization in ESCs with different levels of histone acetylation in ESCs can help us understand the link between epigenetic marks and chromatin compaction. Ultimately, information gained from these experiments would enable us to create models showing the regulation of global chromatin organization in ESCs.


